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Melting of Complexes of Polyribocytidylic Acid Plus Copolymers of Inosinic and Guanylic Acids in Solution

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Three copolymers of riboinosinic acid and riboguanylic acid (poly IG), with guanylic acid contents of 21.6%, 33%, and 45%, have been prepared, as well as three homopolymers of ribocytidylic acid (poly C), riboinosinic acid (poly I), and riboguanylic acid (poly G). On mixing both the poly IG and the poly G with poly C in various mole ratios in an aqueous solution, a marked hypochromicity was observed in the 250 mμ region, and it was shown that there are double helical complexes similar to the well-known poly(I+C) formed in the solutions. The optical melting profile of each complex into random coils was examined, and it was found that the guanine-cytosine pair is 20% (in enthalpy) more stable than the hypoxanthine-cytosine pair. It was also found that actinomycin D, which has no effect on the melting of poly(I+C), stabilizes the helical structure of poly(IG+C).

A 1 mol-to-1 mol mixture of polyriboinosinic acid (poly I) and polyribocytidylic acid (poly C) is known to form a double helical complex, poly-(I+C), in an aqueous solution.(1,2) Its structure(3) is considered to be somewhat similar to those of natural double-helical deoxyribonucleic acid (DNA)(4) and ribonucleic acid (RNA)(5). The two polynucleotide chains (poly I and poly C) are considered to be connected by an I-C pair with the hydrogen bonds(3) shown in Fig. 1 (a). On the other hand, what is involved in the natural DNA and RNA is the G-C pair instead of the I-C pair, and it is considered to have the structure(6,7) shown in Fig. 1(b). These two base pairs are equal excect that in the latter there is an additional hydrogen bond between the 2-amino of guanine and 2-carbonyl of cytosine. The purpose of this investigation is to examine the effect of a partial substitution of the I-C pair with the G-C pair on the stability of the double helical structure.

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

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

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

4) For example, W. Fuller, M. H. F. Wilkins, H. R. Wilson and L. D. Hamilton, *J. Mol. Biol.,* **12**, 60 (1965).

5) For example, T. Sato, Y. Kyogoku, S. Higuchi, Y. Iitaka, M. Tsuboi and K. Miura, *ibid.,* **16**, 180 (1966).

6) F. H. C. Crick and J. D. Watson, *Proc. Roy. Soc.,* **A223**, 80 (1954).

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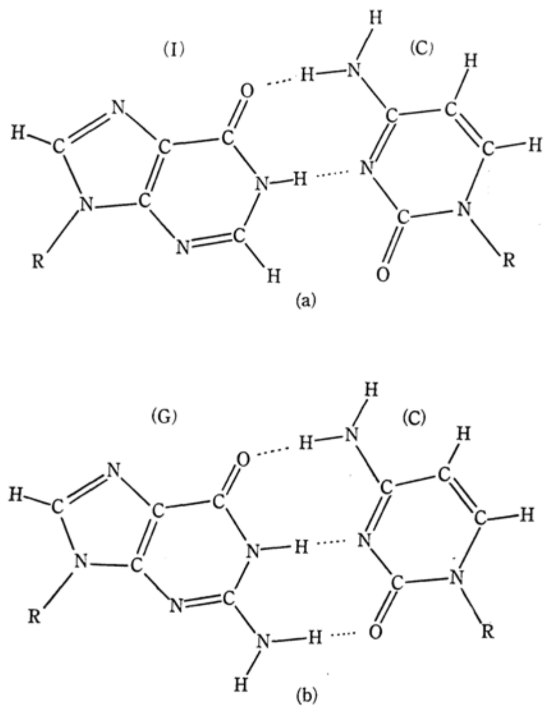

Fig. 1. (a) Hypoxanthine-cytosine pair (I-C pair).
(b) Guanine-cytosin pair (G-C pair).

