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Poly-5-methyldeoxycytidylic Acid and Some Alkylamino Analogs*

Barbara Zmudzka, F. J. Bollum, and D. Shugar

ABSTRACT: 5-Methyldeoxycytidine triphosphate and several alkylamino analog triphosphates have been polymerized with the aid of terminal deoxynucleotidyl transferase to give poly-5-methyldeoxycytidylic acid and the N^4-methyl and N^4-ethyl polymer analogs of 5-methyldeoxycytidylic acid. All three of these were polymerized by the enzyme at the same rate as polydeoxycytidylic acid, and the final products have about the same sedimentation constants, $s_{20,w}$ = 4.5 S. Poly-5-methyldeoxycytidylic acid forms an acid twin-stranded helix similar to that formed by polydeoxycytidylic acid, the 5-methyl substituent being without any appreciable effect on the stability. The alkylamino polymer analogs do not form such structures. All three new polymers are considerably more resistant than polydeoxycytidylic acid to phosphodiesterase I. Poly-5-methyldeoxycytidylic readily complexes with polydeoxyinosinic acid, and the T_m for the twin-stranded helix is 17° higher than that for the corresponding polydeoxyinosinic acid:polydeoxycytidylic acid, thus confirming the significant stabilizing effect of a pyrimidine 5-methyl substituent, even in the absence of a 2′-hydroxyl. An extensive analysis of the complexes between poly-5-methyldeoxycytidylic acid and polydeoxyinosinic acid, as a function of the ionic strength of the medium, demonstrated the existence of four equilibrium reactions between the homopolymers and the twin- and triple-stranded helices formed by them. The alkylamino polymer analogs did not complex with polydeoxyinosinic acid, probably as a result of hindered rotation of the alkylamino groups by the 5-methyl substituent. The nature of the various complexes is discussed with emphasis on the so-called acid form of poly-5-methyldeoxycytidylic acid (and polydeoxycytidylic acid) in relation to the acid form of poly-5-methylribocytidylic acid (and polyribocytidylic acid).

The introduction of a 5-methyl substituent into the base residues of poly rU[1] results in an appreciable enhancement of helix stability, as demonstrated by the higher T_m value of poly rT compared with poly rU (Shugar and Szer, 1962). This effect is also observed in the complexes of the foregoing with poly A (Szer *et al.*, 1963; Barszcz and Shugar, 1968). While the acid and neutral forms of poly rC are not appreciably modified by 5 methylation, for reasons which have been presented elsewhere (Szer and Shugar, 1966), the twin-stranded helix of poly 5MerC with poly rI does exhibit a markedly higher T_m value than the corresponding complex with poly rC. A similar enhancement of helix stability by a pyrimidine 5-methyl substituent has been found in the twin-stranded complexes of poly X with poly rU and poly rT (Fikus and Shugar, 1969).

It thus was of interest to examine the influence of a pyrimidine 5-methyl substituent on the properties of poly dC and its complexes with poly dI, in part because of the absence of any secondary structure in poly dT (Riley *et al.*, 1966) as compared with poly rT (Shugar and Szer, 1962), and in poly dU as compared with poly rU (Zmudzka *et al.*, 1968). The properties of methylated polynucleotides are also of interest because of the widespread presence of methylated base residues as minor or major components in natural nucleic acids.

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[1] For purposes of clarity the prefixes r and d, for ribosyl and deoxyribosyl compounds, respectively, are retained throughout this text where a distinction between the two is necessary. Most of the other abbreviations followed the Revised Tentative Rules of the IUPAC–IUB combined Commission on Biochemical Nomenclature (*Biochemistry 5*, 1445 (1966)). The following nonstandard abbreviations are also employed: poly 5MedC, poly-5-methyldeoxycytidylic acid; poly N^4,5-diMedC, poly-N^4,5-dimethyldeoxycytidylic acid; poly N^4Et,5MedC, poly-N^4-ethyl-5-methyldeoxycytidylic acid; poly rT, poly-5-methylribouridylic acid; poly 5MerC, poly-5-methylribocytidylic acid; poly X, polyriboxanthylic acid 3 → 2, represents a transition from a triple- to a twin-strainded helix, with similar connotations for other types of transitions.

TABLE I: Quantitative Data Regarding Polymerization of Triphosphates.

	μmoles of Triphosphate in Incubn Mixture	μmoles of Polymer Isolated[a]	μmoles of Remaining Monomers[b]	% Polymer Yield	Actual Amt of Triphosphate in Prepn Employed for Polymerization (μM)	% Polymer Yield in Terms of Actual Amt of Substrate Employed	μmoles of Monomer Incorpd/μmoles of $d(pT)_3$ Primer
5MedC	120	93	29	78	96	97	335
N^4,5diMedC	33	27	6	82			355
N^4Et,5MedC	60	41	20	69	43	97	300

[a] Corrected for hyperchromicity determined by enzymatic hydrolysis (see Table III). [b] Calculated with the aid of values of ϵ_{260} for corresponding nucleosides. [c] Determined by paper chromatography and measurement of ultraviolet absorption of eluted triphosphates and other absorbing substances.

Experimental Section

Poly dC (ϵ_{260}^P 5.3×10^3 at pH 8.0; $s_{20,w}$ = 8.41 S at pH 7.2 and 4.93 at pH 8.8) and poly dI (ϵ_{260}^P 5.35×10^3 in 10^{-3} M Tris (pH 8.0); $s_{20,w}$ = 4.57 S at pH 8.0) were obtained with the aid of terminal deoxynucleotidyl transferase as previously described (Bollum, 1966).

Hydrolysis of these, and other polymers to be described below, to mononucleotides was achieved with phosphodiesterase I (Worthington) as follows. (a) To a stoppered 10-mm spectrocuvet containing 0.03 M Tris buffer (pH 8.95), 0.01 M $MgCl_2$, and about 6.5×10^{-5} M polymer, enzyme was added in small portions during the course of hydrolysis to a final concentration of 10–20 μg/ml. The reaction mixture was incubated at 37° and hydrolysis was followed by changes in the absorption spectrum. (b) The incubation medium contained 0.03 M Tris buffer (pH 8.95), 0.01 M $MgCl_2$, about 3×10^{-3} M polymer, 100 μg/ml of enzyme, and was sampled two ways. One sample was subjected to paper chromatography with solvent 2, each of the chromatograms being washed with 5% trichloroacetic acid to remove salt which masked the appearance of nonhydrolyzed polymer under the dark ultraviolet lamp. The second portion provided aliquots which were suitably diluted for absorption measurements with respect to a control. The results obtained by both methods were in satisfactory agreement.

Temperature profiles and melting points, (T_m), were determined on a Unicam SP500 spectrophotometer fitted with a specially constructed thermostatic compartment through which circulated a water–glycerol mixture from a Hoeppler ultrathermostat. Temperatures were measured by means of a thermistor in a dummy cuvet. Changes in absorption as a function of temperature were usually measured at several wavelengths (either at λ_{max} or as given in the figures), several minutes being allowed to elapse for attainment of equilibrium at each temperature. No corrections were made for thermal expansion of the solutions.

Spectral titrations (see Figures 5 and 8) were carried out by two methods. (a) A solution of the polymer in 0.039 M NaH_2PO_4 in a spectrocuvet was titrated with small portions of 10 N, and subsequently, 1 N NaOH. The pH and absorption spectrum were recorded at regular pH intervals. The change in volume of the solution in going from pH 4.75 to 7.82 was only 1.5%, while the Na^+ concentration increased to 0.076 M. (b) A concentrated solution of polymer was diluted into a series of solutions containing 0.01 M buffer and 0.15 M NaCl at various pH values; in this case some of the spectra were taken on a Cary recording instrument.

Control of pH was by means of a Radiometer PHM22 meter fitted with a glass semimicro electrode.

