MODIFICATION OF POLYNUCLEOTIDES BY A FRAGMENT PRODUCED BY ENZYMATIC CLEAVAGE OF S-(1,2-DICHLOROVINYL)-L-CYSTEINE

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SUMMARY: Polyribo- and polydeoxyribonucleotides were allowed to react with ^{35}S-(1,2-dichlorovinyl)-L-cysteine (DCVC) in presence of a bovine kidney lyase yielding products which were substituted to varying degrees with an alkylating thiovinyl fragment (AF) released from DCVC. Polydeoxyribonucleotides were more extensively substituted than polyribonucleotides. Double stranded homopolymer pairs were much less effective as acceptors of (AF) than single stranded polymers. Nucleotide substitution occurred only at the polymer level. Enzymatic hydrolysis of (AF)-substituted polymers yielded dinucleotides which contained an (AF) fragment apparently covalently linked in unknown fashion. (AF)-substituted polynucleotides had reduced ability to form helical complexes with complementary polynucleotides, as revealed by hypochromicity, melting transition and renaturation.

INTRODUCTION: After it was discovered that DNA isolated from hemopoietic tissues of calves which had been treated with a single dose of S-(1,2-dichlorovinyl)-L-cysteine (DCVC) had abnormal physical properties (1) and was devoid of primer activity for DNA (2) and RNA (3) polymerases, we found (4) that the same abnormalities could be observed in DNA from many sources after its exposure *in vitro* to DCVC and a DCVC-lyase (5). One product formed by this enzyme is a very reactive fragment which contains carbon and sulfur of the thiovinyl moiety of DCVC. It has properties of an alkylating agent (5) and is referred to herein as an alkylating fragment (AF) which can react with a variety of acceptors including DNA (4). From enzymatic hydrolyzates of ^{35}S-(AF)-treated DNA, dinucleotides containing the labeled ^{35}S-(AF) could be isolated (4). It is of interest therefore to study the interaction of (AF) with nucleotides and their oligomers and polymers of known structure. We summarize herein experiments in which polynucleotides were used for this purpose.

MATERIALS AND METHODS: Poly(rA), poly(rG), poly(rC), poly(rU) and di-deoxyribonucleoside monophosphates of the type dRpdY were obtained from P-L-

Biochemicals, Inc., Milwaukee, Wis., poly(dA), poly(dT), poly(dG)·poly(dC), di-ribonucleoside monophosphates of the type RpY and oligoadenylates (ApA through $(Ap)_5A$) were purchased from Miles Laboratories, Kankakee, Ill. Free bases and other nucleoside derivatives were from Schwarz BioResearch, Orangeburg, N.Y. and from P-L-Biochemicals.

Poly(dG) and poly(dC) were isolated by alkaline Cs_2SO_4 density gradient centrifugation of the homopolymer pair poly(dG)·poly(dC) according to the procedure of Inman and Baldwin (6). Other homopolymer pairs and hybrid complexes were prepared by mixing equimolar quantities of complementary strands. The conditions were similar to those described by Chamberlin (7) favoring double-strand formation. Each polynucleotide (0.5 µmole P) was allowed to react in 1.0 ml final volume with 5 µmole ^{35}S-DCVC (~8 x 10^5 CPM) (4), 0.6 mg of lyase (5) and 50 µmoles of Tris-HCl buffer, pH 7.5 for 30 mins. at 37°. Bovine serum albumin (1 mg in 0.5 ml) was then added to facilitate precipitation followed immediately by two volumes of ice cold ethanol. The precipitate was centrifuged and washed three times with 2 ml portions of ice cold ethanol. Polynucleotide was then extracted with two 1 ml aliquots of 10^{-3} M Tris-HCl, pH 8.0 according to Bollum (8), centrifuging each time to separate denatured lyase. The same procedure was used to treat polynucleotide pairs with (AF). Aliquots were used to measure phosphorus (9) and radioactivity (5). When the polynucleotides were to be used for enzymatic hydrolysis, the reaction mixture was increased tenfold and the extracted polynucleotide was precipitated by adding two volumes of ethanol (4) before dissolving in appropriate solvent. Before measuring melting profiles, the extracted polynucleotide was dialyzed against the buffer indicated in Fig. 3.

When oligonucleotides, dinucleoside phosphates, nucleoside derivatives and free bases were tested, the reaction mixture (40 µl) contained 0.2 µmole ^{35}S-DCVC (2.15 x 10^4 CPM), 20 µg lyase and 0.2 µmole free base or nucleoside phosphate. After reacting at 37° for 30 mins. a 20 µl aliquot was chromatographed on a DEAE-cellulose paper strip using 0.25 M or 0.75 M ammonium

bicarbonate (10,11). The nucleoside derivatives were located under ultraviolet light and the strip was scanned for radioactivity with a Vanguard recording instrument.