Preparation of the Polynucleotide Samples

All the polynucleotide samples used in the present investigation were prepared by the use of polynucleotide phosphorylase obtained from *Micrococcus lysodeikticus*. After an enzymatic reaction, deproteinization was effected by chloroform, or by sodium lauryl sulfate plus phenol, or by chloroform plus isoamyl alcohol. The polynucleotide product was purified by repeated precipitation with ethanol and dialysis.

The poly C sample used showed a sedimentation coefficient of 4—5S in ultracentrifugal examination. Poly G was prepared in a reaction mixture with urea, which would prevent the formation of a secondary structure of the product, thereby helping the enzyme to produce a longer polymer.[8] An example of the reaction mixture is given below:

Guanosine-5′-diphosphate (Li salt)	10.2 mg (25 μmol)
Tris buffer pH 9.5	700 μmol
$MgCl_2$	10 μmol
EDTA[*2]	5 μmol
Enzyme (specific activity† 29.5)	520 γ protein
Urea	0.4 mol
H_2O	
Total volume	5.0 m*l*

This reaction mixture was incubated at 37°C for 46 hr. After the ethanol precipitation, the product was found not to be easily dissolved in a neutral aqueous solvent. A neutral aqueous solution was prepared by fist dissolving the product in an alkaline solvent and by then dialysing the solution against a pH 7 buffer.

Three samples of poly IG were prepared. The compositions of the substrates (inosine-5′-diphosphate and guanosine-5′-diphosphate) are given in Table 1. An example of the reaction mixture is given below:

Inosine-5′-diphosphate	14 mg
Guanosine-5′-diphosphate	6 mg
Tris buffer pH 9.5	200 μmol
$MgCl_2$	4 μmol
EDTA[*2]	1 μmol
Enzyme (specific activity† 29.5)	200 γ protein
Urea	0.4 mol
H_2O	
Total volume	2.0 m*l*

This reaction mixture was incubated at 37°C for 16 hr. After the ethanol precipitation, the product was dissolved in an alkaline solvent, and then the solution was neutralized; the product was purified by repeated ethanol precipitation. All of the poly IG samples used showed sedimentation coefficients of 4S—6S (see Table 1), and are considered to have degrees of polymerization sufficiently high for our present purposes.

TABLE 1. PREPARATIONS OF COPOLYMERS OF INOSINIC ACID PLUS GUANYLIC ACID

	Substrate composition		Base composition in the product		Sedimentation coefficient
	IDP	GDP	I	G	
(A)	14 mg (70%)	6 mg (30%)	55%	45%	4S
(B)	17 mg (85%)	3 mg (15%)	67%	33%	5S
(C)	18 mg (90%)	2 mg (10%)	78.$_4$%	21.$_6$%	6S

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8) F. Cramer and H. Küntzel, *Biochim. Biophys. Acta*, **80**, 213 (1964).

[*2] Ethylenediamine tetracetic acid.

† Specific activity=(P_i)/(Protein), where (P_i) denotes the μmol of orthophosphate liberated by an enzymatic reaction of 15 min at 37°C, and (Protein), the amount of added enzymes in milligrams.

Base Composition of Poly IG

The base composition of each product was determined by hydrolyzing it with 1 N HCl (at 100°C, for 1 hr), by separating the resulting inosine and guanosine through paper chromatography, and by then determining the relative quantities of these nucleosides through ultraviolet absorption measurements. The base compositions thus determined are given in the last two columns of Table 1. As is seen here, guanylic acid has a greater chance to be incorporated into the polymer than has inosinic acid.

Formation of Two-Stranded Complex

In the present investigation, we did not attempt any detailed examination of poly G. The ultraviolet absorption curve of poly G, the mixing curves of poly G plus poly C, and the optical melting curves of a 1 : 1 complex of poly G plus poly C have already been given by previous experiments.[9] It was shown by these experiments that poly G and poly C form a two-stranded 1 : 1 complex poly(G+C), and that this complex melts into random coils at a relatively high temperature, as can be followed by the observation of the increase in ultraviolet absorption intensity. Our sample of poly G showed an ultraviolet absorption curve equal to what Pochon and Michelson[9] observed. The molar extinction coefficient at 256.5 mμ (λ_{max}) is 9012 (ε_{max}). When the sample was mixed with poly C in a 0.056 M NaCl plus a 0.001 M Na-citrate buffer (pH 7.0), the ε_{max} value decreased to 6289, showing the formation of a secondary structure. In the same solvent, the optical melting profile shown in Fig. 2 was obtained.

