GRADATION OF SPECIFICITY WITH REGARD TO SUGAR AMONG NUCLEASES.*

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Wechter (1966) synthesized several dinucleoside monophosphates each containing an arabinosyl cytosine. These compounds were investigated as substrates for venom exonuclease (phosphodiesterase) by Wechter (1967) and by Richards et al. (1967), and were found to be susceptible. The conclusion was obvious that venom exonuclease does not discriminate against any of the 3 sugars.

The question arose whether all nucleases previously classified as unspecific to sugar (Laskowski, 1959, 1961, 1967) attack the derivatives of arabinose. To this end the trinucleoside diphosphate a-CpCpC was synthesized and was subjected to the action of micrococcal nuclease (MNase), a 3'-monoester former, and mung bean nuclease (MBNase), a 5'-monoester former. These enzymes are endonucleases and are known to hydrolyze both DNA and RNA (Laskowski, 1967). Venom exonuclease was used as a positive control, and pancreatic RNase and DNase I as negative controls.

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Synthesis of arabinosyl-cytidylyl-[3'→5']-arabinosyl-cytidylyl-[3'→5']-arabinosyl-cytosine. N^4,2',3' tribenzoyl-arabinosyl-cytosine was condensed with 5'-O-trityl-N^4-benzoyl-arabinosyl-cytosine-3'-phosphate employing dicyclohexyl carbodiimide [the intermediates and procedures are described in (Wechter, 1967)]. After the usual isolation of the crude protected dinucleoside phosphate it was hydrolyzed in 80% acetic acid for 24 hrs to remove the trityl group. The product, in dry pyridine, was added dropwise to anhydrous ether with vigorous stirring giving a white solid. The identity of this solid was established, by base hydrolysis of a sample to the dinucleoside phosphate followed by chromatographic and n.m.r, comparison with an authentic sample of a-CpC (Wechter, 1967). The protected dinucleoside phosphate was then condensed with 5'-O-trityl-N^4-benzoyl-arabinosyl-cytosine-3'-phosphate as described above followed by both acid (80% acetic acid) and base (anhydrous ammonical methanol) hydrolyses. The water soluble product was chromatographed on AG 1-X2 (formate) resin eluted over a gradient of from 0.08 to 0.4 M ammonium formate (pH 5). Of the 500 fractions, 290-345 were combined, repeatedly freeze-dried to remove salts, then purified further by continuous flow electrophoresis (Ko et al. 1967), run in 0.5 M acetic acid (pH 2.3) at 1760 volts. In this manner three fractions were separated. The desired a-CpCpC (4775 OD units, 278 mμ) was found in tubes 18-23. The contents of these tubes (18-23 were combined and gave a white fluffy solid upon lyophilization. High voltage paper electrophoresis (pH 3.6, 4000 volts, 90 min) gave R_f relative to a-CpC 0.8. Paper chromatography (isopropyl alcohol-conc. NH_4OH-water 7:1:2) gave R_f 0.123 (a-Cp 0.155, a-CpC 0.24). Nuclear magnetic resonance spectrum (D_2O sol.,

SDSS ext. ref.) was consistant with this structure and indicated the unique environment of the 5'-terminal arabinosyl-C*; H6, 7.98δ (J+7) and 7.97δ (7)*: H5, 6.1δ, H1' (under 6.1): 5'-terminal CH_2* 3.95δ (m).

The enzymatic confirmation of structure and purity is presented in Fig. 1 (see below)

DNase I and pancreatic RNase were purchased from Worthington. Exonuclease originating from the venom of Crotalus adamanteus was prepared by Dr. Richards (Richards et al. 1967), mung bean nuclease was prepared according to Sung and Laskowski (1962), and passed through Sephadex G-100 (Johnson and Laskowski, unpublished) phosphatase-free micrococcal nuclease was prepared by Dr. Sulkowski (Sulkowski and Laskowski, 1966).

The incubation mixture consisted of 5.0 A_{271} units of substrate in 50 µl, and of 50 µl of an appropriate buffer (with Ca or Mg when needed) in which a graded amount of enzyme was dissolved. Enzyme was graded from 0.01 µg to 100 µg. The mixture was placed in a water bath at 37° for 2 hrs, heated for 10 min at 100°, and spotted on Whatman No. 1 paper. It was chromatographed in 75 parts of ethanol, 30 parts of 1 M ammonium acetate, pH 7.5, overnight. The spot corresponding to the undigested substrate was eluted and the amount of substrate hydrolyzed, expressed in percent of the original, was recorded in Table 1.

