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Helix-with-Loops Structure of Polynucleotide. I. Poly(C + IC) and Poly(C + GU)

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A copolymer of 57% riboinosinic acid plus 43% ribocytidylic acid has been prepared. The ultraviolet absorbances of aqueous solutions of mixtures of this copolymer and polyribocytidylic acid of various mole ratios were measured. The results indicate that, in these aqueous solutions, a "helix-with-loops" structure is formed; this structure has the hypoxantine-cytosine base pair and the unpaired cytidylic acid residues in the loops. A copolymer of 58% riboguanylic acid plus 42% ribouridylic acid has also been prepared. A similar ultraviolet examination was made of a mixture of this copolymer and polyribocytidylic acid; again the formation of a "helix-with-loops" structure was indicated. This structure is considered to have the guanine-cytosine base pair and the uridylic acid residues in the loops.

We have been engaged for some time in an examination of the double helical structures of polyribonucleotides.[1-4] In these structures, every base residue is involved in one of three specific base-pairs, namely, the adenine-uracil (A-U), hypoxanthine-cytosine (I-C), and guanine-cytosine (G-C) pairs. In the transfer RNA's (ribonucleic acids) and ribosomal RNA's of the biological systems, however, some base residues seem to be involved in some base pairs, while others do not. We have, therefore, attempted a rather detailed examination as to how such an unpaired base residue occurs and what chain conformation is assumed around this residue. In connection with this problem, what Fresco and Alberts[5] found is important. They showed that, on mixing a copolymer of riboadenylic acid plus ribouridylic acid with a homopolymer of ribouridylic acid in an aqueous solution, the structure schematically shown in Fig. 1, (a) is formed, but not the structure shown in Fig. 1, (b). This conclusion, based on the stoichiometric ratio in the interaction of the two polymers, was determined by their experiment with a mixing curve. They called the part of the chain (with the unpaired uridylic acid residue) rotating out of the double helical structure the "loop." They also showed that, in a solvent with 0.05 M magnesium chloride, a triple helical structure with loops (Fig. 1, (c)) is formed. Similar experiments were made by Steiner;[6,7] he showed that not only the triple helix with U-loops (Fig. 1, (c)) but also a triple helix with I-loops (loops with inosinic acid residues, Fig. 1, (d)) can be formed.

On the basis of these findings we programmed the following studies:

(1) To find other examples of the "helix-with-loops" structure, and to examine which is suitable for the studies, (2) and (3), described below.

(2) To find methods to discriminate the helix and loop parts of the polyribonucleotide chain and methods for a quantitative analysis as to how great a fraction of the total G (C, A, U, or I)[*2] is involved in the base pair and how great a fraction of the total G (C, A, U, or I) is involved in the loop.

(3) An X-ray diffraction study of the structure of a synthetic RNA with a regular loop arrangement.

The purpose of this paper is to report the results of the first study. The double helical structure with the A-U pair has a complexity because it is possibly converted into a metastable triple helical structure with a $\overset{\text{U}}{\overset{/}{\text{A}}}$–U-type bridge, even in a solvent without magnesium and with a low salt concentration.[2] On the other hand, the double helical structure with the I–C pair does not seem to have this complexity.[1] Steiner[8] prepared copolymers of inosinic acid plus cytidylic acid (poly IC), and

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1) K. Matsuo and M. Tsuboi, This Bulletin, **39**, 347 (1966).

2) S. Higuchi and M. Tsuboi, *ibid.*, **39**, 1886 (1966).

3) T. Sato, Y. Kyogoku, S. Higuchi, Y. Mitsui, Y. Iitaka, M. Tsuboi and K. Miura, *J. Mol. Biol.*, **16**, 180 (1966).

4) M. Maeda, K. Matsuo, M. Nakanishi and M. Tsuboi, This Bulletin, **40**, 2068 (1967).

5) J. R. Fresco and B. M. Alberts, *Proc. Nat. Acad. Sci.* (U. S. A.), **46**, 311 (1960).

6) R. E. Steiner, *J. Biol. Chem.*, **235**, 2946 (1960).

7) R. F. Steiner, *ibid.*, **236**, 842 (1961).

[*2] G: riboguanylic acid, C: ribocytidylic acid, A: riboadenylic acid, U: ribouridylic acid, I: riboinosinic acid.

