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Poly(5-chlorocytidylic acid)†

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ABSTRACT: 5-Chlorocytidine diphosphate has been obtained in high yield by the chlorination of cytidine diphosphate using tetrabutylammonium iodotetrachloride. 5-Chlorocytidine diphosphate has been polymerized with polynucleotide phosphorylase and the resultant poly(5-chlorocytidylic acid) has been characterized. Poly(5-chlorocytidylic acid), which is more stable toward hydrolysis by pancreatic ribonuclease than the unchlorinated polynucleotide, possesses considerable secondary structure in acid solution and forms a 1:1 hybrid with poly(I). The thermal stability of the hybrid is considerably higher than that of poly(I)·poly(C).

The discovery that double-stranded polyribonucleotides *e.g.*, poly(I)·poly(C) can induce the formation of interferon (Field *et al.*, 1967; Colby, 1971) has encouraged the synthesis of polyribonucleotides which have been modified in the base (Michelson and Monny, 1967), the sugar (Hobbs *et al.*, 1971), and the phosphate backbone (Eckstein, 1970). Little systematic work has, however, been carried out on the relationship between structure and biological activity in these compounds.

Chemical modification of the pyrimidine bases in the polyribonucleotides can be readily accomplished as electrophilic attack occurs at the 5 position in pyrimidines and, for example, halogenation of pyrimidine nucleotides gives the 5-halogenonucleotides. Conversion of the latter to the pyrophosphates followed by polymerization with polynucleotide phosphorylase leads to 5-halogenopyrimidine polyribonucleotides (Michelson and Monny, 1967). The direct bromination of poly(C) to poly(5BrC) has been reported (Means and Fraenkel-Conrat, 1971) and the latter has also been prepared by the polymerization of 5BrCDP (Howard *et al.*, 1969). Introduction of a halogen atom in the 5 position of a pyrimidine has a pronounced effect on the physical properties of the polynucleotide and, for example, poly(5BrC) and poly(5IodoC) possess considerable secondary structure in acid solution (Michelson and Monny, 1967). 5-Substituted cytidine polyribonucleotides form stable hybrids with poly(I) (Ross *et al.*, 1971) and these are inducers of interferon (De Clercq *et al.*, 1970; Colby and Chamberlin, 1969). In the present investigation the hitherto unknown poly(5ClC) has been prepared and the physical properties of the polynucleotide and its complex with poly(I) have been investigated. Biological studies on the poly(5ClC)·poly(I) complex are in progress.

Materials and Methods

Materials. CDP was synthesized from the phosphoromorpholidate (Moffatt and Khorana, 1961) and was converted by ion-exchange chromatography into the trisodium salt before use. UDP, pancreatic ribonuclease type IA (EC 2.7.7.-16), and *Crotalus adamanteus* venom were purchased from Sigma Chemical Corp. Polynucleotide phosphorylase (poly-

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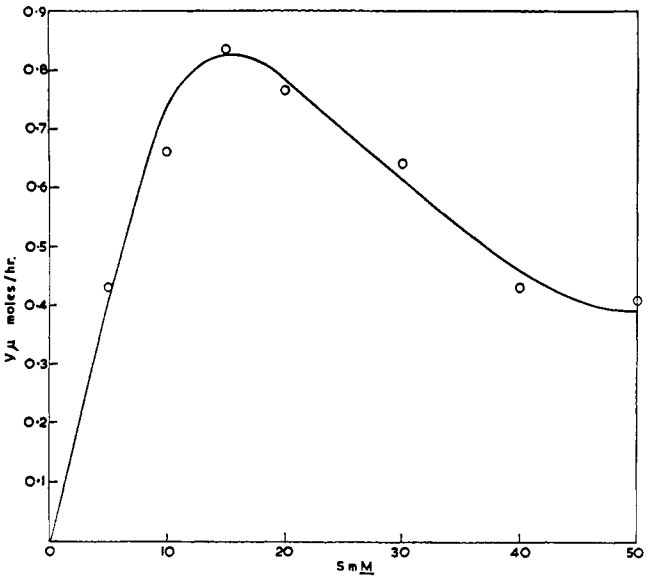

FIGURE 1: Variation of rate of polymerization with substrate concentration in the polymerization of [^{14}C]ClCDP by polynucleotide phosphorylase. The reaction mixture contained 5–50 mM[^{14}C] ClCDP, 5–50 mM $MgCl_2$, 2.5–25 mM EDTA, 0.15 M Tris (pH, 9), and 0.75 mg/ml of enzyme. Incubation was at 37°, and aliquots were removed and assayed as described in Materials and Methods.

