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Poly 2'-O-ethylcytidylate, an inhibitor and poor template for AMV reverse transcriptase.

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ABSTRACT

Poly(2'-O-ethylcytidylate) is a poor template-primer for purified avian myeloblastosis virus reverse transcriptase; the relative activities of the template-primers poly(C)•oligo(dG), poly(Cm)•oligo(dG) and poly(Ce)•oligo(dG) are 23:16:1. A mixture of poly(Ce) and poly(dI) is inactive as template-primer, in agreement with the observed inability of these to form a helical complex. By contrast the inactivity of poly(Ce)• •poly(I) is shown to be due to the influence of the 2'-O-ethyl residue. Poly(Ce) inhibits poly(A)•oligo(dT)-directed polymerase activity, with K_i = 3 μM, but marked inhibition with poly(A)•poly(dT) occurs only at low concentrations of the latter. Poly(Ce) did not inhibit template-primer activity of poly(C)•poly(dI) and poly(dC)•poly(dI). Qualitative physico--chemical studies show only partial complex formation between oligo(dG) and poly(C) and its 2'-O-alkyl analogues. This is discussed in relation to the widespread use of poly(C)•oligo(dG) as a template-primer for reverse transcriptase.

INTRODUCTION

Poly 2'-O-methylcytidylate, poly(Cm)[d], originally synthesized for investigations on the role of the 2'-hydroxyl in nucleic acid structure, and of the role of 2'-O-methyl residues in ribonucleic acids[1], has also proven useful in studies on the specificity of polynucleotide phosphorylase[2], and the role of the 2'-OH in the mechanism of interferon induction by synthetic polyribonucleotides[3]. The subsequent report that poly(Cm)•oligo(dG) is a template-primer apparently specific for RNA-directed DNA polymerases of viruses and of virus-like particles derived from human tumour tissues[4,5], a finding supported by another laboratory[6], provides a means of differentiating this enzyme from cellular DNA polymerases. This prompted us to undertake an examination of the template-primer properties of poly(Ce)•oligo(dG), in part with a view to its subsequent possible utility in studies on the nature of the unexpected specificity of poly(Cm)•oligo(dG) which, if further confirmed, could conceivably prove to be a useful diagnostic tool. The rather surprising specificity of poly(Cm)•oligo(dG) also led us to examine the nature

of the template-primer systems normally employed in such studies, from a physico-chemical standpoint, with results which, although at the moment largely qualitative, are of undoubted relevance.

Furthermore, in view of various reports on inhibition of both RNA- and DNA-directed DNA polymerases by single-stranded synthetic polynucleotides[7,8,9,10,11,12,13,14,15], poly(Ce) was also examined as a potential inhibitor of AMV reverse transcriptase.

MATERIALS AND METHODS

Deoxynucleoside triphosphates were products of Sigma Chemical Co. (St. Louis, Mo., U.S.A.), and ^{3}H-labelled deoxynucleoside triphosphates were obtained from the Radiochemical Centre (Amersham, England).

Oligo(dT)$_{12-18}$ and oligo(dG)$_{12-18}$ were from P-L Biochemicals (Milwaukee, Wis., U.S.A.), and poly(A) and poly(C) from Miles Laboratories (Elkhart, Ind., U.S.A.). The preparation and physico-chemical properties of poly(Cm) and poly(Ce) have been elsewhere described[1,16]; both exhibited sedimentation coefficients, $S_{20,w}$ of about 7 - 8. Poly(dI) and poly(dC), prepared with the aid of terminal transferase[17], were gifts of Dr. F. J. Bollum.

Template-primer duplexes were prepared by annealing the two components at a concentration of 200 µg/ml in 5 mM Tris-HCl buffer pH 7.8 and 10 mM NaCl at 37° for 1 hour and then at 4° for 18 hrs.