8) R. F. Steiner, *J. Biol. Chem.*, **236**, 3037 (1961).

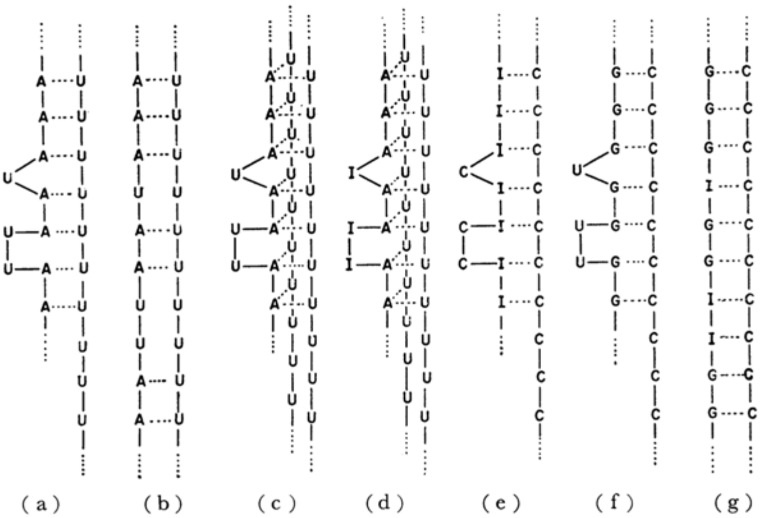

Fig. 1. Schematic drawings of possible structures of polynucleotide complexes, each of which consists of a copolymer and a homopolymer.

- (a) Double-helix-with-loops structure formed by poly AU plus poly U.
- (b) Complete helix formed by poly AU plus poly U. (This structure was excluded by Fresco and Alberts[5])
- (c) Triple-helix-with-loops structure formed by poly AU plus 2 × poly U.
- (d) Triple-helix-with-loops structure formed by poly AI plus 2 × poly U.
- (e) Double-helix-with-loops structure formed by poly IC plus poly C.
- (f) Double-helix-with-loops structure formed by poly GU plus poly C.
- (g) Complete helix formed by poly GI plus poly C.[4]

TABLE 1. ENZYMATIC PREPARATION OF HOMO- AND COPOLYMER OF INOSINIC ACID AND CYTIDYLIC ACID

Polymer product	Substrate[a]		Mg^{2+} μmol	Tris μmol	Enzyme unit[b]	Incubation		Yield %	Sedimentation coefficient	Base composition in the product	
	IDP mg	CDP mg				Temp. °C	Time min			I[c] %	C %
(A) poly C	0	10	7.5	150	10	30	90	23	7.2 S	0	100
(B) poly I	10	0	7.5	150	14	30	180	30	3.0	100	0
(C) poly IC	5	5	7.5	150	14	30	120	33	$3._2$	$18._5$	$81._5$
(D) poly IC	6.5	3.5	7.5	150	14	30	200	35	2.6	$30._5$	$69._5$
(E) poly IC	9	1	7.5	150	14	30	180	15	2.3	$56._5$	$43._5$

a) IDP: inosine-5′-diphosphate. CDP: cytidine-5′-diphosphate.
b) 1 unit = amount of enzyme which can liberate 1 μmol of orthophosphate in the enzymatic reaction of 15 min at 37°C.
c) Hypoxanthine.

he mixed one of them with polycytidylic acid in a 1 : 1 mole ratio. He found evidence that there is interaction between the copolymer and the homopolymer, but he did not examine whether or not a "helix-with-loops" structure such as that shown in Fig. 1, (e) is formed. Therefore, we first did this examination; as will be described in detail below, we found that the "helix-with-loops" structure is formed. This poly IC+poly C system has, however, yet another complexity, because poly IC sometimes forms a secondary structure by itself, probably with the I-C pairs, before being mixed with poly C. Hence, we next prepared a copolymer of guanylic acid plus uridylic acid (poly GU); we found that a "helix-with-loops" structure such as that shown in Fig. 1, (f) is formed when it is mixed with the poly C (see below). This copolymer-homopolymer combination (poly GU+poly C) was considered to be the most promising combination for obtaining a stable "helix-with-loops" structure, because the G-C pair would be the strongest base pair and the U-C pair (if any) would be the weakest one. Thus, the U-residues in poly GU are considered to be readily rotated out from a double helical structure with the GC pair.