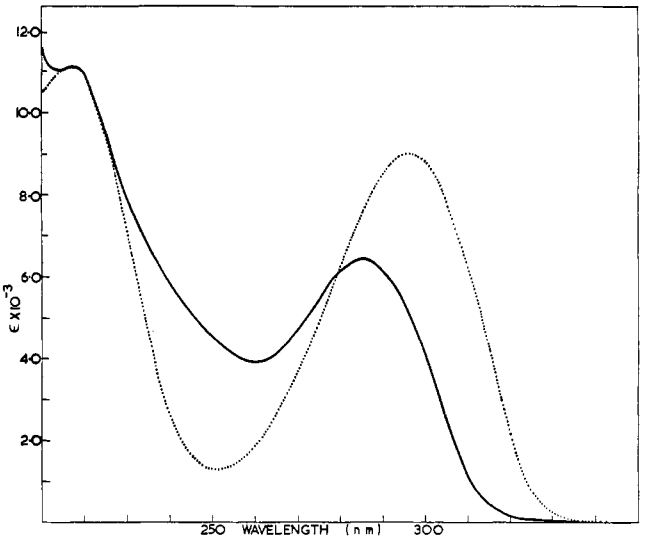

FIGURE 2: Ultraviolet spectra of ClCDP in 0.1 M NaCl–0.05 M sodium cacodylate at 25°: pH 7.0 (—), pH 1.0 (---).

ribonucleotide orthophosphate:nucleotidyl transferase, EC 2.7.7.8) from *Micrococcus luteus* was purchased from Boehringer Corp. Polyinosinic acid was purchased from P-L Biochemicals Inc. Tetrabutylammonium iodotetrachloride was prepared from the corresponding iodide (Popov and Buckles, 1957) and stored in the dark at 0°.

^{14}C-Labeled CDP was purchased from the Radiochemical Centre, Amersham. The ^{14}C content of samples was measured using a toluene-based scintillation medium containing 2,5-diphenyloxazole (5 g/l.) and 1,4-di[2-(4-methyl-5-phenyloxazoylyl)]benzene (0.2 g/l.).

Circular dichroic spectra were recorded by Dr. P. M. Scopes, Westfield College, London, on a Russel-Jouan Dicrograph 185 and the values obtained are expressed in terms of molecular ellipticity θ (Green and Mahler, 1970). Ultraviolet spectra were recorded on a Cary 14 spectrophotometer. Molar extinction coefficients of polynucleotides are based on the molecular weights of the monomers.

5-Chlorocytidine 5'-Diphosphate (ClCDP). To a solution of CDP trisodium salt (100 mg) in dry formamide (3 ml) was rapidly added a solution of tetrabutylammonium iodotetrachloride (300 mg) in dry dimethylformamide (1 ml). The reaction mixture was stirred magnetically in a well-stoppered reaction vessel until all solid has dissolved and then left 36 hr in subdued light. The solution was poured into water (50 ml) which was then extracted with chloroform (three 50-ml portions). The aqueous layer was applied to a Whatman DE-23 cellulose column (1 × 20 cm, HCO_3^- form) and the column eluted with a linear gradient of triethylammonium bicarbonate, ClCDP being eluted at a concentration of 0.15 M. The triethylammonium bicarbonate was removed by repeated evaporation *in vacuo*, and the residue was dissolved in water and passed through a Dowex 50 column (Na^+ form). The eluate was evaporated to an oil *in vacuo* below 40°, lyophilization of which gave colorless trisodium 5-chlorocytidine 5'-diphosphate (92 mg): ultraviolet (uv) spectra (0.01 M NH_4-OAc, pH 7.0) λ_{max} 286.5 nm (ϵ 6460). 5-Chlorocytidine has a λ_{max} of 287 nm (Fukuhara and Visser, 1955). The pK_a of ClCDP determined spectrophotometrically in citrate–HCl buffer was 2.49 ± 0.05 at 18° and the variation in absorbance of ClCDP with pH is shown in Figure 2. *Anal.* Calcd for $C_9H_{11}ClN_3Na_3O_{10}P_2 \cdot 2H_2O$: C, 20.03; H, 2.80; Cl, 6.57; N, 7.78; P, 11.48; Found: C, 20.22; H, 3.45; Cl, 6.85; N, 7.71; P, 11.47. The nuclear magnetic resonance (nmr) (100 MHz, D_2O) showed 8.06 ppm (1 H s) *inter alia.* ^{14}C-Labeled ClCDP was prepared in an identical manner from [^{14}C]CDP.