UV absorption spectra of template-primers, and their dependences on temperature, were measured with the aid of a Unicam SP-500 spectrophotometer fitted with a specially constructed temperature-controlled cuvette compartment, through which circulated an aqueous glycol mixture fed from a Hoeppler ultrathermostat. Cuvette temperatures were measured by means of a thermistor in a dummy cuvette.

AMV reverse transcriptase, purified according to Kacian & Spiegelman[18], was a gift of Dr. J. W. Beard. The enzyme was further dialyzed against 50% glycerol, 50 mM Tris-HCl buffer pH 7.5, 2 mM mercaptoethanol, 0.5% Triton X-100. It then contained 41 units/ml.

Reverse transcriptase activity was assayed in 50 µl reaction volumes, as indicated in individual experiments, for 20 min at 37°. Acid-precipitable radioactivity was then collected and washed on Whatman No. 1, or Whatman GF/C glass fiber, filters as described by Bollum[19]. One unit of enzyme activity is defined as that leading to incorporation of 1 nmol of dTMP into acid precipitable material with poly(A)·oligo(dT) as template-primer, in 20 mins at 37°.

RESULTS AND DISCUSSION

Interaction of poly(Ce) with oligo(dG), poly(dI) and poly(I).

Although a number of investigations have been reported on the interactions between synthetic homopolynucleotides and potentially complementary oligonucleotides, largely with the use of optical methods, less attention appears to have been devoted to an examination of template-primer complexes employed in polymerase reactions[20].

Mixing of equimolar solutions of poly(Ce) and oligo(dG) at neutral pH led to only slight modifications in absorption in the region 270-280 nm, indicative of partial formation of the complex poly(Ce)•oligo(dG). Analogous small, but clearly defined changes were observed when poly(Ce) was replaced by poly(C) or poly(Cm). It is clear that only a portion of the oligo(dG) is involved in complex formation, and this was unaltered when the salt concentration was increased to 0.1 M, when the solution was heated to 80° and cooled, or brought to pH 12 and then neutralized.

The foregoing is probably due to the known "aggregation" or self-association of the oligo(dG) employed, the resulting strong structure preventing interaction with poly(Ce). This result is by no means surprising if it is recalled that the oligo(dG) preparation employed has a mean residue length of 15. It had earlier been shown that oligo(dG)$_3$ and oligo(dG)$_4$ exhibit strong self-association[21], a result subsequently extended to embrace poly(dG)[22]. Attempts to quantitatively evaluate the amount of poly(Ce)• •oligo(dG) from changes in the UV absorption spectrum following mixing, from sedimentation analyses and by dialysis, were unsuccesful, and demonstrated only that complex formation was appreciably lower than that anticipated from the concentrations of the components. Additional measurements showed that an equally low degree of complex formation occurs between oligo(dG) and poly(Cm) and poly(C), and this is now the subject of further investigation.

The results of a mixing experiment involving poly(Ce) and poly(dI) in the ratio 1:1 are shown in Fig. 1. It will be noted that the resulting absorption spectrum almost coincides with that calculated for the sum of the two components. Heating of the solution led to an increase in absorption in the region about 245 nm (insert to Fig. 1). The extent of this increase, and the temperature at which it occurs, show that it is due to melting of the self structure of the poly(dI) component alone[23]. This result recalls earlier unsuccessful attempts to demonstrate complex formation between poly(Cm) and poly(dI)[24]. In the case of poly(Ce), the analogous absence of complex cannot be ascribed to its lower stability as due to the 2'-O-

ethyl substituent, since poly(Ce)•poly(I) readily forms a complex with a sharp helix-coil transition and with a T_m 6° higher than that for poly(C)•poly(I)[16].