Preparation of the Polynucleotide Samples

All the polynucleotide samples used were prepared by the use of polynucleotide phosphorylase obtained from *Micrococcus lysodeikticus*. The compositions of the reaction mixtures, the incubation times, and the incubation temperatures are given in Tables 1 and 2. After the enzymatic reaction, deproteinization was made by the use of chloroform and isoamyl alcohol, and then the polynucleotide was purified by repeating the precipitation with ethanol and by dialysis.

TABLE 2. ENZYMATIC PREPARATION OF A COPOLYMER OF GUANYLIC ACID PLUS URIDYLIC ACID

Reaction mixture:	
Guanosine-5′-diphosphate (Li-salt)	3.5 mg
Uridine-5′-diphosphate (Li-salt)	7.5 mg
Tris (pH 9.5)	300 μmol
EDTA[a]	8 μmol
$MgCl_2$	16 μmol
Urea	0.4 mol/*l*
Enzyme	10 unit
H_2O	
Total volume	2.0 m*l*
Incubation Temperature	37°C
Incubation Time	18 hr
Product:	
Yield	820 μg
Base composition	G 58% U 42%
Degree of polymerization: We did not examine ultracentrifugal sedimentation with this particular product. We did it, however, with a product obtained from another reaction mixture having almost the same composition as given above. Its sedimentation coefficient was found to be 4.4 S, and, therefore, the product in question has probably a degree of polymerization sufficiently high for the present purpose.	

a) Ethylenediamine tetraacetic acid.

Base Analyses of the Copolymers

The base compositions of the poly IC samples were kindly determined by Mr. K. Shuto in this laboratory by the method described in our previous paper.[9] The results are given in the last two columns of Table 1. As may be seen there, the cytidylic acid residue is found to have a much greater chance of being incorporated into the polymer than does the inosinic acid residue. The base composition of the poly GU sample was determined by hydrolyzing it with 1 N HCl, by the paper-chromatographic separation of the resulting guanine and uridylic acid, and by ultraviolet absorption measurements of them. The composition thus determined is given in Table 2. It has been found that the guanylic acid residue has a greater chance to be incorporated into the polymer than does the uridylic acid residue.

Experimental

Ultraviolet absorption measurements were made by the use of an Ito Spectrophotometer, Model QU-3, and a Cary-14 Spectrophotometer. The temperature of the sample solution was controlled by a method described in a previous paper.[1]

The polynucleotide concentration in each standard solution was determined by measuring the phosphorus content.[10]

Secondary Structures of Poly IC and Poly GU

Absorption *vs.* temperature profiles were observed at 248 mμ in the three copolymer of inosinic and cytidylic acids. The results are given in Fig. 2. As may be seen here, the copolymer with I $18._5$% and C $81._5$% has the greatest secondary structure and shows a marked elevation in the absorption intensity when broken by heat. The copolymer with I $30._5$% and C $69._5$% has less secondary structure, while the copolymer with I $56._5$% and C $43._5$% has almost none. This result is apparently contradictory to what Steiner[8] observed. He found that the optical density increment at 250 mμ caused by heat is equally big in the poly IC with I 45% and C 55%, and in the

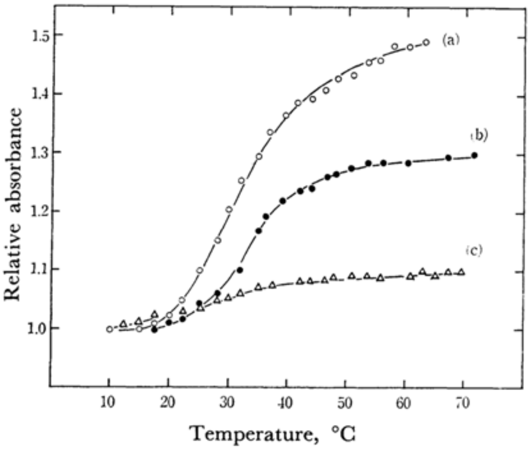

Fig. 2. The variation of the absorbance at 248 mμ of poly IC solutions with temperature. Solvent: 0.05 M NaCl + 0.001 M Na-citrate buffer, pH 7.0.
(a) Copolymer with I $18._5$% and C $81._5$%.
(b) Copolymer with I $30._5$% and C $69._5$%.
(c) Copolymer with I $56._5$% and C $43._5$%.