5-Chlorouridine 5'-Diphosphate. ClUDP was prepared as the tripotassium salt in an analogous manner from UDP in 94% yield: uv spectra (H_2O, pH 7.0) λ_{max} 277 nm (ϵ 8300), lit. (Massoulié *et al.*, 1966) λ_{max} (H_2O, pH 7.0) 276 nm; nmr spectra (100 MHz, D_2O) 8.09 ppm (1 H s) *inter alia. Anal.* Calcd for $C_9H_{10}ClK_3N_2O_{11}P_2$: C, 19.55; Cl, 6.41; H, 1.82; N, 5.07; P, 11.20,; Found: C, 19.75; H, 2.01; Cl, 6.23; N, 4.93; P, 10.92.

Dephosphorylation of ClCDP. [^{14}C]ClCDP (10 μmoles) in 0.1 M Tris-acetate buffer (0.1 ml, pH 8.0) was incubated with *Crotalus adamanteus* venom (100 μg) for 12 hr at 37°. Examination of the reaction mixture by paper chromatography (Howard *et al.*, 1969) showed only one radioactive compound with the same R_F as authentic 5-chlorocytidine.

Synthesis of Poly(5-chlorocytidylic acid) (Poly(ClC)). The polymerization of ClCDP by polynucleotide phosphorylase was followed both by release of inorganic phosphate (Fiske and Subbarow, 1925) and by incorporation of ^{14}C into polymeric material over a range of substrate concentrations to determine the optimum conditions for polymerization. In a typical case, the reaction medium which contained 20 mM ClCDP, 1.24 mg/ml of polynucleotide phosphorylase, 10 mM $MgCl_2$, and 5 mM EDTA in 0.15 M Tris-chloride buffer (pH 9.0) was incubated for 10 hr at 37°. After deproteinization by repeated extraction with chloroform–isoamyl alcohol (5:2, v/v) (Scheit and Gaertner, 1969), the aqueous phase was desalted by dialysis over 36 hr at 5° against 0.1 M NaCl–0.001 M NaEDTA, 0.001 M NaEDTA, and then twice against water. Lyophilization at 0° gave poly(ClC) in 53% yield (62% by release of phosphate).

Polymerization reactions with ^{14}C-labeled ClCDP (2 × 10^4 dpm/μmole) were monitored as follows. Aliquots were removed at different times applied to strips of Whatman No.

