Heteropolydeoxynucleotides Synthesized with Terminal Deoxyribonucleotidyltransferase. II. Nearest Neighbor Frequencies and Extent of Digestion by Micrococcal Deoxyribonuclease*

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ABSTRACT: Nearest neighbor frequency determinations and extent of digestion by micrococcal deoxyribonuclease (DNase) have been done on all 11 possible heteropolymers prepared with deoxyribonucleoside 5′-triphosphates and terminal deoxyribonucleotidyltransferase. The results have shown that the heteropolymers are indeed copolymers and not a mixture of homopolymers and that their nucleotide sequences are largely random. Those dimer sequences that departed from random expectation did so almost to the same extent in all the polymers, and there was observed a marked similarity in frequency for inverted sequence dimer pairs. Data from the extensive digestion with micrococcal DNase of the four homopolymers and the six two-nucleotide heteropolymers were used along with their nearest neighbor frequencies to obtain per cent hydrolyses for each of the 16 dimer sequences. These values were used with high success to calculate the per cent polymer hydrolyses for the three- and four-nucleotide heteropolymers. In addition to providing further evidence for the greater extent of hydrolysis of d(XpA) and d(XpT) than d(XpC) and d(XpG), it was found that d(CpX) and d(GpX) were to a similar extent preferentially hydrolyzed over d(ApX) and d(TpX).

The synthesis of polydeoxyribonucleotides containing two or more of the different monomer units supplies important model compounds to enhance our understanding of native DNA structure and reactivity. Previous efforts toward synthesis of heteropolymers with terminal deoxyribonucleotidyltransferase (addase) have dealt with the incorporation of various deoxynucleotides from mixtures of their triphosphates (Ratliff *et al.*, 1967; Kato *et al.*, 1967). The present studies initially involved determination of nearest neighbor frequencies in the heteropolymers to show the extent of order in base sequence. A secondary profit of great value has been elucidation of relative cleavage susceptibility of the 16 possible dimer sequences to micrococcal DNase. This information has come from combination of polymer digestion data with nearest neighbor frequencies. The possibility for having and recognizing unique site cleavage of DNA by endonucleases is suggested.

Materials and Methods

Unlabeled deoxynucleoside triphosphates were purchased from Calbiochem, Nutritional Biochemicals Corp., and P-L Biochemicals, Inc. The labeled deoxyribonucleoside 5′-phosphates containing an α-^{32}P label were prepared as described by Lehman *et al.* (1958), and after separation of each of the monophosphates on a Dowex 1 (chloride form) column they were converted to the triphosphates by the procedure of Hoard and Ott (1965). The initiator d(pT)$_6$[1] was synthesized according to Khorana and Vizsolyi (1961). The addase was purified by the procedure of Yoneda and Bollum (1965). Micrococcal DNase was purchased from Miles Laboratories, Inc., and had a specific activity of 6000 units/mg of protein. Calf spleen phosphodiesterase was isolated by Hilmoe's (1960) procedure through step 5 and was further purified by the method of Richardson and Kornberg (1964) by chromatography on phosphocellulose. Alkaline phosphatase was obtained from Worthington Biochemical Corp.

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[1] Abbreviations used: dA, dC, dG, and dT are 2′-deoxyadenosine, 2′-deoxycytidine, 2′-deoxyguanosine, and 2′-deoxythymidine, respectively; dX and dY are used for collective representation of all four deoxynucleosides; dAp and dTp are 3′-nucleotides; 5′-triphosphates are represented as in dXTP; d(TpA) is thymidylyl-(3′,5′)-deoxyadenosine, with other dinucleoside monophosphates similarly designated; d(pT)$_6$ is the linear hexamer of 5′-thymidylic acid; d(A-T)$_n$ is the strictly alternating copolymer; d(C-C-T-T)$_n$ and d(A-T-C-A-A-T-T-C-C)$_n$ are similar polymers with more extended repeating units; (dC)$_n$·(dG)$_n$ is the hydrogen-bonded pair of homopolynucleotides; d(A,C)$_n$ is the essentially random copolymer, with other such heteropolymers similarly designated.

Experimental Procedure and Results

Enzymatic Synthesis of α-^{32}P-Labeled Heteropolymers. The reaction mixture was the same as that described previously by Ratliff *et al.* (1967), using 40 mM potassium phosphate (pH 7.0), 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 60 μg of addase, 1-μmole total of deoxynucleoside 5′-triphosphate, and 0.01 μmole of $d(pT)_6$ as initiator, except that α-^{32}P-labeled deoxyribonucleoside 5′-triphosphates (specific activity 0.5–1.0 × 10^7 cpm/μmole) were used in place of ^{14}C-labeled triphosphates. To obtain the 11 possible heteropolymers, each with a specific ^{32}P-labeled nucleotide, 28 reactions were required; the four homopolymers were also synthesized. Incubation was at 37° for 24 hr. Each mixture was then heated in a boiling water bath for 2 min, chilled in an ice bath, and centrifuged. The reaction mixtures were dialyzed against four changes of distilled water, 4 l. each.