Sedimentations constants were measured in the Beckman Model E ultracentrifuge. The polymer solutions were dissolved in 0.01 M phosphate buffer (pH 6.54) or Tris buffer (pH 8.8) which contained 10^{-3} M sodium versenate and 0.15 M NaCl.

Paper chromatography made use of Whatman No. 1 paper with solvents 1 (isobutyric acid–1 M NH_4OH–0.1 M EDTA, pH 8.2, 100:60:1.6, v/v) and 2 (isopropyl alcohol–concentrated NG_4OH–H_2O, 7:1:2, v/v) and Whatman DEAE paper with solvent 3 (0.4 M NH_4HCO_3 in 10^{-3} M sodium versenate, pH 8.5).

Polymer Syntheses. The 5′-triphosphates of 5MedC and its N^4-methyl and N^4-ethyl analogs (Kulikowski *et al.*, 1969) were freed from impurities by adsorption and elution from charcoal. Each of the substrates was prepared as a 1 mM solution in 0.01 N HCl and adsorbed on Baranby Cheney Grade SA charcoal (4 ml of charcoal for 0.1 mmole of substrate). The adsorbent was washed with water and the substrates were then eluted with ethanol–concentrated ammonia–H_2O (250:15:235, v/v). The eluates were concentrated under vacuum at 30° (yields about 75%). Paper chromatography with solvent 1 demonstrated the continued presence of about 25% impurities (Table I).

These partially purified substrates were utilized for small-scale polymerizations (about 0.5–1.0 μM) under the conditions described below for larger scale preparations. The course of polymerization was monitored by the following methods. (a) Aliquots of 50 μl were withdrawn and added to 2.5 ml of 0.05 M acetate buffer (pH 4.5) containing 0.5 M NaCl. The spectrum was then determined and the hypochromicity was measured. (b) Polymerization was carried out in the presence of inor-

TABLE II: Properties of Polymers.

Poly	$\epsilon_{260}^{P} \times 10^{-3}$	$\epsilon_{250}/\epsilon_{260}$	$\epsilon_{280}/\epsilon_{260}$	$S_{20,w}$ at pH 8.43 (S)	$S_{20,w}$ at pH 6.5 (S)
5MedC	4.70	1.03	1.33	4.58	7.58
N^4,5diMedC	6.47	0.96	1.08	4.55	4.44[a]
N^4Et,5MedC	7.71	0.98	1.13	4.33	4.14[b]

[a] At pH 4.2 this value was 5.06. [b] At pH 4.2 this was 4.40.

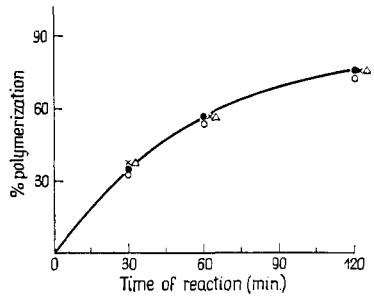


FIGURE 1: Course of polymerization, measured by release of inorganic phosphate, of (O—O) poly dC, (●—●) poly 5MedC, (×—×) poly N^4,5diMedC, and (△—△) poly N^4Et,5MedC (polymerization conditions as described in Experimental Section).

ganic pyrophosphatase and aliquots were withdrawn for measurement of liberated orthosphosphate by the method of Fiske and Subbarow (1925). (c) The incubation mixture, after 2-days incubation, was applied to a column of Sephadex G-50 equilibrated with 0.01 M cacodylate buffer (pH 7) containing 0.01 M NaCl and eluted with the same solvent. The column effluent was monitored continuously at 276 mμ, 4-ml fractions being collected at 5-min intervals.

For the larger scale syntheses, substrate was added during incubation in three equal portions, the totals being presented in Table I. The incubation mixtures contained: 0.2 M potassium cacodylate (pH 7.5), 1 mM mercaptoethanol, 1 mM $CoCl_2$, 1–3 mM substrate, $d(pT)_3$ (about 10 μM), terminal transferase (40 μg of protein/ml), and inorganic pyrophosphatase (50 μg/ml). Incubation was at 35° and the second and third portions of substrate were added after 2 and 10 hr, respectively. Aliquots were removed from the reaction mixture after 0.2- and 10-hr incubation for determinations of hypochromicity, and after 3 days for chromatography with solvents 1 and 2. The incubation was terminated after 3 days by addition of 1 mM sodium versenate and the mixture was heated for 10 min at 60°, followed by removal of the precipitate. The supernatant was then dialyzed against 0.01 M sodium versenate (pH 8.5) containing 0.2 M NaCl and finally against distilled water. The amount of final dialyzed product was estimated by ultraviolet absorption measurements and the dialyzed solutions were lyophilized.[2] The absence of monomers and short oligonucleotides was established by chromatographic methods. Phosphate analyses on the dialyzed preparations, performed by an Auto-Analyzer method, were used to estimate the molar extinctions of the polymers.

Results and Discussion

Synthesis of Polymers. The synthetic 5′-triphosphates of 5MedC, N^4,5diMedC, and N^4Et,5MedC (Kulikowski *et al.*, 1969) were utilized for polymerization with the aid of deoxynucleotidyl terminal transferase (Yoneda and Bollum, 1965; Kato *et al.*, 1967). Although the final purification step for these substrates was based on DEAE column chromatography, they were found to be nonhomogeneous on paper, and contaminated with ultraviolet-absorbing material corresponding to mono- and diphosphates and traces of inorganic polyphosphates. Since such contaminants may inhibit the terminal transferase enzyme, the substrate preparations were further purified by adsorption on charcoal. Even this treatment left about 25% of material other than the 5′-triphosphates (monophosphate, ~5%; diphosphate, ~15%; nonspecific absorbing impurities, <5%). However, as can be seen from Table I, this was without major effect on the final yields in the polymerization reactions, since more than 90% of the triphosphates were converted into polymer. Furthermore the R_F values and absorption spectra of both the mono- and pyrophosphates corresponded to those for 5-methyldeoxycytidine or its alkylamino analogs; and enzymatic hydrolysis of the isolated polymers (see below) showed the absence of foreign residues. Attention has been drawn to the foregoing because of the fact that commerical preparations of triphosphates frequently require such purification in order to obtain reasonable polymerization yields.

Polymerization conditions were first established in small-scale experiments. Since the above substrates are analogs of dCTP, the conditions employed were those for the preparation of poly dC (Bollum, 1966; Kato *et al.*, 1967), *i.e.*, in cacodylate buffer in the presence of Co^{2+} ions. Several methods were used for following the course of polymerization, with no essential difference from that observed for poly dC (*cf.* Figure 1).

Larger scale polymerizations gave yields of over 90% (Table I). From the ratio of 5′-triphosphate incorporated to the amount of initiator used, the chain lengths of the resulting polymer preparations were estimated to be 335, 355, and 300 for 5MedC, N^4,5diMedC, and N^4Et,5MedC, respectively. The corresponding sedimentation constants are given in Table II. The sedimentation boundaries were all sharp, testifying to the homogeneity of the preparations.

The ability of terminal transferase to polymerize analogs of natural nucleotides enhances both the utility and significance of this enzyme. Apart from its possible biological function, it is worth emphasizing the role of this enzyme as a tool for the preparation of synthetic polydeoxynucleotides. The remarkable progress achieved in the study of ribopolymers has been due largely to the availability of polynucleotide phosphorylase, which made possible the synthesis of numerous ribopolymer analogs (Michelson *et al.*, 1967; Felsenfeld and Miles, 1967). The first homopolydeoxynucleotides were obtained as a

[2] Isolation of the polymers by precipitation with 1 N perchloric acid or with alcohol was impractical because of appreciable losses due to solubility. Poly dC was readily precipitable under these conditions, whereas the ability of poly 5MedC, poly N^4,5-diMedC, and poly N^4Et,-5MedC to precipitate decreased in the order given.