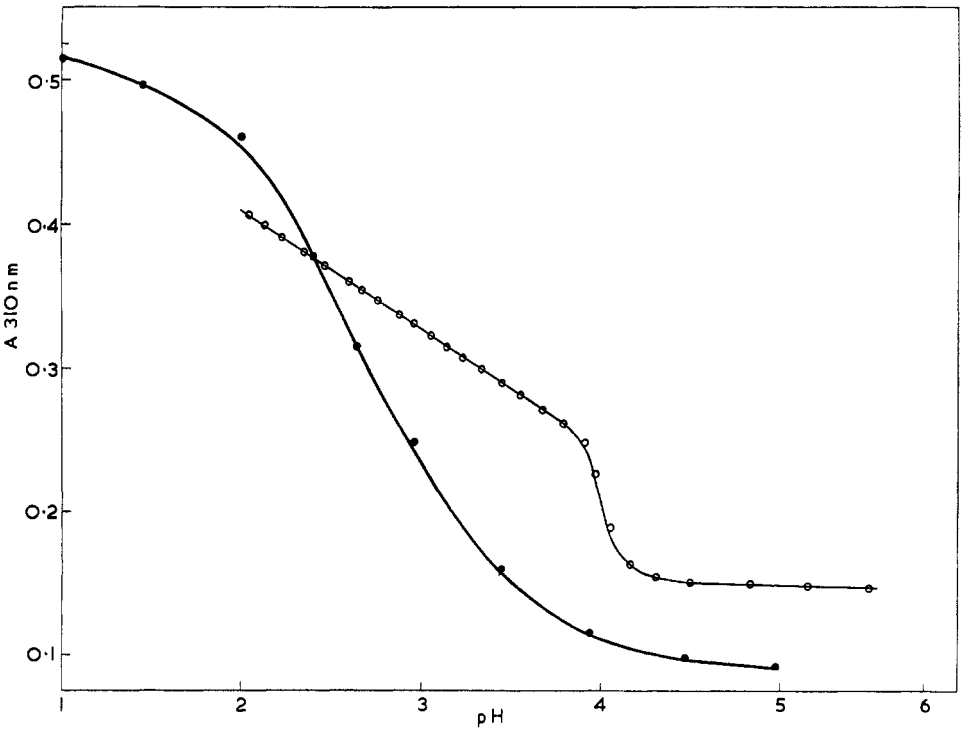

FIGURE 3: Spectrophotometric titration at 310 nm of 1.85×10^{-4} M solution of poly(ClC) (○) and ClCDP (●) in 0.3 M NaCl at 25°.

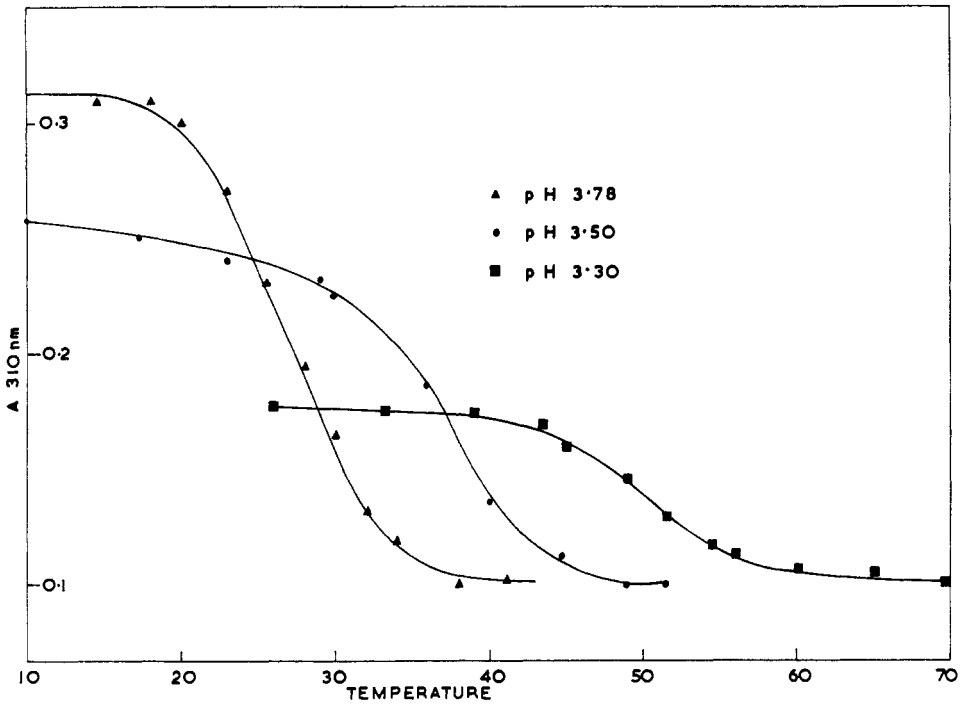

FIGURE 4: Temperature–ultraviolet absorption profiles at 310 nm of poly(ClC) at different pH values. Concentration of polymer, 1.35×10^{-4} M; buffer 0.1 M NaCl–0.05 M sodium acetate.

3MM paper and the reaction was stopped by the addition of a little acetone. The papers were developed in 0.5 M ammonium acetate in EtOH–H_2O (1:1, v/v). After drying, appropriate sections of the paper were excised and their radioactivity was measured. The polymerization data are summarized in Figure 1 and from this K_m and V_{max} can be estimated to be approximately 5.0 mM and 0.95 μmole/hr, respectively.