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[9] K. Shuto, K. Matsuo and M. Tsuboi, *Chem. Pharm. Bull.,* **14**, 915 (1966).

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

poly IC with I 31% and C 69%. The difference may be caused by the fact that he used a solvent of 0.1 M KCl plus 0.01 M cacodylate (pH 6.5), while we used a solvent of 0.05 M NaCl plus a 0.001 M Na-citrate buffer (pH 7.0). It may also be caused by a difference in the base arrangements between his poly IC and ours.

Poly GU was considered to form no secondary structure by itself, because it cannot involve any of the specific base pairings. Actually, however, a small increment of absorbance at 255 mμ is observed at about 70°C in a 0.01 M Na-cacodylate buffer, pH 7.0. This is probably caused by a secondary structure formed by a G-G pairing and a G-on-G stacking.[11,12]

Mixing Curve of Poly IC and Poly C

Poly IC and poly C (or poly I) were mixed in a solution at various mole ratios, and the ultraviolet absorbance *vs.* mole-ratio profiles were observed. From such a profile we may judge whether the two polymers in question have no interaction or whether they form a complex. When a complex is formed,

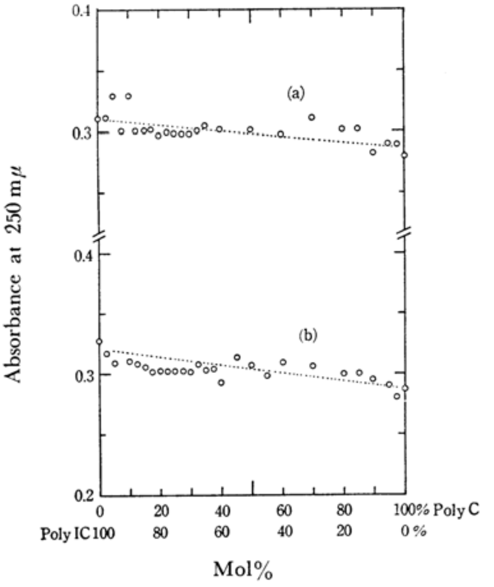

Fig. 3. Mixing curves for poly IC + poly C.
Solvent: 0.05 M NaCl + 0.001 M Na-citrate buffer, pH 7.0.
Polymer concentration: 4×10^{-5} M.
(a) Copolymer with I 18.$_5$% and C 81.$_5$% plus homopolymer of C.
(b) Copolymer with I 30.$_5$% and C 69.$_5$% plus homopolymer of C.
Absorbance at 250 mμ was observed after each mixture solution was kept at 6°C for 48 hr.

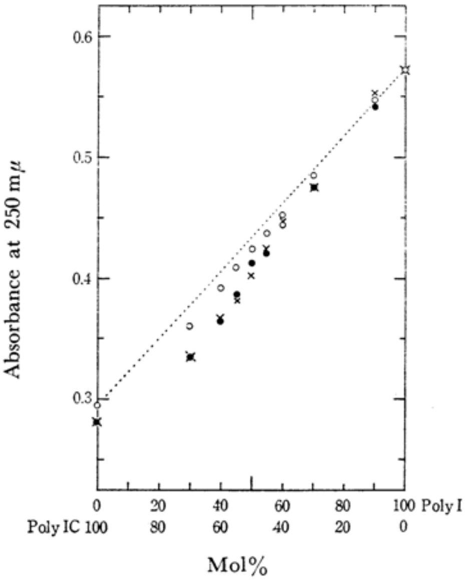

Fig. 4. Mixing curve for copolymer with I 18.$_5$% and C 81.$_5$% plus homopolymer of I.
Polymer concentration is always 4×10^{-5} M.
Solvent: 0.05 M NaCl + 0.001 M Na-citrate buffer, pH 7.0.
Absorbance at 250 mμ was observed after each mixture solution was kept at 6°C for 6 hr (○), for 48 hr (●), and for two weeks (×).