Polynucleotides, treated as indicated above, were subjected to enzymatic hydrolysis according to a method adopted from the procedure of Bollum (8). A specimen containing about 2 μmoles of polynucleotide phosphorus was digested for 4 hours at 37° with 100 μg pancreatic DNase I in the presence of 2 μmoles $MgCl_2$ buffered with 10 μmoles Tris-HCl, pH 7.0. For poly(dC) and poly(dG)·poly(dC) the pH was increased to 7.5 and 0.05 μmole $CaCl_2$ was also added (8). To stop this reaction the mixture was then held at 70° for 5 mins. and the pH was adjusted to 8.8 with dilute NH_4OH. One hundred microgram of snake venom phosphodiesterase (Worthington) was added and the digestion was continued for another 4 hours at 37°. Aliquots were chromatographed on DEAE-cellulose paper strips with 0.25 M ammonium bicarbonate (10). The dried strips were scanned with a Vanguard instrument to locate radioactive areas, which were then eluted with saturated ammonium bicarbonate (12). The eluates were evaporated under reduced pressure and dissolved in a suitable solvent. Aliquots were analyzed for radioactivity, phosphorus, chain length and base contents. Methods used for these analyses as well as for ultraviolet absorption and melting experiments have been cited previously (4).

RESULTS AND DISCUSSION: Fig. 1 shows that all polynucleotides used in this study combined with (AF) released from DCVC by the lyase. The uptake of ^{35}S-(AF) was much greater in single stranded homopolymers than in the double stranded homopolymer pairsor in hybrid complexes. Of the single stranded homopolymers polydeoxynucleotides were about twice as susceptible to attack than the polyribonucleotides. The reason for this is unknown. The greater susceptibility of single stranded, denatured DNA than of native DNA has also been noted before (4). A highly ordered, unbroken DNA structure does therefore not favor its interaction with (AF). Free bases, nucleosides and mono-, di-, and triphosphates of ribo- and deoxyriboside derivatives of A, G, C and T were

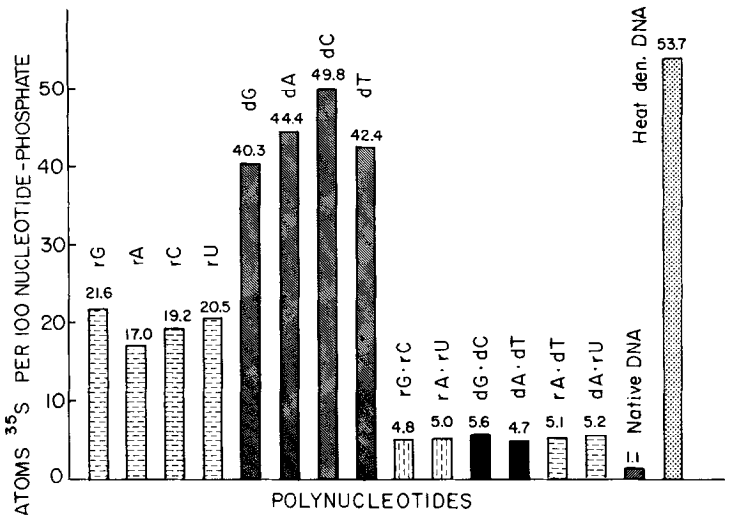

Fig. 1: Extent of reaction of polynucleotides with ^{35}S-(AF) from ^{35}S-DCVC.

also tested as potential acceptors of ^{35}S-(AF). As judged by chromatographic separation of each reaction mixture, none of these compounds reacted with (AF). Several dinucleoside phosphates, oligoadenylates and partially digested homopolymers also did not react.

The site of substitution in the polymer molecule has not been determined because chemical hydrolysis completely destroyed the (AF) fragment. The (AF)-substituted polymer products were resistant to phosphodiesterase attack. Sequential hydrolysis of ^{35}S-(AF)-substituted polydeoxyribonucleotides with pancreatic DNase I followed by venom phosphodiesterase yielded evidence for the presence of ^{35}S-(AF)-nucleotides. Their separation by paper chromatography showed only one radioactive spot from each polymer which was found to be a dinucleotide. The evidence used for identification of the products of hydrolysis is summarized in Table 1. The corresponding dinucleotides obtained after digestion of poly(dA), poly(dT), poly(dG) and poly(dC) all contained one radioactive fragment. Similar sequential digestion of the ^{35}S-(AF)-homopolymer pairs of poly(dA)·poly(dT) and poly(dG)·poly(dC) in each case produced two different dinucleotides, each complementary strand yielding one. No mixed dinucleotides could be detected. This indicated that (AF) did not produce