Characterization of Poly(ClC). Poly(ClC) has a $s_{20,w}$ of 4.20 S determined by ultracentrifugation in an isokinetic gradient of sucrose containing 0.1 M sodium acetate (pH 7.5). The uv maximum of poly(ClC) at 25° in 0.01 M ammonium acetate (pH 7.0) was 286 nm (ϵ 5050 ± 50), heating for 10 min at 95° caused a hypochromic change in absorption of 11.5% ($(A_{95°} - A_{25°})/A_{95°}$). Pancreatic RNase digestion of poly(ClC) gave a hypochromicity of 30% ($(A_{monomer}$ –

$A_{polymer})/A_{monomer}$) which agrees well with the value 27.5% calculated using A_{ClCDP} in place of $A_{monomer}$. The ultraviolet absorption spectra of poly(ClC) in acid and in neutral solution are shown in Figure 2. Spectrophotometric titration at 310 nm of poly(ClC) in 0.3 M NaCl solution showed an abrupt transition in the region of pH 4 (Figure 3) and the T_m of the polymer was also dependent on pH in this region (Figure 4).

Hydrolysis of Poly(ClC) by Pancreatic RNase. The polymer (0.2 μM) in 0.1 M ammonium acetate (pH 7.0) (2 ml) was treated with 0.5 μg of RNase (Massoulié *et al.*, 1966). Under these conditions poly(C) has $t_{1/2} \approx 5$ sec, poly(ClC) has $t_{1/2} =$ 50 sec, and poly(BrC) (Howard *et al.*, 1969) has $t_{1/2} = 80$ sec determined spectrophotometrically at room temperature.

Preparation and Properties of a Poly(I)·Poly(ClC) Hybrid. Equimolar quantities of poly(I) ($s_{20,w} = 6.64$ S) and poly(ClC) were dissolved in 0.01 M sodium acetate (pH 7.0) at 37° to give a 1:1 hybrid. The solution was left at room temperature for 2 hr and then applied to a Sepharose 4B 200 column. Elution of the column with acetate buffer gave double-stranded poly(I)·poly(ClC) in the void volume followed by a small amount of unannealed material. The ultraviolet spectrum in 0.01 M sodium acetate (pH 7.0) is shown in Figure 5 and the polymer has a λ_{max} of 280 (ϵ 6600) and 245nm (9500).

The stoichiometry of the hybridization was determined by the method of continuous variations (Job, 1928) in 0.1 M NaCl–0.005 M sodium cacodylate (pH 7.0) at 25° (Figure 6). At 245 nm there is a break in the curve at 50% corresponding to the formation of a 1:1 hybrid, at 286 nm no break can be observed. The melting profiles of poly(I)·poly(ClC) are summarized in Figure 7, the T_m's in 0.01, 0.1, and 0.3 M Na^+ are 65, 75, and 82°, respectively. Poly(I)·poly(BrC) has T_m values of approximately 65, 83, and 88° under the same conditions (Howard *et al.*, 1969).

The circular dichroic spectra of poly(ClC) and its 1:1 hybrid with poly(I) are shown in Figure 8. The polymers were dissolved in 0.1 M sodium chloride–0.005 M sodium cacodylate (pH 7).

Discussion

Cytidine nucleosides have been chlorinated in the 5 position of the pyrimidine ring using *N*-chlorosuccinimide (British Patent, 1969) or chlorine and ultraviolet irradiation (Fukuhara and Visser, 1955; Frisch and Visser, 1959). The yields of chlorinated nucleoside are only moderate and we have found that when these methods are applied to the chlorination of CDP, decomposition of the pyrophosphate residue occurs. Tetrabutylammonium iodotetrachloride has been used to chlorinate olefins and phenols under mild conditions (Buckles and Knaack, 1960), and we have found that this reagent will chlorinate CDP and UDP in virtually quantitative yield. An advantage of this method is the ease with which excess iodotetrachloride can be removed from the reaction mixture by extraction with chloroform. Dephosphorylation of ^{14}C-labeled ClCDP by *Crotalus adamanteus* venom indicated that chlorination of the pyrimidine was complete. The only radioactive material which could be detected by paper chromatography was 5-chlorocytidine. The position of chlorination in the cytidine ring was confirmed by ^1H nmr spectroscopy, as the signal due to H_5 which appears as a doublet at 6.05 ppm in CDP was absent. The signal due to H_6 in ClCDP appeared as a singlet at 8.06 ppm. Similarly, in the nmr spectrum of ClUDP the signal due to H_5 of the uridine was absent while the signal due to H_6 appeared at 8.09 ppm.