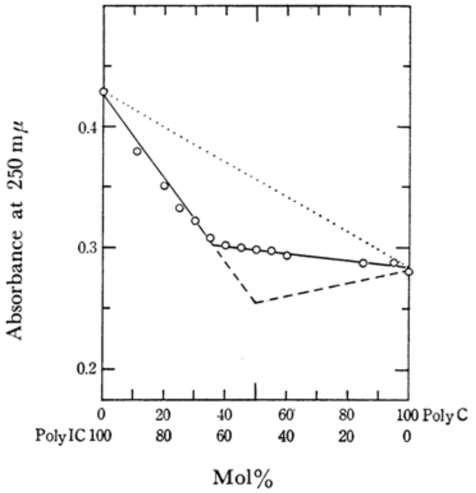

Fig. 5. Mixing curve for copolymer with I 56.$_5$% and C 43.$_5$% plus homopolymer of C.
Polymer concentration is always 4×10^{-5} M.
Solvent: 0.05 M NaCl + 0.001 M Na-citrate buffer, pH 7.0.
Absorbance observed at 250 mμ after each solution was kept at 6°C for 48 hr is given by ○.
(——): Mixing curve expected for the formation of a " helix-with-loops " structure.
(- - -): Mixing curve expected for the formation of a complete helix similar to what is shown in Fig. 1, (b).
(……): Mixing curve expected for no interaction.

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[11] M. Gellert, M. N. Lipsett and D. R. Davies, *Proc. Nat. Acad. Sci.* (*U. S. A.*), **48**, 2013 (1962).

[12] J. R. Fresco and J. Massoulié, *J. Am. Chem. Soc.*, **85**, 1352 (1963).

its stoichiometric ratio may also be determined from the observed profile of the mixing curve.

As is illustrated in Figs. 3 and 4, the poly IC with I 18.$_5$% and C 81.$_5$% shows practically no interaction when mixed with the poly C or with the poly I. It was found also that the poly IC with I 30.$_5$% and C 69.$_5$% shows no interaction when being mixed with poly C (see Fig. 3).

The poly IC with I 56.$_5$% and C 43.$_5$%, on the other hand, shows evidence of interaction with the poly C (see Fig. 5). The mixing curve consists of two straight lines which intersect at the poly IC/poly C mole ratio of 64/36. As has been described above, we refer to the moles of phosphorus (or the moles of nucleotide residues) as the "moles of polynucleotides." Therefore, the poly IC/poly C mole ratio means the ratio of the moles of I residues plus the moles of C residues in the poly IC now in question *vs.* the moles of C residues in the poly C now in question. If the complex molecule formed by the poly IC and the poly C is a "complete helix," with both the I-C base pair and the C-C base pair, then the stoichiometric poly IC/poly C mole ratio in this complex molecule should be 1/1 or 50/50. On the other hand, if the complex molecule has a "helix-with-loops" structure in which only the I residues (56.$_5$%) in the copolymer form base pairs with the C residues in the homopolymer (see Fig. 1, (e)), the stoichiometric poly IC/poly C mole ratio in this complex molecule should be 100/56.$_5$=64/36. What is actually observed shows that the poly IC and the poly C form a complex molecule with the stoichiometric mole ratio of 64/36 rather than 50/50. This is in accord with the view that the complex molecule has the "helix-with-loops" structure shown in Fig. 1, (e) rather than a complete helix.

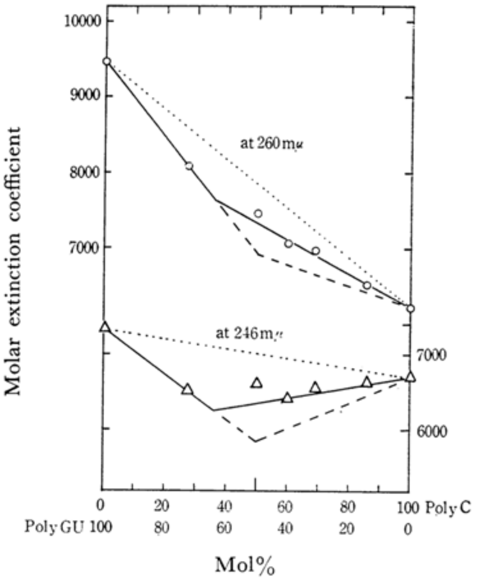

Fig. 6. Mixing curves for copolymer with G 58% and U 42% plus homopolymer of C.
Solvent: 0.01 M Na-cacodylate buffer, pH 6.7. After the preparation of each solution, it was kept at room temperature (22—24°C) for 4 days and then at 31°C for 19 hr before it was subjected to the ultraviolet absorption measurement.