The ultraviolet absorption curve of one of our poly IG sample is shown in Fig. 3. On mixing with poly C, the absorption intensity at 250 mμ (maximum) greatly decreases in every case (see Fig. 3). The mixing curves shown in Fig. 4 indicate that a two-stranded 1 : 1 complex is formed between poly C and poly IG. Thus, there may be considered to be a double-helical complex molecule formed in which I-C and G-C base pairs (probably with the structures shown in Fig. 1, (a) and (b)) coexist.

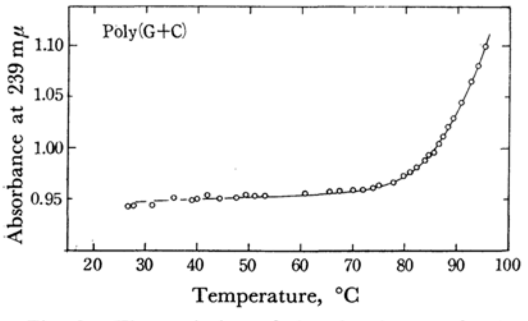

Fig. 2. The variation of the absorbance of poly-(G+C) solution at 239 mμ with temperature. Solvent: 0.056 M NaCl plus 0.001 M Na-citrate buffer, pH 7.0.

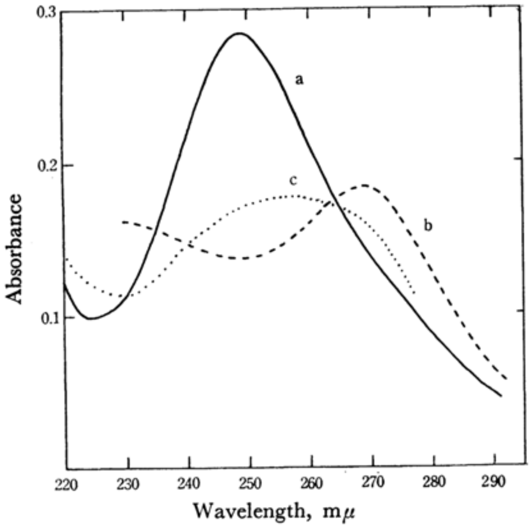

Fig. 3. Ultraviolet absorption curves (a) Copolymer of inosinic (67%) and guanylic (33%) acids $0.28_2\times10^{-4}$ M, (b) Poly C $0.28_2\times10^{-4}$ M, (c): 1:1 mixture of (a) and (b). Solvent: 0.1 M NaCl plus 0.01 M cacodylate buffer, pH 7.0.

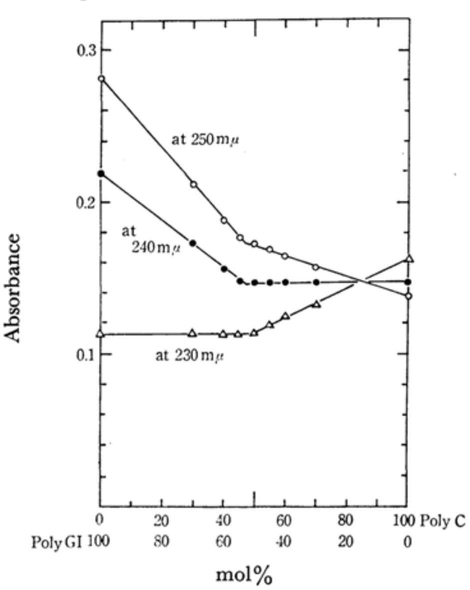

Fig. 4. Mixing curves for copoly (IG) (G 33%) and homopoly C. Solvent: 0.1 M NaCl plus 0.01 M cacodylate buffer, pH 7.0. Every mixture solution was prepared so that it contains an equal amount of phosphorus.