TABLE 1

CHARACTERISTICS OF HYDROLYZED PRODUCTS FROM

(AF)-SUBSTITUTED POLYDEOXYNUCLEOTIDES

Substituted Polynucleotides (Hydrolyzed)	Nature of Products Formed				
	R_f	Total P / Terminal P	Total Base / ^{35}S	Total P / ^{35}S	Identified as ^{35}S-bound nucleotides
Poly(dG)	0.14	2.08	2.10	2.14	d(pGpG)
Poly(dA)	0.30	2.02	2.04	1.95	d(pApA)
Poly(dC)	0.44	1.92	2.00	2.00	d(pCpC)
Poly(dT)	0.71	1.95	1.81	1.87	d(pTpT)
Poly(dG)·poly(dC)	0.14	2.07	2.00	2.13	d(pGpG)
	0.44	1.95	2.06	2.24	d(pCpC)
Poly(dA)·poly(dT)	0.30	2.13	1.88	1.70	d(pApA)
	0.71	2.04	1.90	1.97	d(pTpT)

interstrand cross linking. A product formed from interstrand linked polymers would contain two different bases. The terminal phosphate from each dinucleotide could be removed by treatment with E. coli alkaline phosphate. The products thus formed still contained radioactivity and were highly resistant to further hydrolysis by spleen phosphodiesterase. These findings are analogous to those obtained with (AF)-treated, denatured DNA (4). The nature of the binding of (AF) in these phosphodinucleosides remains to be ascertained.

When synthetic polynucleotides associate under appropriate conditions (13-15) there occurs a progressive decrease in absorbance of their solutions. When double stranded complexes are formed, the hypochromicity is greatest when the mole ratio of the reactants is 1:1. To test the effect of substitution of polynucleotides with (AF) we measured the change in absorbance of mixtures of poly(dT) with either poly(rA) or poly(dA) before and after treatment of each

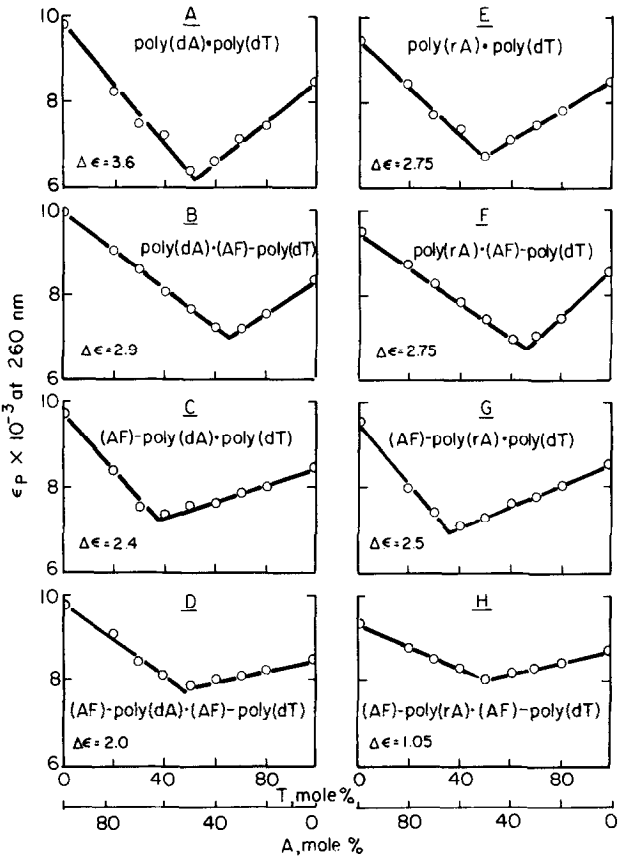

Fig. 2: Hypochromicity of mixtures of normal and (AF)-treated poly(dA) and poly(rA) respectively with poly(dT). The absorbance at 260 nm from which the mixing curves (17) were constructed was measured in 0.15 M sodium chloride-0.015 M sodium citrate pH 7.0, at 22°. The change in the extinction coefficient of the mixtures was calculated by subtraction of ε at the intercept from ε of 100% polyadenylate, as shown in each diagram.

with (AF). As shown in Fig. 2 A-D, in the series of paired homopolymers poly(dA)·poly(dT), substitution of either component with (AF) prior to pairing lessened the hypochromicity; this effect was greater when each member of the pair was pretreated with (AF). In the hybrid series of poly(rA)·poly(dT) (Fig. 2 E-H) the effect of (AF) substitution did not become strong unless both members of the hybrid were pretreated. In both series, however, when (AF)-treated polynucleotide was used as one member of a paired complex, maximum hypochromicity was not reached until approximately 2 moles of the (AF)-treated polymer were present per mole of normal polymer (Figs. 2 B,C,F,G). This would