Polymerization of ClCDP with polynucleotide phosphorylase gave poly(ClC). Little phosphorolysis appeared to occur and the polymerization was allowed to proceed for 10 hr after which time poly(ClC) was isolated and found to have $s_{20,w} = 4.20$ S. Poly(ClC) possessed considerable secondary structure in acid solution, and a sharp change in uv absorption was observed at 310 nm around pH 4 in 0.3 M salt solution. In contrast to the gradual change in the spectrum of ClCDP with pH (Figure 2) the change in absorption of poly(ClC) occurred over a narrow pH range (*ca.* 0.2 pH unit). Furthermore the shape of the melting curve for poly(ClC) changes abruptly in shape in this pH range. This indicates that a cooperative phenomenon is taking place and that poly(ClC) has a helical structure in acid solution. Similar behavior has been observed with poly(BrC) (Michelson and Monny, 1967) and poly(BrdC) (Inman, 1964).

Halogenation of the cytidine ring renders the polymer more resistant to hydrolysis by pancreatic ribonuclease. At pH 7.0 in the presence of an excess of enzyme the half-life for poly(ClC) hydrolysis was approximately ten times greater

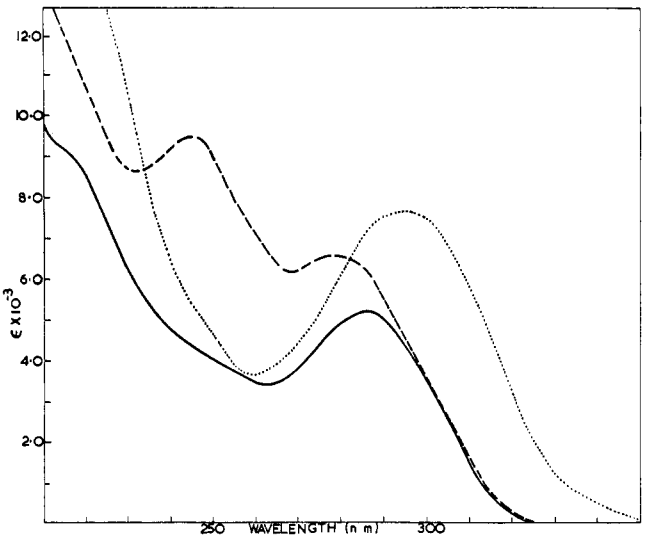

FIGURE 5: Ultraviolet spectra of poly(ClC) at pH 7.0 (—) and pH 2.0 (. . .), and the 1:1 hybrid poly(I)·poly(ClC) at pH 7.0 (- - -) in 0.1 M NaCl–0.005 M sodium cacodylate at 25°.

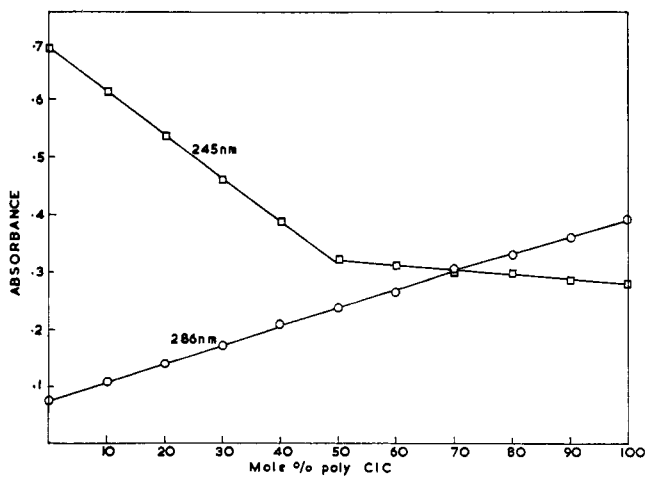

FIGURE 6: Variation in ultraviolet absorption on mixing poly(ClC) and poly(I) in 0.1 M NaCl–0.005 M sodium cacodylate (pH 7.0) at 25°. Total polymer concentration 0.96×10^{-4} M. Readings were taken 6 hr after mixing.