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[9] J. R. Fresco and D. F. Su, *J. Biol. Chem.*, **237**, 3305 (1962); J. R. Fresco and J. Massoulié, *J. Am. Chem. Soc.*, **85**, 1352 (1963); F. Pochon and A. M. Michelson, *Proc. Nat. Acad. Sci. (U. S. A.)*, **53**, 1425 (1965).

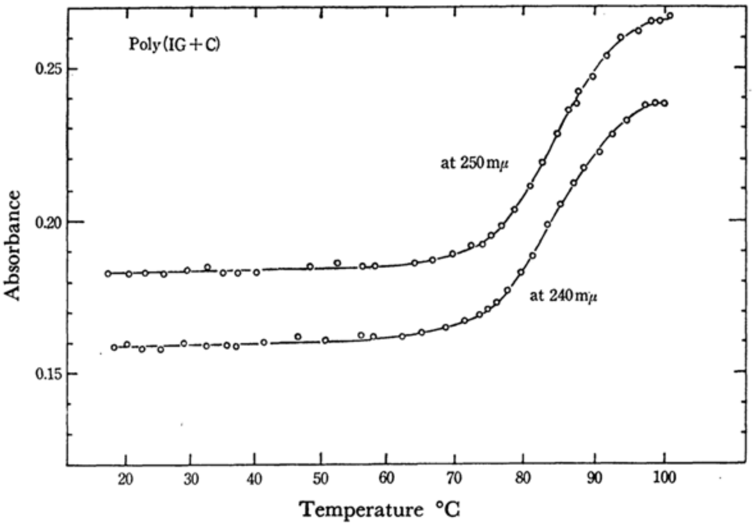

Fig. 5. The variation of the absorption of a 1 mol : 1 mol mixture solution of copoly(IG) (G 33%) and homopoly C with temperature. Solvent: 0.1 M NaCl plus 0.01 M cacodylate buffer, pH 7.0.

Melting of the Complex Molecules

The optical melting profile was observed in a 0.1 M NaCl+0.01 M cacodylate buffer (pH 7.0), in a 0.05 M NaCl+0.001 M Na-citrate buffer (pH 7.0), in a 0.025 M NaCl+0.001 M Na-citrate buffer (pH 7.0), and in a 0.01 M NaCl+0.001 M Na-citrate buffer (pH 7.0), for each of the three complexes now in question. The results for the solution of pH 7.0 are illustrated in Fig. 5. The melting takes place at much higher temperatures and over a wider temperature range for poly-(IG+C) than for poly (I+C).[2] The midpoint temperature of this temperature range was defined as the melting temperature, T_m; T_m is plotted against the guanine content in the copolymer in Fig. 6.

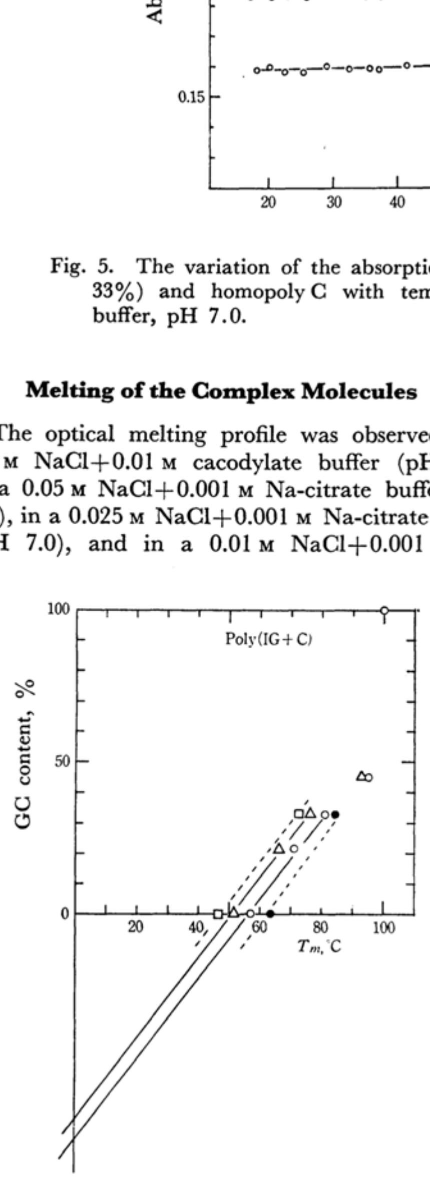

Fig. 6. Dependence of T_m on the content of the gaunine-cytosine pair in a double helical poly-(IG+C).