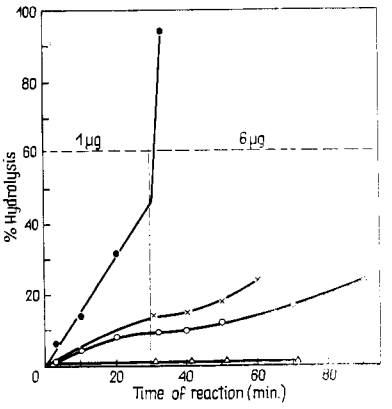


FIGURE 2: Course of hydrolysis by phosphodiesterase I of (●—●) poly dC, (○—○) poly 5MedC, (×—×) poly N^4,5diMedC, and (△—△) poly N^4Et,5MedC. Reaction conditions: 0.03 M Tris buffer (pH 8.95), 0.01 M $MgCl_2$, and 6.5×10^{-5} M polymer; at zero time, enzyme was added to a concentration of 1 μg/ml, and after 30 min to a concentration of 6 μg/ml; reaction was carried out in 10-mm spectrocuvets at 37° and followed by increase in optical density at absorption maximum.

result of the observation of some peculiarities in the mode of action of *E. coli* DNA polymerase, *e.g.*, synthesis without primer (Radding *et al.*, 1962) or on a ribopolymer matrix (Lee-Huang and Cavalieri, 1963, 1964). Both of these processes have major limitations. The calf thymus terminal transferase permits the synthesis of model polydeoxynucleotides analogous to those prepared in the ribose series.

Enzymatic Hydrolysis of Polymers. From Figure 2 it will be seen that poly 5MedC and its alkylamino analogs are much

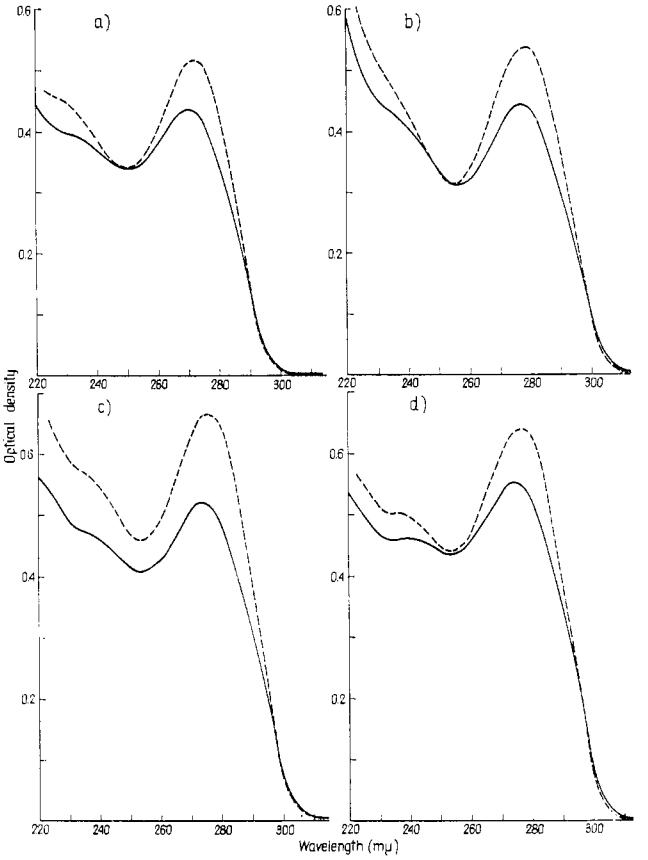


FIGURE 3: Absorption spectra in 0.03 M Tris buffer (pH 8.95) and 0.01 M $MgCl_2$ prior to (———) and following enzymatic hydrolysis to monomers (— — —) of (a) poly dC, (b) poly 5MedC, (c) poly N^4,5-diMedC, and (d) poly N^4Et,5MedC. Conditions of enzymatic hydrolysis as in the Experimental Section.

TABLE III: Total (following Enzymatic Hydrolysis to Monomers), Thermal (following Heating from 20 to 80°), and Residual (Difference between Total and Thermal) Hyperchromicities, Measured in all Instances at λ_{max}, of Neutral Single-Stranded Forms of Poly rC, Poly dC, and Some of Their Analogs.

Polymer	Total Hyperchromicity (%)	Thermal Hyperchromicity (%)	Residual Hyperchromicity (%)
Poly rC	40[a]	19,[a] 23[b]	20
Poly 5MerC	52[a]	18[a]	34
Poly N^4MerC		30[b]	
Poly N^4,N^4diMerC		35[b]	
Poly N^4,5diMerC	51[c]	15[c]	36
Poly dC	18	2, 1[d]	17
Poly 5MedC	21	5	16
Poly N^4,5diMedC	28	11	17
Poly N^4Et,5MedC	16	2	14

[a] Szer and Shugar (1966). [b] Brimacombe and Reese (1966). [c] Rabczenko and Szer (1967). [d] Ts'o *et al.* (1966).

less susceptible to phosphodiesterase I than poly dC. Complete hydrolysis of the former required larger concentrations of enzyme, the course of hydrolysis being followed by changes in hyperchromicity (Table III), and by paper chromatography, which revealed the appearance of only one nucleotide, under conditions (using 200-μg samples of polymer) where less than 1% impurity could be detected.

A similar resistance to phosphodiesterase hydrolysis is exhibited by the corresponding ribopolymers. Poly 5MerC (Rabczenko and Szer, 1967), the mono- and dimethylamino analogs of poly rC (Brimacombe and Reese, 1966), and the methylamino analogs of poly 5MerC (Rabczenko and Szer, 1967) are still appreciably more resistant than poly rC. All of these analogs of poly rC are also less susceptible to pancreatic ribonuclease. From observations on the phosphodiesterase I hydrolysis products of tRNA, Wenkstern (1966) has concluded that 5MerU residues are more resistant to hydrolysis than rU residues. It would clearly be of value to compare poly rT and poly rU, which can exist in both the random coil and structured forms. Poly dT and poly dU, the latter of which has recently become available by direct polymerization and by deamination of poly dC (Zmudzka *et al.*, 1968), which do not exhibit secondary structure, are also of interest.

Hyperchromicities of Neutral Forms of Polymers. The ultraviolet spectra of the various polymers and their monomer hydrolysis products are represented in Figure 3. The hyper-

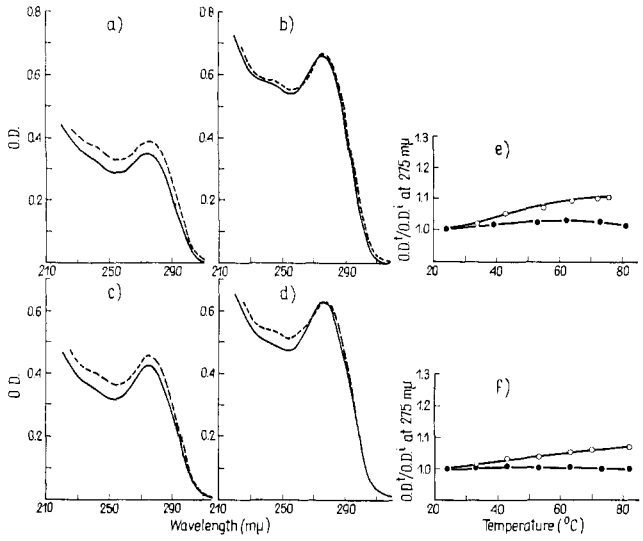


FIGURE 4: Influence of temperature on the spectra of (a) poly N^4,5diMedC and (b) poly N^4Et,5MedC in 0.02 M Tris buffer (pH 8.82) and 0.1 M Na^+ and (c, d) in 0.02 M acetate buffer (pH 5.0) and 0.4 M Na^+; (———) 24°; (— — —) 82°; and melting profiles of poly N^4,5diMedC (O—O) and poly N^4Et,5diMedC (●—●) at pH 8.82 (e) and pH 5.0 (f).