Two-Step Nuclease Digestion of Heteropolymers to 3′-Mononucleotides. After dialysis, each ^{32}P-labeled heteropolynucleotide was treated with 8 μmoles of Tris buffer (pH 8.6), 4 μmoles of $CaCl_2$, and 1800 units of micrococcal DNase (total volume, 2 ml). The mixture was incubated at 37° for 20 hr and assayed, according to the procedure of Josse *et al.* (1961), for ^{32}P which had become susceptible to the action of alkaline phosphatase (Table I). The number at the bottom of each of the columns of figures represents the average cleavage by the DNase of the 5′-^{32}P bond from the same nucleoside in the various polymers. These values are dependent to different extents upon the base and nearest neighbor frequencies in each polymer. Later data will include consideration of these points.

The pH of each micrococcal DNase digest was then adjusted to 7.0 with 0.1 N HCl, 0.5 unit of calf spleen phosphodiesterase was added, and the mixture was incubated at 37°. Another 0.5 unit was added at the end of the first and second hours.[2] The total time of incubation was 3 hr. At this point, aliquots were removed from each digestion mixture and assayed for completeness of digestion as judged by the fraction of ^{32}P which had become susceptible to the action of alkaline phosphatase. After the calf spleen phosphodiesterase digestions, 95% or more of the radioactivity was phosphomonoesterase sensitive. The digests were then lyophilized and dissolved in 0.1–0.2 ml of water.

Each sample was subjected to electrophoresis according to the procedure of Markham and Smith (1952): Whatman 3MM paper, 0.05 M ammonium formate buffer (pH 3.5), 4000 v, and 2–2.5 hr. The radioactive spots were located with a Vanguard Autoscanner (Model 880), cut out, and counted in a Packard Tri-Carb liquid scintillation counter.

Nearest Neighbor Frequency Calculations. Addase acts by adding nucleotides to the 3′-hydroxyl end of the growing nucleotide chain with the elimination of pyrophosphate. Both micrococcal DNase and calf spleen phosphodiesterase yield 3′-nucleotides. Therefore, the phosphorus atom initially on the 5′ position of the incoming nucleotide after digestion is attached to the 3′ position of the adjacent nucleotide. Therefore, with the aid of α-^{32}P-labeled triphosphates the frequency with which one nucleotide is adjacent to another can be determined. All the results from nearest neighbor frequency calculations appear in Tables II–IV; the methods of calculation are according to Josse *et al.* (1961). The radioactivity recovered in each 3′-nucleotide in a digest was normalized so that the sum of the radioactivity in all those labeled nucleotides was equal to unity. The normalized radioactivity for each nucleotide in a digest appears under the column entitled Radioactivity Fraction. The radioactivity fraction data are averages of at least three different replicative sets of syntheses and hydrolyses, and the fraction data within each set agreed within 5%.

Mole fractions of each of the nucleotides in a polymer were calculated by combining all the radioactivity fraction data for that polymer through the relationship that the total amount of a base incorporated as a 5′-nucleotide should equal the total amount recovered after digestion as 3′-nucleotides. The radioactivity fraction multiplied by its corresponding mole fraction gave the nearest neighbor frequency.

The prediction of complete randomness on the basis that the frequency of a given nearest neighbor or dinucleotide sequence [*e.g.*, fd(TpA)] in a particular polynucleotide would be equal to the product of the mole fractions of the two constituent mononucleotides [*e.g.*, (fdTp × fdAp)] in that polymer, as proposed

TABLE I: Per Cent Hydrolysis of ^{32}P-Labeled Polymer Bonds by Micrococcal DNase.

Polymer	Nucleotide with 5′-^{32}P			
	dpA	dpC	dpG	dpT
$(dA)_n$	78			
$(dC)_n$		72		
$(dG)_n$			46	
$(dT)_n$				72
$d(A,C)_n$	86	39		
$d(A,G)_n$	89		32	
$d(A,T)_n$	69			79
$d(C,G)_n$		74	52	
$d(C,T)_n$		50		82
$d(G,T)_n$			49	88
$d(A,C,G)_n$	91	60	49	
$d(A,C,T)_n$	82	38		82
$d(A,G,T)_n$	81		41	80
$d(C,G,T)_n$		63	55	89
$d(A,C,G,T)_n$	82	53	44	88
Av	82	56	46	83

[2] The total units of spleen phosphodiesterase used for the hydrolysis of the heteropolymers was the amount necessary for the complete hydrolysis of the most resistant heteropolymers: those prepared with α-^{32}P-labeled dGTP and dCTP.