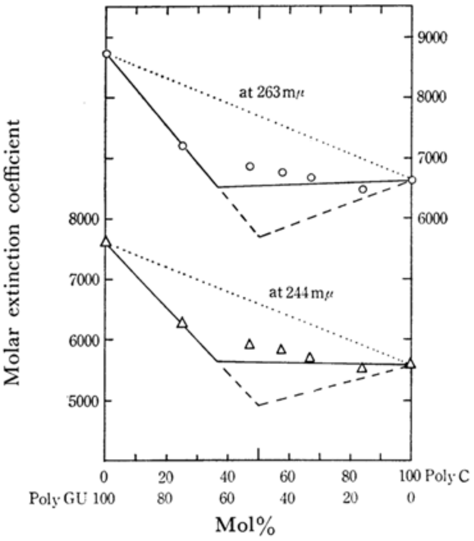

Fig. 7. Mixing curves for copolymer with G 58% and U 42% plus homopolymer of C obtained by another series of experiments than that shown in Fig. 6.
Solvent: 0.01 M cacodylate buffer, pH 6.7. After preparation of each solution, it was kept at room temperature (22—24°C) for 3 days and then at 31°C for 19 hr.

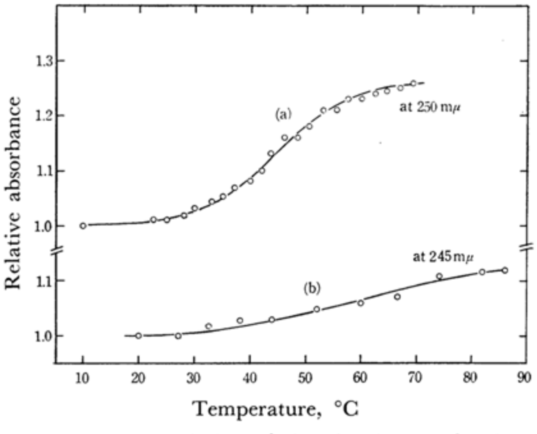

Fig. 8. The variation of the absorbance of polynucleotide solution with temperature.
(a) Copolymer with I 56.$_5$% and C 43.$_5$% plus homopolymer of C. Mole ratio of copolymer/homopolymer is 65/35. Solvent: 0.05 M NaCl+0.001 M Na-citrate buffer, pH 7.0.
(b) Copolymer with G 58% and C 42% plus homopolymer of C. Mole ratio of copolymer/homopolymer is 73/27. Solvent: 0.01 M Na-cacodylate buffer, pH 6.7.

Mixing Curve of Poly GU and Poly C

A similar observation was made by mixing GU and the poly C. Two series of experiments were made; both sets of results give evidence for an interaction of these two polymers, as may be seen in Figs. 6 and 7. In addition, every mixing curve appears to have a minimum or a bend at the poly GU/poly C mole ratio of 100/58 (=63/37), rather than at 50/50. This is in contrast with the case when the poly GI and the poly C were mixed. In the latter case a bend is always observed at poly GI/poly C=50/50.⁴⁾ What has been found here is in agreement with the view that the poly GU plus poly C forms a complex molecule with the "helix-with-loops" structure shown in Fig. 1, (f), whereas the poly GI plus the poly C forms a complete helix such as that shown in Fig. 1, (g).

Melting of the "Helix-with-Loops" Structure

A "helix-with-loops" structure is considered to be less stable than the corresponding helix-without-loop structure. This is found to be actually the case for poly(IC+C). As may be seen in Fig. 8, (a), the "IC-helix with C-loops" melts at about 45°C in 0.05 M NaCl plus a 0.001 M Na-citrate buffer, pH 7.0. In the same solvent, the "IC-helix without loop" melts at 57°C.¹⁾ For the "GC-helix with U-loop," only a gradual increase in the absorbance at 245 mμ is observed (Fig. 8, b) in a 0.01 M Na-cacodylate buffer, pH 6.7, over the temperature range from 30 to 80°C.

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