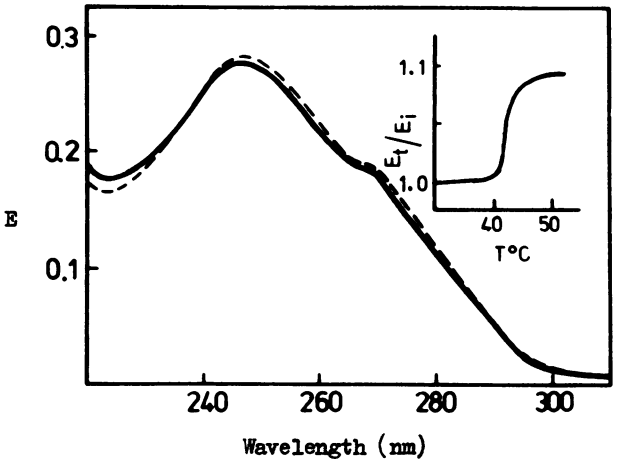

Fig. 1. Absorption spectrum, in Tris-HCl buffer pH 7.8 and 0.1 M NaCl, of equimolar mixture of poly(Ce) and poly(dI): ——, observed; - - - - -, arithmetic sum of the two components. Insert: Temperature-dependence of spectrum of 1:1 mixture of poly(Ce) and poly(dI), in Tris-HCl buffer pH 7.8 and 1 M NaCl, measured at 245 nm. The observed profile corresponds to melting of poly(dI), and no further change occurs above 50°.

Poly(Ce) directed replication. In agreement with the spectrophotometric data, pointing to the absence of complex formation between poly(Ce) and poly(dI), no detectable replication was observed with a mixture of these two homopolymers in the presence of AMV reverse transcriptase with dGTP as substrate. This result was unchanged when the NaCl concentration was increased to 0.1 M, and the incubation temperature reduced to 20°, with a view to facilitating complex formation (Table 1, Exp. b).

Replication with the aid of the template-primer poly(Ce)•oligo(dG) was carried out at a pH, and in the presence of mono- and divalent cation concentrations, described by others[25] as optimal for replication of poly(C)•oligo(dG) and poly(Cm)•oligo(dG). A 20-minute incubation period and a dGTP concentration of 10 µM was found to give a reaction linear with time and a saturation concentration of substrate. Under these conditions

Table 1. Influence of a 2'-O-ethyl substituent on poly(C) directed replication by purified AMV reverse transcriptase at 37°.[x]

Exp.	Template	Primer	Comments	Incorporation (pmole)
(a)	Poly(C)	Poly(dI)		300
	Poly(dC)	Poly(dI)		130
(b)	Poly(Ce)	Poly(dI)	20°; 0.1 M KCl	0.01
(c)	Poly(C)	Oligo(dG)		23
	Poly(Cm)	Oligo(dG)		16
	Poly(Ce)	Oligo(dG)		1
	Poly(Ce)	Oligo(dG)	20 mM EDTA	0.2
	Poly(Ce)	----		0.1
	----	Oligo(dG)		0.1
(d)	Poly(Ce)	Poly(I)		0
(e)	Poly(A)	Oligo(dT)		40

[x]The constituents of the reaction mixtures (final volume 0.05 ml) and their concentrations were as follows: Tris-HCl buffer pH 8.0, 40 mM; KCl, 60 mM; DTT, 1.6 mM; Triton X-100, 0.05%; enzyme, 0.04 units; template, 30 µM;

plus

Primer: poly(I) or poly(dI), 30 µM; oligo(dG), 10 µM; oligo(dT), 15 µM.
Substrate: ^{3}H-dGTP, 10 µM (300 cpm/pmole) in (a), (c) and (d), plus dCTP, 10 µM in (a); ^{3}H-dGTP, 0.5 µM (9300 cpm/pmole) in (b); ^{3}H-dTPP, 10 µM (320 cpm/pmole) in (e),
Cation: $MgCl_2$, 12 mM in (a), (b) and (d); $MnCl_2$, 0.2 mM in (c) and (e).

the extent of incorporation of dGTP was low, although clearly well above the background obtained with the template or primer alone (Table 1, Exp. c). The relative activities of poly(C)·oligo(dG), poly(Cm)·oligo(dG) and poly(Ce)·oligo(dG) were 23:16:1.