The results are self-explanatory. Of the two nucleases unspecific to deoxyribose and ribose, the mung bean nuclease is also unspecific to arabinose, and hydrolyzes a-CpCpC. However, micrococcal nuclease does not hydrolyze the derivatives of arabinose regardless of the amount of enzyme used. Among the unspecific nucleases different degrees of "blindness to

TABLE I

DIGESTION OF a-CpCpC BY DIFFERENT NUCLEASES

ENZYME / AMOUNT	0.01µg	0.1µg	1.0µg	10.0µg	100.0µg
PERCENT OF HYDROLYZED SUBSTRATE					
MBN-ase	0.0	16.9	46.5	62.3	
MN-ase	0.0	0.0	0.0	0.0	0.0
RN-ase	0.0	0.0	0.0	0.0	0.0
DN-ase	0.0	0.0	0.0	0.0	0.0
VENOM EXO	7.3	24.0	100.0		

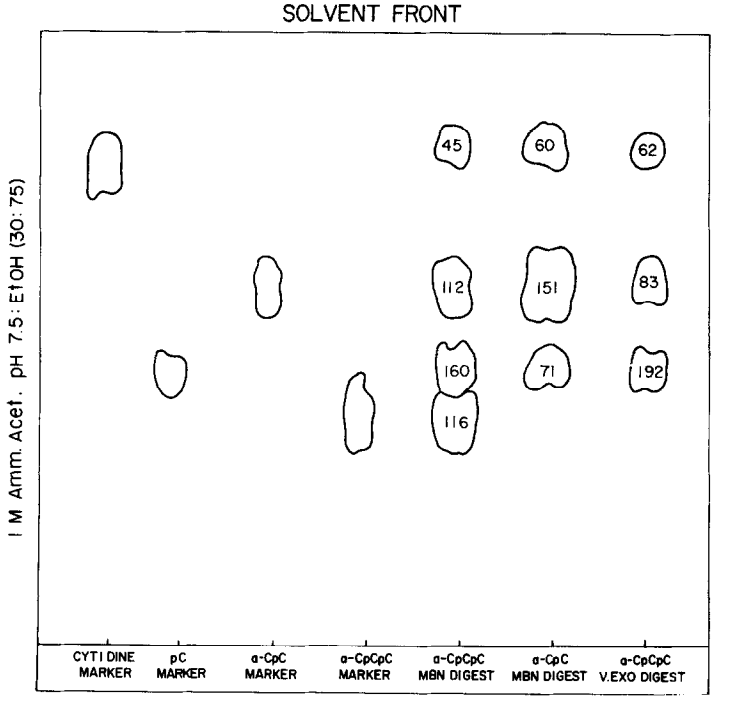

Fig. 1 Paper chromatography of digests. Markers: C, r-pC, a-CpC, a-CpCpC. Digests of a-CpCpC, 400 µmoles, and of a-CpC, 300 µmoles, with 1.63 µg of purified mung bean nuclease (MNB), 2 hrs at 37°, pH 5.0, 0.05 M Na-acetate. Digest of a-CpCpC, 400 µmoles, with 1 µg of venom exonuclease, 2 hrs, 37°, pH 8.9, 0.1 M Tris, 0.01 M $MgCl_2$ chromatography on Whatman No.1 paper, 17 hrs, 24°, in 1 M ammonium acetate: ethanol 30:75.

sugar" occur. Two sub-groups are: 1) totally unspecific e.g. venom exonuclease, mung bean nuclease, 2) unspecific to deoxyribose and ribose, but incapable of attacking arabinose e.g. micrococcal nuclease.

Fig. 1 illustrates the mechanism of action. Mung bean nuclease attacks this substrate in the same manner as venom exonuclease. Of the two possible cleavages the nucleotide with the free 3'-hydroxyl group is cleaved first. The reaction proceeds in two steps: a-CpCpC$\rightarrow$a-CpC + a-pC

a-CpC$\rightarrow$a-C + a-pC.

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