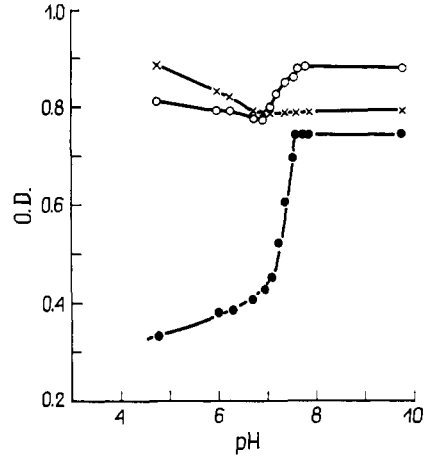


FIGURE 5: Absorbance–pH curves for poly 5MedC measured at 245 mμ (●—●), 276 mμ (O—O), and 282 mμ (×—×). See Experimental Section for details of measurements.

chromicity values at λ_{max} accompanying hydrolysis to monomers, along with the analogous values for the corresponding polyribonucleotides, are listed in Table III. In all instances the values for the deoxy polymers are lower than those for the ribose series, as noted earlier for other polymer pairs like rT and dT, and rI and dI. However, this correlation is not general; it does not apply to the pair rA and dA, for example. The lower over-all hyperchromicities of the deoxy, as compared with the ribo, polymers is suggestive of a lower degree of base stacking for the neutral forms of the polydeoxynucleotides (*cf.* Ts'o *et al.*, 1966).

The thermal hyperchromicities for the neutral forms of all of the foregoing, over the temperature range 20–80°, are also listed in Table III (*cf.* Figure 4). At first sight, the lower values for the polydeoxynucleotides, as compared with the ribo, polymers might indicate a greater resistance to "melting" of the former, in apparent contradiction with the statement made in the previous paragraph as to the lower degree of base stacking for the neutral forms of the deoxy polymers. This anomaly disappears, however, upon examination of the residual hyperchromicities (last column of Table III), which are a measure of the degree of base stacking resistant to melting out at elevated temperatures. It may be seen that these values are consistently higher for the polyribonucleotides.

Finally, the low degree of thermal hyperchromicity observed for the polydeoxynucleotides is noncooperative (illustrated for two polymers in Figure 4), as it is for the corresponding ribopolymers, and this is in agreement with the suggestion originally advanced by Fasman *et al.* (1964) that the neutral forms of these polymers are single stranded, the structure being due solely to base stacking.

Poly 5MedC in Acid Medium. It was anticipated at the outset that the behavior of poly 5MedC in acid medium might closely parallel that of acid poly dC reported on earlier by Inman (1964a; *cf.* Ts'o *et al.*, 1966), since it had previously been shown that the acid forms of poly rC and poly 5MerC bear a striking resemblance to each other (Szer and Shugar, 1966). On the other hand, while the acid form of poly dC is similar to the corresponding form of poly rC, there remain some differences which call for reflection before concluding that both do, in fact, possess identical structures. These differences may also be of interest in relation to the differences between RNA and DNA resulting from the presence of rC or dC residues. In order to facilitate a direct comparison with poly dC, experiments with poly 5MedC were carried out essentially under the conditions previously employed for the former (Inman, 1964a).

Evidence for an increase in ordered structure with decrease in pH was apparent from the observation that the sedimentation constant of poly 5MedC increased from 4.58 S at pH 8.4 to 7.6 S at pH 6.5 (Table II).

Subsequently, spectral titration at λ_{max} of a sample of poly 5MedC over the pH range 9.8–4.5 (Figure 5) demonstrated the existence of a sharp transition in the pH range 7.6–7.0. This transition was accompanied by a modification of the entire absorption spectrum, completely analogous to that undergone by poly dC under the same conditions (Inman, 1964a). The initial slow increase in absorption at about 245 mμ (Figure 5) is clearly due to partial deprotonation of the acid form just prior to its collapse, as observed for poly dC and in agreement with the potentiometric titration curve for this latter polymer (Inman, 1964a). The midpoint for the cooperative transition of poly 5MedC in Figure 5 is $pH_{1/2}$ 7.3. This is to be compared with a $pH_{1/2}$ of 7.4 reported for poly dC at approximately the same ionic strength (~0.05 M Na^+). This is far removed from the pK_a of 5MedCMP, about 4.7.

When the salt concentration was increased to an initial value of 0.2 M, the $pH_{1/2}$ for poly 5MedC decreased to 7.1, in qualitative agreement with the observation that the pK_a of cytidine decreases by 0.2 pH unit when the ionic strength is increased from 0.0 to 0.2 (Lewin and Humphreys, 1966). A more quantitative comparison would require the pK_a values for dCMP and 5MedCMP over a range of ionic strengths, but such data are as yet not available.

Additional evidence for the highly ordered structure of poly 5MedC in "acid" medium is seen in its melting profile (Figure

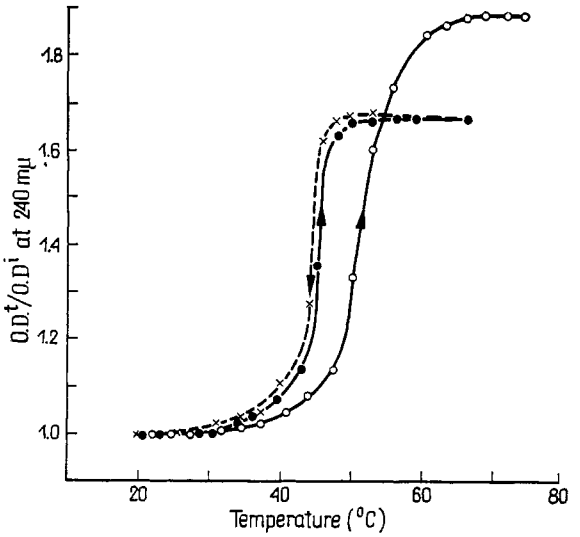


FIGURE 6: Melting profiles for poly 5MedC in 0.41 M Na^+ (pH 6.52). (●—●) Heating profile, (×—×) cooling profile, and (○—○) heating profile for poly dC in 0.42 M Na^+ (pH 6.57).

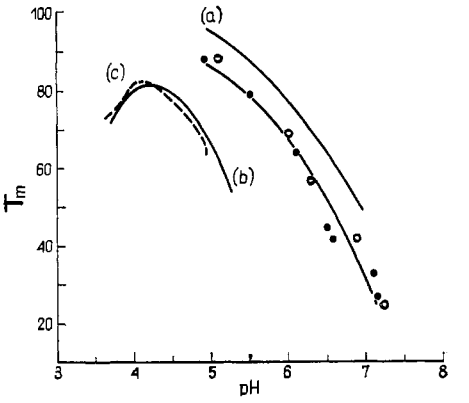


FIGURE 7: Influence of pH on T_m of acid forms of poly dC (○—○) and poly 5MedC (●—●) in 0.4 M Na^+ and of (a) poly dC in 0.1 M Na^+ (from Inman, 1964a), (b) poly rC in 0.13 M Na^+ (from Guschlbauer, 1967), and (c) poly rC in 0.1 M Na^+ (from Akinrimisi *et al.*, 1963). All solutions were appropriately buffered.