In view of the low extent of complex formation between poly(C) and its 2'-O-alkyl analogues with oligo(dG), it might be argued that the low template activity is due to a considerably lower extent of complex formation by poly(Ce). Attention was consequently directed to the use of poly(Ce)·poly(I), where formation of a helical complex has been well established[16]. It has been shown by Spiegelman et al.[26] that complexes of the form poly(C) poly(I) are amongst the most active template-primers for reverse

transcriptase. The total absence of activity with poly(Ce)•poly(I) (Table 1, Exp. d) suggests that it is the presence of the 2'-O-ethyl substituent which is responsible for the low activity of poly(Ce)•oligo(dG). An analogous result has been reported for poly(Ae); poly(Ae)•oligo(dT) is not a template-primer[8]. The low activity of poly(Ce) cannot, in any event, be ascribed to the influence of the RNase H associated with AMV reverse transcriptase, since the former is known to exhibit a specificity for purine residues[27].

Poly(Ce) as inhibitor. Poly(Ce) was found to act as an inhibitor in the reverse transcriptase system, the extent of inhibition depending on the template-primer employed. The most pronounced effect was noted with poly(A) oligo(dT) (see Fig. 2). For poly(Ce) in this system, the K_i (3 μM), determined by extrapolation of the initial linear portions of the curves representing the dependence of $1/V_i$ on [I], was comparable to the values previously reported with the same purified enzyme system for poly(C) and poly(dCcl), 12 μM and 2.6 x 10^{-2} μM, respectively[14]. It is nonetheless clear that, while replacement of the 2'-OH in poly(C) by 2'-O-ethyl increases the inhibitory effect of poly(C), it is less pronounced than substitution of a

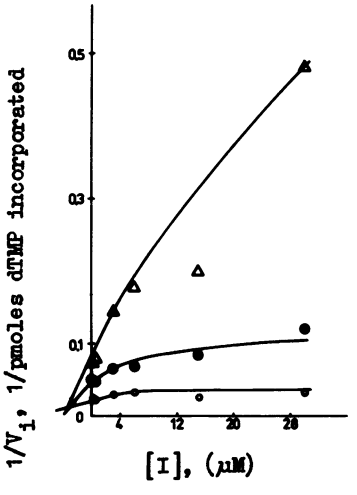

Fig. 2. Kinetics of inhibition ($1/V_i$ vs [I]) of poly(A)•oligo(dT)-directed AMV reverse transcriptase by poly(Ce). Reaction conditions as in legend to Table 1. Reaction mixture (total volume 50 μl), contained 0.04 units of enzyme and: o o o o, 30 μM; ● ● ● ●, 10 μM; Δ Δ Δ Δ, 3 μM poly(A)•oligo(dT).

2'-deoxy-2'-chloro. Comparison of the value of K_i for poly(Ce) with those for other polyribonucleotides and their 2'-O-alkyl derivatives[8,9] can only be approximate, since the latter were measured with the use of disrupted virions as an enzyme source; Erickson & Grosch[14] have shown that, relative to a purified enzyme preparation, this leads to a 6 to 40-fold increase in K_i values. Nonetheless the available data demonstrate that the influence of a 2'-O-ethyl on the inhibitory effect of poly(C) is analogous to that with poly(A), where the K_i values for poly(A), poly(Am) and poly(Ae), with a disrupted MLV virion system, were 36, 22 and 8.5 uM respectively[8].

An examination of the kinetic data for replication of poly(A)·oligo(dT) in the presence of poly(Ce) points to a partially competitive nature of the inhibition. The dependence of $1/V_i$ on [I] is linear only at low concentrations of the inhibitor. With increasing concentrations there is a marked departure from linearity, similar to that observed with other polynucleotide inhibitors[8,9,13,14].