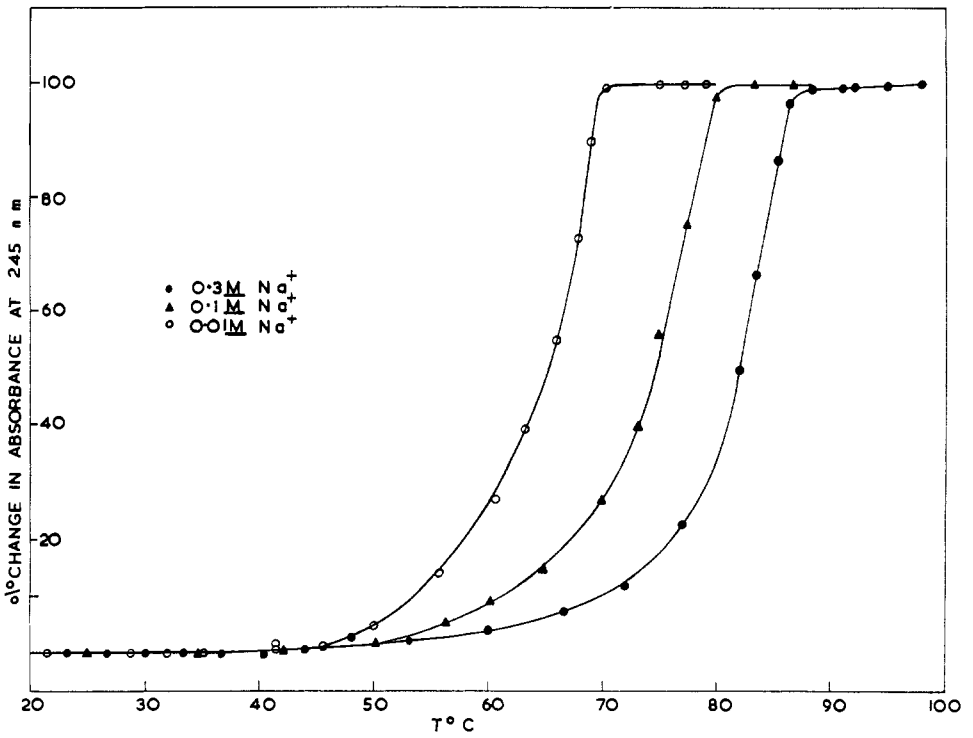

FIGURE 7: Ultraviolet melting curves of poly(I)·poly(ClC) in 0.005 M sodium cacodylate at differing Na^+ concentrations. Total concentration of poly(I)·poly, (ClC), 0.96×10^{-4} M.

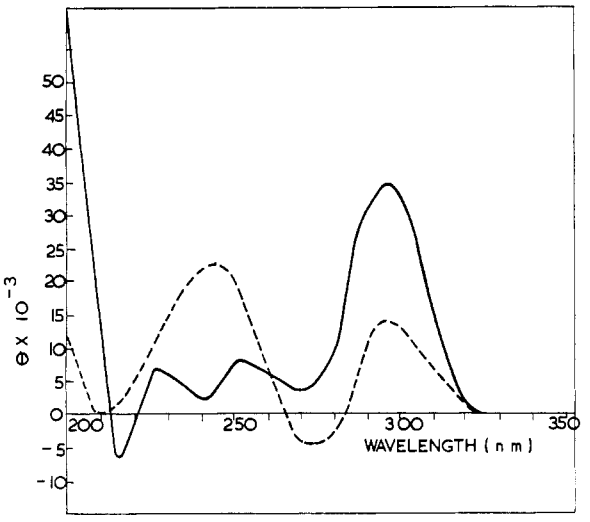

FIGURE 8: Circular dichroic spectra of poly(ClC) (—) and poly(I)·poly(ClC) (- - -) in 0.1 M NaCl–0.005 M sodium cacodylate solution, pH 7.0. The concentrations of the polymers were determined spectrophotometrically and were poly(ClC), 5.7×10^{-4} M, and poly(I)·poly(ClC), 9×10^{-4} M. Cell path length 0.1 cm.

than the half-life for poly(C) hydrolysis while poly(BrC) was even more stable under the same conditions.

The circular dichroism (CD) spectrum of poly(ClC) in neutral solution consists of a maximum at 296 nm (θ 34,700), and thus resembles the CD spectrum of poly(C) which consists of a maximum at 277 nm (θ 59,000) (Green and Mahler, 1970). The CD spectrum of the 1:1 complex of poly(ClC) with poly(I) shows two principal maxima, one at 294 nm (θ 14,000) the other at 244 nm (θ 23,250). This resembles the CD spectrum of poly(I)·poly(C) which consists of two maxima one at 277 nm (θ 15,550) the other at 245 nm (θ 17,200). It has been shown (Brahms and Sadron, 1966) that the CD spectrum of poly(I) consists of a maximum at 248 nm. Hence it appears that the CD spectra of poly(I)·poly(C) and poly(I)·poly(ClC) are made up of components due to the purine and pyrimidine residues which is not the case for poly(A)·poly(U), where the CD spectrum consists of a single maximum at at 262–265 nm (Brahms, 1965).