●: Observed in 0.1 M NaCl
○: in 0.05 M NaCl
△: in 0.025 M NaCl
□: in 0.01 M NaCl

Let us assume that the enthalpy (ΔE) of the melting of the complex, poly(IG+C), now in question is given as:

$$\Delta E = n[\Delta\varepsilon_a(1-g)+\Delta\varepsilon_b g] \qquad (1)$$

where $\Delta\varepsilon_a$ is the enthalpy of melting assigned to one hypoxanthine-cytosine bridge (see Fig. 1 (a)); $\Delta\varepsilon_b$, that assigned to one guanine-cytosine bridge; g, the guanine content in the poly(IG) copolymer; and n, the mean number of bridges in the double-helix structure of poly(IG+C). We then obtain the relation[10]:

$$g = \frac{\Delta S - \boldsymbol{R}\ln 0.5a}{n(\Delta\varepsilon_b - \Delta\varepsilon_a)}\,T_m - \frac{\Delta\varepsilon_a}{\Delta\varepsilon_b - \Delta\varepsilon_a}. \qquad (2)$$

Here, ΔS is the entropy of the melting of the complex, and a is the total concentration of the complex. If ΔS, $\Delta\varepsilon_a$, and $\Delta\varepsilon_b$ are assumed to be constants independent of T_m, then Eq. (2) shows that the melting temperature, T_m, of poly(IG+C) should be a linear function of the guanine content, g, of the copolymer, poly IG. As may be seen in Fig. 6, a linear relation is actually obtained in the low g range. Therefore, in this range the assumptions made above may be valid. The slope and

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

intercept of the line for the solutions with 0.05 M NaCl are found to be, respectively, 0.013 deg^{-1} and -4.3. From Eq. (2) we obtain:

$$\frac{\Delta S-\boldsymbol{R}\ln 0.5a}{n(\Delta\varepsilon_b-\Delta\varepsilon_a)}=0.013\ \mathrm{deg}^{-1} \tag{3}$$

$$\frac{\Delta\varepsilon_a}{\Delta\varepsilon_b-\Delta\varepsilon_a}=4.3 \tag{4}$$

At present the values of $\Delta\varepsilon_a$, $\Delta\varepsilon_b$, $\Delta\varepsilon_b-\Delta\varepsilon_a$, and $(\Delta S-\boldsymbol{R}\ln 0.5a)/n$ are all unknown. However, if one of these values is fixed, all of them can be determined by means of these relations. For example, if $(\Delta S-\boldsymbol{R}\ln 0.5a)/n$ is assumed to have the same value as that (32 e. u.) estimated for double helical polyriboadenylic acid,[10] then the values of $\Delta\varepsilon_a$ and $\Delta\varepsilon_b$ may be calculated to be 9.7 and 11.9 kcal/mol respectively. At any rate, it may be concluded from Eq. (4) that the guanine-cytosine bridge (Fig. 1(b)) makes roughly $(4.3+1.0)/4.3=1.2$ times the contribution to the double-helix stability given by the hypoxanthine-cytosine bridge (Fig. 1 (a)).

As may be seen in Fig. 6, the linear relation just mentioned cannot be extrapolated into the high *g* range. This fact shows that the assumptions presented above are not valid over the whole range of *g*. This is understandable because poly(G+C) probably takes a somewhat different conformation from that of poly(I+C), therefore, the values of $\Delta\varepsilon_a$ and $\Delta\varepsilon_b$ in Eq. (1) would not be constant, but would depend greatly on *g*, and also on the manner of the IG arrangement. Before we can go into detail on this problem, we need to obtain a few more copolymers with higher guanine contents, and also we need to obtain some information about the base sequence.