imply formation of triple stranded complexes as described by Felsenfeld and Rich (13). If such triple complexes were formed in the present experiments, they did not produce the expected (13), much greater hypochromicity. It is likely that in these complexes the changed conformation of the (AF)-substituted polynucleotides produced loose structures in which the individual components were not intertwined in a well defined, replicated arrangement (14,15). Our results indicate nevertheless that through substitution with (AF) the ability of polynucleotides to form complementary complexes is impaired. Troll and Berkowitz (16) reported that poly(G), alkylated with the proximal carcinogen β-propiolactone, formed a triple helix with poly(C), and also showed "ambiguous" behavior in reaction with poly(U). In our studies, however, the (AF)-substituted polymer did not react with any non-complementary nucleotide polymer. That the modification of a polynucleotide interferes with its interaction with a complementary strand has also been recently recorded by Shapiro and Braverman (17). They showed that partial saturation of poly(U) by bisulfite, which converts uracil to 5,6-dihydro uracil-6-sulfonate, sharply reduced its ability to form a helical complex with poly(A).

The inability of (AF)-substituted polynucleotides to form ordered, paired structures has been confirmed by melting and annealing experiments. Several homologous and RNA hybrid pairs were formed and then treated with DCVC and lyase to introduce (AF)-substitution. Their melting profiles were recorded. After heating, the change in absorbance was also recorded while the solutions were cooled very slowly and during a second course of melting. As illustrated in Fig. 3 the (AF)-substituted homopolymer and hybrid pairs in each case showed T_m values which were 4-5° higher than the normal pairs. Although there is no evidence for interstrand cross linking, an increased T_m value was always observed with all (AF)-treated homopolymer and hybrid pairs. This is consistent with our previous findings with (AF)-treated DNA from various sources (4).

After complete denaturation of normal duplexpolymers, the separated

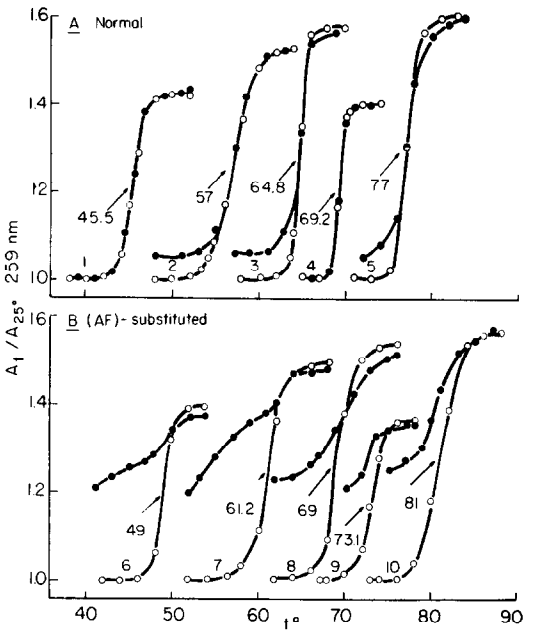

Fig. 3: Melting profiles of polynucleotide pairs prepared from normal and (AF)-treated components. 0 First heating, ● second heating after slowly cooling to room temperature. Solvent: 0.1 M sodium phosphate buffer, pH 7.8 containing 10^{-4} M EDTA except that for poly(dG)·poly(dC) it was in 0.01 M sodium phosphate buffer, pH 7.8 containing 10^{-4} M EDTA. Concentration of polynucleotide was 0.05 µmole P/ml. Tm of first heating as indicated by arrow for each curve. Data from the following polynucleotide pairs as shown. 1. poly(dA)·poly(rU); 2. poly(rA)·poly(rU); 3. poly(rA)·poly(dT); 4. poly(dA)·poly(dT); 5. poly(dG)·poly(dC); 6-10. Corresponding (AF)-substituted pairs.

strands associated again on slow cooling as shown by identical melting profile of the renatured polymers (Fig. 3A). Under similar treatment, however, the absorbance of (AF)-substituted polymers remained very high, so that the second melting curves (Fig. 3B) were entirely different than the first ones. This implied that the separated strands could not form proper base pairings (15) due to the presence of the (AF) fragments in the polymers. This observation supports our other evidence that the thiovinyl fragment does not form covalent bonds between complementary chains, since reversal of thermal denaturation is favored in chemically cross linked DNA (18-21).

The data presented above indicate that single stranded DNA species modified by reaction with (AF), as it may happen in vivo, have impaired

ability for complex formation. Biologically, the changes recorded above would indicate interference with replication of DNA and its transcription into RNA. We have in fact observed such loss in the biological properties of DNA which was either modified in vitro with DCVC and lyase (4) or which had been isolated from target tissues of calves after treatment with DCVC (2,3). Modification of messenger RNA species would also imply a change in translation process. Many studies (16,17,22-24) have indicated that synthetic RNA species modified by alkylating, carcinogenic and mutagenic agents have abnormal coding properties. It will be interesting to see if m-RNA can be modified by treatment with (AF) and if its functional properties become impaired.

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