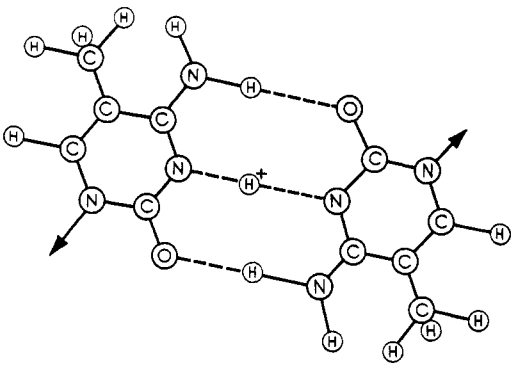
6), which is cooperative in character, fully reversible on running a cooling profile, and similar to that for poly dC shown in the same figure. The T_m for our poly dC preparation shown in Figure 6 is 51°, almost identical with that reported by Inman under the same conditions. It will be noted, however, that the T_m for poly 5MedC is 6° lower than that for poly dC, but it should be recalled that acid poly 5MerC exhibits a T_m about 3° below that for poly rC (Szer and Shugar, 1966). This point is referred to again, below.

The higher hyperchromicity of poly dC as compared with poly 5MedC (Figure 6) is more apparent than real and is dependent upon the wavelength at which measurements are made. When measurements are made at 270 mμ, the temperature hyperchromicities for both polymers are almost the same, about 19%; whereas at 220 mμ, the hyperchromicity of poly dC is 0 and that of poly 5MedC, 31%. These differences result from the complex spectral modifications accompanying the melting of the acid helical forms (in which some of the cytosine residues are protonated) to the neutral single-stranded forms. In more acid medium (pH ~ 6.5), where the melted forms also contain protonated cytosine residues, the spectral changes are even more complex (*cf.* Hartman and Rich, 1965; Guschlbauer, 1967).

Attention should be drawn to the fact that the melting profile for poly dC is appreciably broader than that for poly 5-MedC (Figure 6). This might be ascribed to the sedimentation constant of our poly dC, which is lower than that of Inman's (1964a). The latter author shows only one profile for poly dC, measured at 270 mμ, and melting over a temperature range of almost 20° at pH 6.9 in 0.41 M Na^+; the profile for our poly dC sample under these conditions extended over a range of 18°, and hence was certainly not inferior to that of Inman. It is also of interest, in this connection, that the melting profiles for poly 5MedC, at pH 6.52 (Figure 6) and at other pH values, exhibit a more cooperative character than those for poly dC.

The dependence of T_m upon pH, along with the corresponding values for dC taken from Inman (1964a), both presented graphically in Figure 7, are seen to be very similar. While there is tendency toward higher values of T_m for poly dC (as noted above, at pH 6.5), there is some scatter to the points suggesting that this difference is not consistent. This may be related to the breadth of some of the profiles, particularly for poly dC, which renders accurate estimates of T_m difficult. For this reason a single curve has been drawn through both sets of points at a concentration of Na^+ of 0.4 M.

The close similarities between poly dC and poly 5MedC imply that, as for the corresponding ribopolymers, the 5-methyl substituent does not contribute to the stabilization of the structure of the acid form. The absence of any stabilizing effect of the 5-methyl substituent, in going from acid poly rC to acid poly 5MerC, has been interpreted as due to maximal stacking of the nitrogenous bases in both of these polymers by the strong ionic bond between the N_3 ring nitrogens of two "complementary" cytosine residues, in addition to the two normal hydrogen bonds, as



It is clear that a similar situation prevails between poly dC and poly 5MedC. Reference should, however, be made to the following comparative analogies between poly rC (or 5MerC) and poly dC (or 5MedC). (a) Referring to Figure 7 it will be noted that, in the pH range between 5.25 and 4.90, where the T_m *vs.* pH curves for poly rC and poly dC (as well as poly 5-MedC) overlap, the T_m values for poly dC are about 25° higher than those for poly rC, *i.e.*, the converse of that normally encountered in a comparison of stabilities between corresponding ribo- and deoxyribopolynucleotide helices. This

apparent higher stability of poly dC (and poly 5MedC) as compared with poly rC (and presumably 5MerC) is likewise reflected in the higher $pH_{1/2}$ values at room temperature for poly dC (7.4; Inman, 1 964a)and poly 5MedC (7.3; see Figure 5) as compared with poly rC (about 5.6 in 0.1 M Na^+; Hartman and Rich, 1965, Akinrimisi *et al.* 1963).

(b) The potentiometric titration curves for poly rC (Hartman and Rich, 1965) and poly dC (Inman, 1964a) show that, *at room temperature*, half-protonation of the cytosine residues in each of these occurs only at values well below the $pH_{1/2}$ values, *viz.*, for poly rC at pH 4.7 (as *cf.* to $pH_{1/2}$ of 5.6), and for poly dC at pH 6.4 (as compared with $pH_{1/2}$ of 7.4). From Figure 7 the T_m of poly rC at pH 4.7 (obtained by extrapolation of curve b and with help of analogous data from Hartman and Rich, 1965) is about 74–75°. For poly dC at pH 6.4 the T_m (at the same ionic strength, 0.1 M Na^+) is 69°. We see, therefore, that under conditions where each cytosine base pair in poly rC and poly dC possesses *exactly* one proton, the thermal stabilities of both helices are similar; in fact the ribopolymer is somewhat more stable. We regard this as additional evidence for the identity of the structures of the helical forms of acid poly rC (or 5MerC) and poly dC (or 5MedC).

(c) From a comparison of the spectral and potentiometric titration curves for both poly rC and poly dC, it is also clear that formation of a twin-stranded helix occurs even when the average number of protons per cytosine base pair is *less* than one. In fact, at the $pH_{1/2}$ for both polymers, the average number of protons per cytosine base pair is only 0.50. As the number of protons per cytosine base pairs increases, the T_m increases, as might be expected. But, what appears surprising is that, when the average number of protons exceeds 1.0/cytosine pair, the T_m still continues to increase up to 1.16 protons/cytosine pair in poly dC (calculated from potentiometric titration curve of Inman, 1964a) and to 1.10 proton/cytosine pair in poly rC (data taken from Hartman and Rich, 1965). This apparent anomaly had been noted for poly dC by Inman (1964a), at a time when the potentiometric titration data for poly rC had not yet become available, and was regarded by him as inconsistent with the formulation of an analogous structure for the acid forms of poly rC and poly dC. This anomaly disappears, however, if we take cognizance of the fact that potentiometric measurements of proton uptake by the polymers were carried out at *room temperature*, while the spectral modifications accompanying heating lead to deprotonation (see above), as already noted by numerous observers. The deprotonation during the heating process must obviously be related to the decrease in pK_a of the cytosine residues with increase in temperature. And, in fact, it has also been demonstrated by Lewin and Humphreys (1966) that the pK_a (for protonation of the ring N_3 nitrogen) of cytidine, estimated both spectrally and potentiometrically, decreases by 0.26 pH unit between 20 and 50°.[3]

(d) The lower "stability" (or what we have referred to above as the apparent lower stability) of poly rC, as compared with poly dC, has been interpreted by Ts'o *et al.* (1966) in terms of hydrogen bonding of the 2'-hydroxyls of the former to the 2-carbonyl of the aglycon; and the evidence for such hydrogen bonding in ribonucleosides is extensively reviewed by these authors. According to their reasoning, such hydrogen bonding leads to a lower T_m for poly rC, as against poly dC, (a) by decreasing the pK of the cytosine residue and, (b) by preventing participation of the 2-carbonyl in the hydrogen-bonding scheme of poly rC. In the absence of further experimental data, we do not feel we have much to offer by discussing the foregoing postulates in detail. It seems to us that the experimental facts regarding the acid form of poly rA (Holcomb and Timasheff, 1968) and those for acid poly rC (and poly 5MerC) might have appeared perfectly normal if poly dC (and poly 5MedC) had not appeared on the scene. In other words, what *really* requires explanation is why what we have referred to throughout the above text as "acid" poly dC and "acid" poly 5MedC, in which up to half the bases are protonated, are readily formed at *slightly alkaline pH*. It is clear that this will require additional experimental and, perhaps, theoretical investigations for clarification.