TABLE II: Nearest Neighbor Frequency Calculations for Two-Nucleotide Heteropolydeoxynucleotides.

Polymer	Labeled Triphosphate	Mole Fraction	Sequence	Radioactivity Fraction	Nearest Neighbor Frequency (%)		Deviation (ppt)
					Calcd	Found	
$d(A,C)_n$	dATP	0.56	d(ApA)	0.567	0.314	0.319	+5
			d(CpA)	0.433	0.246	0.244	−2
	dCTP	0.44	d(ApC)	0.558	0.246	0.244	−2
			d(CpC)	0.442	0.194	0.193	−1
							3
$d(A,G)_n$	dATP	0.52	d(ApA)	0.496	0.270	0.259	−11
			d(GpA)	0.504	0.250	0.263	+13
	dGTP	0.48	d(ApG)	0.551	0.250	0.263	+13
			d(GpG)	0.449	0.230	0.215	−15
							13
$d(A,T)_n$	dATP	0.33	d(ApA)	0.404	0.109	0.134	+25
			d(TpA)	0.596	0.221	0.197	−24
	dTTP	0.67	d(ApT)	0.295	0.221	0.197	−24
			d(TpT)	0.705	0.449	0.472	+23
							24
$d(C,G)_n$	dCTP	0.49	d(CpC)	0.570	0.240	0.280	+40
			d(GpC)	0.430	0.250	0.212	−38
	dGTP	0.51	d(CpG)	0.416	0.250	0.211	−38
			d(GpG)	0.584	0.260	0.297	+37
							38
$d(C,T)_n$	dCTP	0.51	d(CpC)	0.500	0.260	0.254	−6
			d(TpC)	0.500	0.250	0.254	+4
	dTTP	0.49	d(CpT)	0.517	0.250	0.254	+4
			d(TpT)	0.483	0.240	0.238	−2
							4
$d(G,T)_n$	dGTP	0.66	d(GpG)	0.710	0.436	0.469	+33
			d(TpG)	0.290	0.224	0.192	−32
	dTTP	0.34	d(GpT)	0.566	0.224	0.192	−32
			d(TpT)	0.434	0.116	0.147	+31
							32

by Josse *et al.* (1961) in studies of DNA, was applied to give the calculated nearest neighbor frequencies of Tables II–IV. The deviations (in parts per thousand) of each nearest neighbor frequency from the calculated for complete randomness are entered in Tables II–IV. In order to have a single number to describe each polymer, the absolute values of each set of deviations have been averaged. These average deviations appear to the right just below the line under each set.

Another use made of the deviations in Tables II–IV was to compile the data of each of the 16 possible dimer sequences one at a time through all the polymers in which it occurred by summing the actual values of the deviations and dividing by the number of polymers that gave the data. Since the signs exert either a cancelling or reenforcement during the addition, the result is called the average net deviation. It indicates the over-all prevalence of a given dimer sequence in the polymers. The results of this operation are shown in Table V.

Extent of Cleavage of Dimer Sequences by Micrococcal DNase. The data of Tables I and II were combined to calculate per cent hydrolysis by micrococcal DNase of the 16 dimer sequences (Table VI). For a polymer synthesized using a specific α-^{32}P-labeled nucleoside 5′-triphosphate, there are both a micrococcal DNase digestion value in Table I and a set of nearest neighbor frequencies in Tables II–IV. These numbers are related to the individual dimer sequence hydrolyses in that the sum of the products of individual per cent hydrolysis times nearest neighbor frequency is equal to the product of the polymer per cent digestion times the sum of the nearest neighbor frequencies [*e.g.*, for d(CpA), $78(0.319) + X(0.244) = 86(0.563)$, $X = 96$]. Thus it required only the one-

TABLE III: Nearest Neighbor Frequency Calculations for Three-Nucleotide Heteropolydeoxynucleotides.