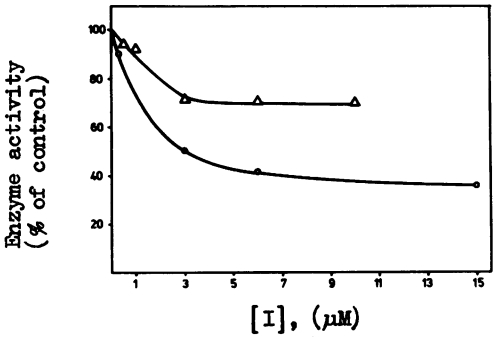

Fig. 3. Effect of poly(Ce) on AMV reverse transcriptase catalyzed replication of: o o o, poly(A)·oligo(dT), 3 μM; Δ Δ Δ , poly(A)·poly(dT), 3 μM. Reaction conditions as in legend to Table 1, with 0.04 enzyme units in 50 μl incubation volume.

With poly(A)·poly(dT) as template-primer, and dTTP as substrate, poly(Ce) inhibited the reaction, but only at low template-primer concentrations (3 μM). The extent of inhibition was only 30% as compared to 64% with poly(A)·oligo(dT) (Fig. 3). A rough estimate of the K_i in this case gave a value of 5 μM, hence similar to the K_i value with poly(A)·oligo(dT), as might have been anticipated since these depend largely on the enzyme and inhibitor, and not on the template-primer. The less pronounced inhibition observed with the longer primer is undoubtedly due to stronger binding of poly(dT) to the poly(A) template, leading to stronger interaction with the

enzyme, which is consequently less accessible to the inhibitor. This is also reflected in the values of K_m, 0.75 and 0.15 μM, respectively for poly(A)·oligo(dT) and poly(A)·poly(dT)[28].

Finally poly(Ce) was examined as a potential inhibitor of the replication of poly(C) itself. This proved feasible, with the use of poly(C)·poly(dI) and poly(dC)·poly(dI), because of the fact that poly(Ce) does not complex with poly(dI).[e] With 3 - 30 μM template, and 30 μM poly(Ce), there was no detectable inhibition. One might expect poly(Ce) to compete more effectively for the enzyme with a template containing the same base, as compared to another such as poly(A). Furthermore the stability of poly(A)·poly(dT) is higher than that of poly(C)·poly(I) and poly(C)·poly(dI). The absence of inhibition in this instance is consequently due to the marked affinity of poly(C) for the enzyme. No K_m values appear to have been reported for these template-primers. The value of 4 μM for the K_m of poly(C)·oligo(dG)[9] is undoubtedly unduly high, bearing in mind the low extent of interaction between these two constituents (see above) and the fact that it pertains to disrupted virions as a source of reverse transcriptase. If, however, the above value is compared with that of 20 μM for poly(A)·oligo(dT) as found for the same enzyme preparation[9], this is in agreement with our interpretation.

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[d]Abbreviations employed conform to those recommended by the Commission on Biochemical Nomenclature, IUPAC. In addition: poly(Cm), poly 2'-O-methylcytidylate; poly(Ce), poly 2'-O-ethylcytidylate; poly(Ae), poly 2'-O-ethyladenylate; poly(dCcl), poly 2'-deoxy-2'-chlorocytidylate; AMV, avian myeloblastosis virus; DTT, dithiothreitol.

[e]The inhibitory effect of poly(Ce) on poly(C)·oligo(dG) may also be readily measured, and amounts to 50% when the concentrations of poly(Ce), poly(C) and oligo(dG) are 10 μM, 3 μM and 1 μM, respectively. However, in this case it is not possible to evaluate K. for poly(Ce) because of the lack of

quantitative data as to the extent of complex formation between poly(C) and oligo(dG), as well as the probable complexing of the latter with poly(Ce).

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