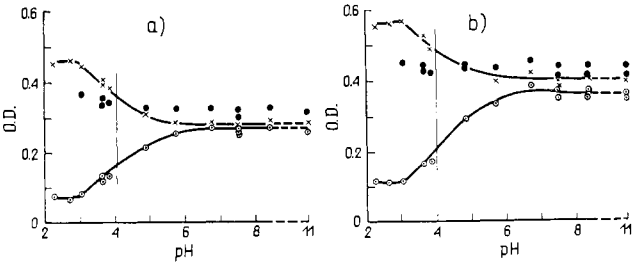


FIGURE 8: Absorbance–pH curves for (a) poly N^4,5diMedC and (b) poly N^4Et,5MedC measured at 245 mμ (○—○), 273 mμ (●—●), and 281 mμ (×—×). The vertical lines are located at the pK values for the corresponding monomers.

Acid Titration of Alkylamino Analogs of Poly 5MedC. Acid titration curves for poly N^4,5diMedC and poly N^4Et,5MedC down to about pH 2, and measured at two wavelengths for each polymer, are exhibited in Figure 8. It is clear that these curves bear no resemblance to that for poly 5MedC (Figure 5), and there is no indication whatever of the formation of an acid twin-stranded helix. The titration curves are, in fact, closely similar to those of the monomers themselves; and, in going from neutral to acid pH, the accompanying hypochromicity at 245 mμ and hyperchromicity at 273 (and 281) mμ point to normal protonation of the monomer residues. The behavior of these polymers is fully analogous to that of poly N^4MerC and poly N^4,N^4diMerC described by Brimacombe (1967), and neither of which forms an acid structure. From the titration curves in Figure 8, the apparent pK for protonation of the monomer residues of both the alkylamino deoxy polymers is 4.4. This value is fairly close to that for the monomers, again indicative of a low degree of interaction between base residues in the single-stranded forms. It should be recalled that the sedimentation constants for these polymers at acid and alkaline pH (Table II) point to the absence of a twin-stranded form in acid medium.

Melting profiles for both polymers were run at three pH values: 6.55, 5.01, and 3.05 (*cf.* Figure 4). At the two former values, both polymers show only slight hyperchromicity as at

[3] A somewhat analogous situation prevails for poly rA, which undergoes a transition at about pH 6 to form the so-called acid, twin-helical form, in which a proton on each of the ring N_1 nitrogens forms an electrostatic bond with a phosphate hydroxyl on the complementary chain. The titration curve from the neutral to the acid form is similar to that for poly C, and has only recently been studied in considerable detail by Holcomb and Timasheff (1968).

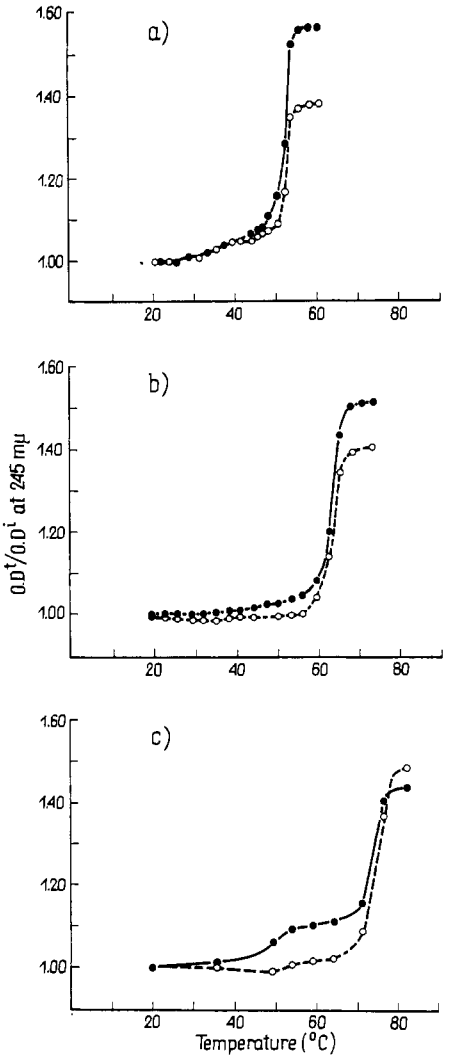


FIGURE 9: Absorbance–temperature curves (measured at 245 mμ) for mixtures of poly 5MedC and poly dI in molar ratios of 1:1 (●—●) and 1:2 (○—○) in 0.01 M phosphate buffer (pH 7.8) and Na^+ concentrations of (a) 0.03, (b) 0.15, and (c) 0.45 M.

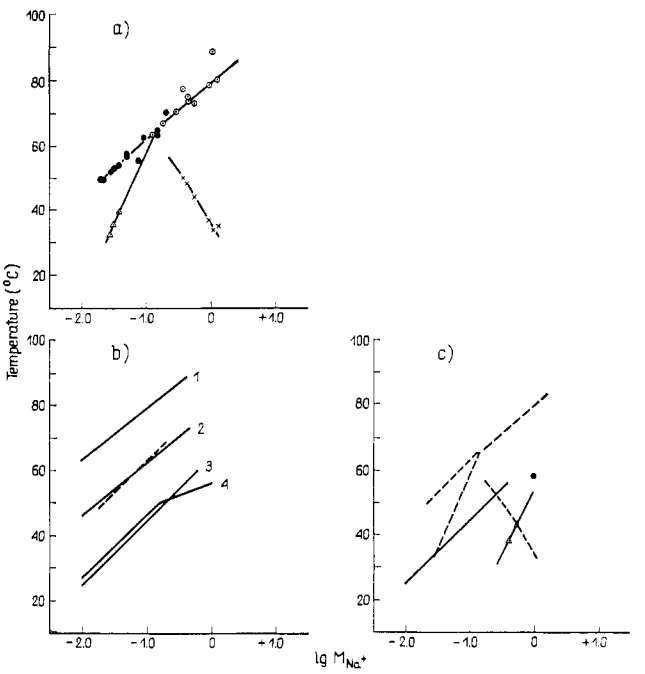


FIGURE 10: Effects of Na^+ concentration. (a) Influence of Na^+ concentration (including that due to buffer cation) at pH 7.8 (phospate buffer) on the T_m values of complexes between poly 5MedC and poly dI. (●—●) Melting of twin-stranded helix to homopolymers, (⊙—⊙) melting of triple-stranded helix to homopolymers, (△—△) melting of triple-stranded to twin-stranded complex, and (×—×) rearrangement of twin-stranded to triple-stranded helix. The T_m values were calculated from 5MedC:dI mixtures of 1:1 and 1:2. (b) Relative comparison of dependence of T_m upon Na^+ concentration for the twin-stranded helices 5MedC:dI (— — —) and (1) 5MerC:rI (Szer and Shugar, 1966), (2) rC:rI (Szer and Shugar, 1966), (3) dC:dI (Inman, 1964b), and (4) dC:dI (Chamberlin and Patterson, 1965). (c) Comparison of influence of Na^+ concentration on stability of twin- and triple-stranded complexes of poly dI with poly 5MedC (– – – –, values transferred from part) and of poly dI and poly dC (———, melting of twin-stranded complex to homopolymers); (△—△) melting of triple-stranded to twin-stranded complex; (●) melting of triple-stranded helix to homopolymers (Inman, 1964b).

slightly alkaline pH, suggestive of temperature-induced decrease in base stacking, and similar to the behavior of poly N^4MerC, poly N^4,N^4diMerC (Brimacombe, 1967), and poly N^4,5diMerC (Rabczenko and Szer, 1967).