Poly(ClC) forms a 1:1 complex with poly(I) and like other 5-substituted polycytidylic acids the thermal stability of this complex is greater than that of poly(I)·poly(C) over a range of salt concentrations. It has been shown (Ross *et al.*, 1971) that substitution in the cytidine ring by bromine produces a large enthalpic stabilization of the complex with poly(I) probably due to the introduction of the polarizable bromine atom into the heterocyclic system. Chlorine is less polarizable than bromine and if polarizability were an important feature of the stabilization forces, poly(ClC) should form a less stable complex with poly(I) than poly(BrC). We have found that at a given salt concentration the melting temperatures of poly(I)·poly(ClC) complexes are slightly lower than those of poly(I) poly(BrC) complexes. Furthermore, it has been shown (Michelson and Monny, 1967) that melting temperatures of polyinosinic acid·polyiodocytidylic acid complex is higher than the poly(I)·poly(BrC) complex.

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A Comparison of Steady- and Presteady-State Kinetics of Bovine and Human Plasmins†

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ABSTRACT: A comparison of steady-state and presteady-state kinetics on plasmins derived from a streptokinase-insensitive plasminogen (bovine) and a streptokinase-sensitive plasminogen (human) has been undertaken. Steady-state kinetics on bovine plasmin using α-*N*-tosyl-L-arginine methyl ester as the substrate at 22° yielded K_m and V_{max} values of 8.2×10^{-3} M and 17.5 μmoles min^{-1} mg^{-1}, respectively. Benzamidine hydrochloride was found to be a competitive inhibitor of plasmin esterase activity with a K_I value of 5.1×10^{-4} M. The corresponding values for human plasmin were 7.7×10^{-3} M, 12.4 μmoles min^{-1} mg^{-1}, and 10.6×10^{-4} M. Presteady-state kinetics using *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride as the substrate for bovine plasmin at 22° yielded k_2 and K_s values of 0.543 sec^{-1} and 22.2×10^{-6} M and for human plasmin the corresponding values were 0.064 sec^{-1} and 5.42×10^{-6} M. Similar presteady-state experiments were performed with another acylating agent, *N*-(*p*-carboxybenzyl)pyridinium *p*-nitrophenyl ester bromide. This reagent yielded k_2 and K_s values for bovine plasmin at 22° of 0.085 sec^{-1} and 2.12×10^{-3} M and values of 0.084 sec^{-1} and 5.34×10^{-4} M for human plasmin. These studies indicate that marked differences occur in the active sites of bovine and human plasmins.

Plasmin (EC 3.4.4.14) is a proteolytic enzyme which functions, physiologically, in the dissolution of the fibrin clot and exists in the plasma as the inactive precursor, plasminogen. The conversion of plasminogen to plasmin has been widely studied and has some well recognized features. Activation of human plasminogen by a urinary protease, urokinase, or by a variety of other tissue proteases proceeds through cleavage of a single arginyl–valine bond in plasminogen, yielding a two-chain plasmin structure stabilized by a single disulfide bond (Robbins *et al.*, 1965, 1967; Summaria *et al.*, 1967a,b; Groskopf *et al.*, 1969a). Activation of human plasminogen also occurs with a bacterial endotoxin, streptokinase. However, since streptokinase possesses no inherent proteolytic activity its induced cleavage of a peptide bond in plasminogen occurs through its combination with "proactivator" and subsequent formation of "activator," which possesses the necessary proteolytic activity for cleavage of a peptide bond in plasminogen (see Kline and Fishman (1963), Davis *et al.* (1964), Ling *et al.* (1967), Summaria *et al.* (1969), and Reddy and Marcus (1972)). It has also been found (see Wulf and Mertz, 1969) and in part confirmed on purified plasminogens (Brockway and Castellino, 1972) that streptokinase is species specific, only activating the plasminogens of man, monkey, and cat, whereas urokinase or "activator" activated the plasminogens of all species so far studied.

The plasmin obtained by activating plasminogen as described above is a proteolytic enzyme which possesses trypsin-like esterase activity, catalyzing the hydrolysis of α-NH_2-substituted lysine and arginine esters (Sherry *et al.*, 1966). Human plasmin possesses an active-site serine and histidine

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