Effect of Actinomycin D

It has been found that actinomycin D exerts no effect upon the melting of poly(I+C). Upon the melting of poly(IG+C), however, a marked effect has now been observed. Thus, as is shown in Fig. 7, the optical melting profile of poly(IG+C) in a solvent (pH 7.0) with actinomycin D (30 γ/m*l*) is quite different from that in a solvent without actinomycin D. Only a small fraction of the molecules melts at a relatively low T_m value; the rest remains unmelted up to at least 95°C. When the pH of the solution is elevated, however, the absorption intensity greatly increases, showing that practically all of the molecules melt at room temperature. (The elevation of pH causes also the color of actinomycin D to disappear.)

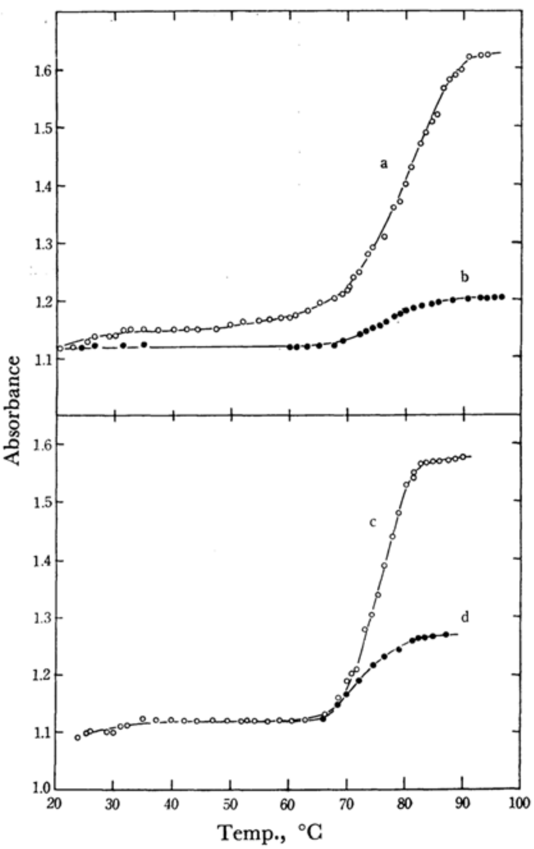

Fig. 7. The variation of the absorbance of poly-(IG+C) solution at 245 mμ with temperature. (a): Poly IG (G 33%) $1.8_8\times10^{-5}$ M plus poly-C $1.7_2\times10^{-5}$ M in 0.05 M NaCl plus 0.001 M Na-citrate buffer, pH 7.0. (b): Solution (a) plus actinomycin D 30 γ/m*l*. (c): Poly IG (G 21.6%) $1.8_0\times10^{-5}$ M puls poly C $1.7_2\times10^{-5}$ M in 0.05 M NaCl plus 0.001 M Na-citrate buffer, pH 7.0. (d): Solution (c) plus actinomycin D 30 γ/m*l*.

On the basis of the previous data,[11-13] it has been considered that actinomycin D is bound specifically to the guanine residue of DNA and that it stabilizes the DNA structure. For double helical RNA, however, no effect of actinomycin D has so far been observed. This seems to be the case even with an RNA which has a number of guanine residues. Thus, Mr. Shigesada Higuchi of this laboratory found that 7 γ/m*l* actinomycin causes only a slight elevation (from 73.5°C to 75.0°C) of the T_m of the rice dwarf rivus RNA with the GC content of 43.4% in a 0.001 M Na-citrate buffer. Therefore, what we have found here is the first example of RNA which is markedly stabilized by actinomycine D.

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[12] I. H. Goldberg, M. Rabinowitz and E. Reich, *Proc. Nat. Acad. Sci (U. S. A.)*, **48**, 2094 (1962).

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