By contrast, the melting profiles at pH 3.05, where virtually all the base residues are protonated at room temperature, exhibited up to 20% hyperchromicity at λ_{max} and 6–9% hypochromicity at λ_{min}. This is clear evidence for deprotonation at elevated temperatures.

Complexes of Poly 5MedC with Poly dI. The nature of these complexes was studied by comparing the temperature profiles of 1:1 and 2:1 mixtures of poly dI and the potentially complementary poly 5MedC at various salt concentrations and at a pH of about 7.8, *i.e.*, above the transition point for the acid form of poly 5MedC. From the results to be described it will be seen that, under most conditions, the complexes formed are not unique but are accompanied by rearrangements, observed under certain conditions with the complementary pair poly dI and poly dC (Inman, 1964b; Inman and Baldwin, 1964).

At low ionic strengths (0.02–0.03 M Na^+) both the 1:1 and 2:1 mixtures of dI with 5MedC exhibit sharp transition profiles with identical T_m values of about 50°. Since the 1:1 mixture has a higher temperature hyperchromicity than the 2:1 (Figure 9a), it appears reasonable to assume that the profiles for both mixtures correspond to the melting of a twin-stranded helix.

It should, on the other hand, be noted that the 2:1 mixture, at these low salt concentrations, displays a minor transition in the temperature range 30–40°, which probably corresponds to the melting of a triple-stranded helix to give the more stable twin strand. However, the extent of formation of the triple-stranded complex cannot be appreciable; if maximal formation of a triple strand had occurred, then the subsequent thermal rearrangement to a twin strand should have been accompanied by an increase in optical density of 18%, whereas the observed increase is only 3–5%.

Furthermore the 2:1 mixture exhibits a profile with a temperature hyperchromicity of 27–30%. On the assumption that such a mixture contains a 1:1 complex, plus a 1 molar excess of poly dI, the calculated hyperchromicity is 26%, thus pro-

viding additional evidence for the 2 → 1 nature of the observed melting profile.

As the ionic strength is increased, a 1:1 mixture continues to exhibit the melting characteristics of a twin strand. By contrast, a 2:1 mixture exhibits overlapping profiles of 3 → 2 (triple strand to twin strand) and 2 → 1 (twin-strand to single strands) transitions. This is due to the fact that the T_m for the 3 → 2 transition increases more rapidly with ionic strength than that for the 2 → 1. At moderate salt concentrations the stabilities of the triple- and twin-stranded complexes are similar and they melt over the same temperature range. This is illustrated graphically in Figure 10, and the sharp transition profiles seen in Figure 9b do not necessarily represent the melting of one type of complex in a given mixture, nor that we are dealing with the identical complex in mixtures of 1:1 and 2:1. On the contrary, whereas the hyperchromicity of the 1:1 mixture testifies to the melting only of a twin-stranded complex, that of a 2:1 mixture points to the presence, and melting, of a mixture of twin and triple strands.

At higher ionic strengths (>0.2 M Na^+) both 1:1 and 2:1 mixtures exhibit cooperative melting with a T_m above 70°, but the hyperchromicity is greater for the 2:1 mixture and is also higher than that for the same mixture at lower ionic strengths (where the evidence supports the formation largely of a twin-stranded helix). These data point to the formation at higher ionic strengths of a triple-stranded complex which melts out directly to its single-stranded components.

The melting of a 1:1 mixture proceeds in two steps, the second of which (with a $T_m \sim 70°$) coincides with the melting of the triple-stranded complex in a 2:1 mixture. The first step, which occurs in the temperature range 30–55°, differs from the previously described transitions (2 → 1, 3 → 1, 3 → 2) in that the T_m of the melting profile *decreases* with increased ionic strength (Figures 9c and 10a). This suggests that, as in the case of the complex of poly rU with poly rA (Stevens and Felsenfeld, 1964; Blake *et al.*, 1967), the transition involves the rearrangement of a twin-stranded complex to a triple strand. And, in fact, if we assume that the initial twin-stranded complex is transformed to a triple strand plus a free excess of poly 5MedC, then the resulting calculated hyperchromicity of this transformation should be 10%, which is identical with that observed experimentally (Figure 9c).[4]

It may be concluded from the foregoing that, in dilute salt solution, irrespective of the ratio of the components, poly dI and poly 5MedC combine to form mainly a twin strand. At higher salt concentrations (>0.2 M) the triple strand is more stable and is the only one formed in a 2:1 mixture; whereas in a 1:1 mixture there is initial formation of the twin strand which, on warming, rearranges to form the more stable triple strand. And, finally, irrespective of the salt concentration, it is the triple strand which eventually melts out to the component homopolymers.

Hyperchromicity Values. The foregoing experiments demonstrated appreciable variations in temperature hyperchromicity, resulting from the complex nature of the transitions. A perusal of the over-all results suggested that the transitions 2 → 1 and 3 → 1 are best represented, respectively, by a 1:1 mixture in 0.032 M Na^+ (Figure 9a) and a 2:1 mixture in 0.457 M Na^+ (Figure 9c). The respective hyperchromicities for these transitions are 56 and 50%; the lower hyperchromicity observed in, *e.g.*, 0.15 M Na^+ (Figure 9c) is most likely due to the presence in the starting mixture of both twin- and triple-stranded helices, with a resultant decrease in degree of ordered structure, and a consequent decreased hyperchromicity on melting.

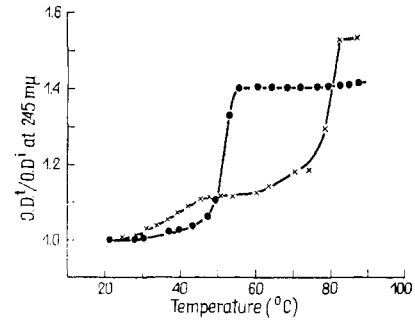


FIGURE 11: Absorbance–temperature curves (measured at 254 mμ) for 1:1 mixtures of poly 5MedC and poly dI in 0.95 M Na^+ (×—×) and of poly dC and poly dI in 1.23 M Na^+ (●—●) both in phosphate buffer (pH 7.8).

The influence of salt concentration on all four types of transitions of the complexes of poly dI and poly 5MedC are exhibited graphically in Figure 10 which, in some respects, is similar to a phase diagram that divides the plane into four regions in each of which, according to the salt concentration and temperature, there exists a given complex or polymer. A similar diagram has been elaborated for poly rA, poly rU, and their various complexes (Blake *et al.*, 1967).

Influence of a Pyrimidine 5-Methyl Substituent on a Twin-Stranded Complex. Figure 10b exhibits graphically the values of T_m for the twin-stranded dI:5MedC compared with those for dI:dC and the analogous rI:5MerC and rI:rC. The identical slopes for all the curves is in agreement with the twin-stranded nature of all the complexes exhibited. Note that the T_m for dI:5MedC is 17° higher than that for dI:dC, whereas the corresponding value for rI:5MerC is 16° above that for rI:rC. It is clear that the presence of a 5-methyl substituent influences in the same way the interaction between the complementary cytosine and hypoxanthine bases in twin-stranded ribo or deoxyribo helices, notwithstanding the differences between these two which are reflected in the lower stability of the deoxyribo polymers (Figure 10).

Influence of a 5-Methyl Substituent on a Triple-Stranded Helix. The stabilizing effect of a cytosine 5-methyl substituent on the complexes of poly rI and poly rC previously reported (Szer and Shugar, 1966) referred exclusively to the twin-stranded helix, since the range of salt concentrations employed (0.01–0.3 M) was such that in neutral medium, poly rI does not form a triple strand either with poly rC or poly 5-MerC. By contrast, poly dI gives rise to triple-stranded helices such as dI:dI:dC (Inman, 1964b), dI:dI:rC (Chamberlin and Patterson, 1965), and dI:dI:dBrC (Inman, 1964b), as established by means of mixing curves, as well as by the disappearance of free dI with the simultaneous appearance of a new complex which could be distinguished from a twin strand by its melting behavior.

[4] It should be emphasized that at the pH of these experiments, which is beyond the range of the acid form of poly 5MedC, the T_m values for the ordered form of poly dI are far removed from the T_m's for the transition 2 → 3 and, furthermore, *increase* with increasing ionic strength (Inman, 1964a).

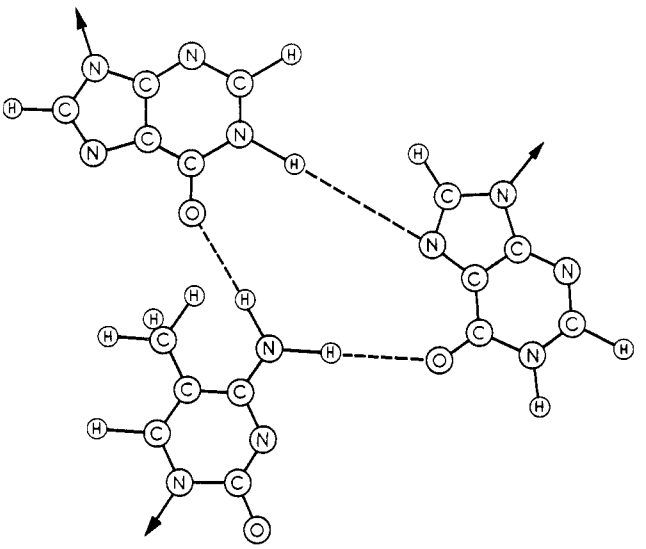
The addition of the third, dI, strand to dI:dC or dI:rC only at relatively high salt concentrations is undoubtedly due, at least in part, to the necessity of suitable shielding of the negative phosphate charges by the counterions. At such high ionic strengths, the study of these triple-stranded complexes was of necessity limited to a narrow range of salt concentrations. More extensive data was obtained with triple-stranded complexes involving 5BrdC, which are more stable than those with dC. An even better model system is that involving 5MedC, the triple-stranded complexes of which with dI are even more stable than those with 5BrdC.

It was obviously of special interest to evaluate the stabilizing effect, if any, of a cytosine 5-methyl substituent on the binding of the third, dI, strand as compared with the effect on a twin strand. The appropriate data are presented in Figure 10c along with those of Inman (1964b) for dI:dI:dC and show that, for the transition from a triple to a twin strand (3 → 2), the difference in T_m values between 5MedC and dC is much larger than for the melting of a twin-stranded complex (2 → 1).[5] In line with the foregoing is the observed transformation of dI:5MedC to a triple-stranded helix, with the release of excess poly 5MedC, on raising the temperature at elevated salt concentrations. This type of dismutation reaction was not observed for dC either by Inman (1964b) or in the present investigation (Figure 11). In general, then, the stability of the triple-stranded complex, measured in terms of the T_m of the transition 3 → 1, is greater for poly 5MedC as compared with poly dC than in the case of the transition 2 → 1. It may be concluded that the 5-methyl substituent exerts a more pronounced effect on the binding of the second dI strand, to form a triple-stranded helix, than it does on the first strand to form the twin-stranded helix.

A direct comparison between the stabilizing effects of a cytosine 5-bromo, as compared with a 5-methyl, substituent cannot readily be made because of the profoundly different effects of the two substituents on the electron distribution and polarizability of the pyrimidine ring, as well as on the pK_a for protonation of the ring N_3 nitrogen which is involved in base pairing to the hypoxanthine residues. It should, nonetheless, be noted that, whereas a 5-bromo exerts a greater stabilizing effect than a 5-methyl on a twin-stranded helix, the influence of a 5-methyl is more marked in the case of the triple-stranded form.

Nature of Triple-Stranded Helix of Poly I and Poly C. There is no direct evidence as to the manner in which a second chain of poly dI attaches to an existing dI:dC twin strand. Chamberlin (1965) has proposed a hydrogen-bonding scheme in which the original dI:dC pair is left intact and the second dI chain adds in such a manner that the C_6 carbonyl of the incoming dI residue bonds to the second amino hydrogen of the dC residue, and the N_1 of the incoming dI residue to the C_6 carbonyl of the original dI residue. An examination of a model of this base-pairing scheme shows, however, that while the orientation of the glycosidic bonds is acceptable, the angles of the hydrogen bonds from the two dC amino hydrogens are rather unorthodox. Another conceivable hydrogen-bonding scheme, more in line with the lower stability of the triple-stranded, compared with the twin-stranded, helix is

[5] This difference can only be evaluated qualitatively, because of the lack of adequate data for dC.



Complex Formation between Poly dI and Alkylamino Poly 5MedC. Possible complex formation between poly dI, on the one hand, and poly N^4, 5diMedC and poly N^4Et,5MedC, on the other, was sought for by examining 1:1 mixtures of both of these. In neither instance was there any evidence of hypochromicity following mixing. Furthermore no heating profile characteristic of a helical complex could be found.

The failure of the 5-methyl-4-alkylamino polymer analogs to form a complementary helix with poly dI is probably understandable in view of the effect of the 5-methyl substituent on the rotation of the alkylamino group. Complex formation would be expected only when the amino hydrogen is so directed as to hydrogen bond to the 4-keto of the hypoxanthine residue in poly I. In the absence of the 5-methyl substituent, there is relatively little hindrance to such rotation above 5° (Martin and Reese, 1967).

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The Reaction of Nucleic Acid Components with *m*-Chloroperoxybenzoic Acid*

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ABSTRACT: We have studied the kinetics of the reactions of the commonly occurring nucleic acid bases, nucleosides, and nucleotides with *m*-chloroperoxybenzoic acid as a function of pH. The pH–rate profiles are bell-shaped curves with maxima which fall into one of two groups: cytosine, adenine, and their derivatives show maxima on the acid side of the pK_a of the peroxy acid, while uracil, thymine, guanosine, and their derivatives show maxima on the alkaline side. The products from the first group are *N*-oxides, while the second group gives ring-cleavage products. Substituent effects allow us to characterize the predominant mechanism for *N*-oxide formation from cytosine as nucleophilic attack of the peroxy acid anion on the cationic substrate, while *N*-oxide formation from adenine occurs *via* electrophilic attack of the un-ionized peroxy acid on the neutral substrate. The initial attack leading to ring cleavage of the uracil group occurs *via* nucleophilic attack of the peroxy acid anion at the 5,6-double bond of the neutral substrate.

The reactions of peroxides with nucleic acids and their components have been studied previously in a number of laboratories. Peroxycarboxylic acids have been used to convert cytosine, adenine, guanine, and some of their derivatives into *N*-oxides (Cramer and Seidel, 1963; Delia *et al.*, 1965; Seidel, 1967; Cramer *et al.*, 1963; Delia and Brown, 1966); hydrogen peroxide in acetic acid has also been used for the synthesis of adenine 1-*N*-oxide and some derivatives (Stevens *et al.*, 1958, 1959; McCormick, 1966; Sigel and Brintzinger, 1965). In addition to *N*-oxide formation, however, a number of other reactions of peroxides with nucleic acid components have been observed, and these include addition of hydrogen peroxide to the 5,6-double bond of the pyrimidines (see Rhaese *et al.*, 1968), pyrimidine ring cleavage in alkaline solutions of hydrogen peroxide (Priess and Zillig, 1965), and both cleavage of the *N*-glycosidic bond and breakage of the sugar–phosphate backbone (Rhaese and Freese, 1968).

Peroxides are capable of reacting in a number of fundamentally different ways (Behrman and Edwards, 1967). They may undergo polar reactions in which the peroxide serves either as nucleophile or electrophile, or, as is usually assumed, but fre-

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