Polymer	Labeled Triphosphate	Mole Fraction	Sequence	Radioactivity Fraction	Nearest Neighbor Frequency (%) Calcd	Found	Deviation (ppt)	
$d(A,C,G)_n$	dATP	0.33	d(ApA)	0.298	0.109	0.097	−12	
			d(CpA)	0.308	0.099	0.101	+2	
			d(GpA)	0.393	0.122	0.129	+7	
	dCTP	0.30	d(CpC)	0.362	0.090	0.109	+19	
			d(ApC)	0.303	0.099	0.092	−7	
			d(GpC)	0.335	0.111	0.101	−10	
	dGTP	0.37	d(GpG)	0.376	0.137	0.139	+2	
			d(ApG)	0.375	0.122	0.138	+16	
			d(CpG)	0.249	0.111	0.092	−19	
								10
$d(A,C,T)_n$	dATP	0.31	d(ApA)	0.340	0.096	0.106	+10	
			d(CpA)	0.313	0.102	0.098	−4	
			d(TpA)	0.347	0.112	0.109	−3	
	dCTP	0.33	d(CpC)	0.331	0.109	0.107	−2	
			d(ApC)	0.268	0.102	0.087	−15	
			d(TpC)	0.401	0.119	0.130	+11	
	dTTP	0.36	d(TpT)	0.341	0.130	0.123	−7	
			d(ApT)	0.330	0.112	0.119	+7	
			d(CpT)	0.329	0.119	0.119	0	
								7
$d(A,G,T)_n$	dATP	0.33	d(ApA)	0.284	0.109	0.092	−17	
			d(GpA)	0.347	0.106	0.113	+7	
			d(TpA)	0.369	0.116	0.120	+4	
	dGTP	0.32	d(GpG)	0.386	0.102	0.125	+23	
			d(ApG)	0.349	0.106	0.113	+7	
			d(TpG)	0.265	0.112	0.086	−26	
	dTTP	0.35	d(TpT)	0.414	0.123	0.145	+22	
			d(ApT)	0.342	0.116	0.120	+4	
			d(GpT)	0.244	0.112	0.086	−26	
								15
$d(C,G,T)_n$	dCTP	0.33	d(CpC)	0.394	0.109	0.131	+22	
			d(GpC)	0.339	0.125	0.113	−12	
			d(TpC)	0.267	0.096	0.089	−7	
	dGTP	0.38	d(GpG)	0.479	0.144	0.183	+39	
			d(CpG)	0.297	0.125	0.113	−12	
			d(TpG)	0.224	0.110	0.086	−24	
	dTTP	0.29	d(TpT)	0.388	0.084	0.111	+27	
			d(CpT)	0.310	0.096	0.088	−8	
			d(GpT)	0.302	0.110	0.086	−24	
								19

and two-base polymers to derive a complete set of numbers to fill Table VI. However, in each case of a specific second base in the nearest neighbor sequence, there were also three three-nucleotide and one four-nucleotide polymers with which to test all the preliminary values. Good agreement was observed between the calculated and found hydrolysis values with the exception of $d(A,C,G\text{-}^{32}P)_n$ (calcd 40%; found 49%) and $d(A,G,T\text{-}^{32}P)_n$ (calcd 87%; found 80%) and are given in Table VII. An alternative method was available for calculating d(XpX) per cent hydrolysis by solving simultaneous equations using data from two-

TABLE IV: Nearest Neighbor Frequency Calculations for $d(A,C,G,T)_n$.

Polymer	Labeled Triphosphate	Mole Fraction	Sequence	Radioactivity Fraction	Nearest Neighbor Frequency (%)		Deviation (ppt)
					Calcd	Found	
$d(A,C,G,T)_n$	dATP	0.22	d(ApA)	0.225	0.048	0.047	−1
			d(CpA)	0.196	0.053	0.041	−12
			d(GpA)	0.350	0.064	0.072	+8
			d(TpA)	0.229	0.055	0.047	−8
	dCTP	0.24	d(CpC)	0.288	0.058	0.069	+11
			d(ApC)	0.160	0.053	0.038	−15
			d(GpC)	0.269	0.070	0.064	−6
			d(TpC)	0.283	0.060	0.068	+8
	dGTP	0.29	d(GpG)	0.317	0.084	0.093	+9
			d(ApG)	0.288	0.064	0.085	+21
			d(CpG)	0.209	0.070	0.061	−9
			d(TpG)	0.186	0.073	0.055	−18
	dTTP	0.25	d(TpT)	0.326	0.063	0.085	+22
			d(ApT)	0.201	0.055	0.052	−3
			d(CpT)	0.249	0.060	0.064	+4
			d(GpT)	0.224	0.073	0.058	−15
							11

and three-nucleotide polymers. The d(XpX) data from this treatment were in good agreement (3.5% average deviation) with the homopolymer data. The number at the bottom of each of the columns of Table VI is the average micrococcal DNase per cent hydrolysis of the dimer frequencies with the same 5′-linked nucleoside, and the averages at the right hand of Table VI are hydrolysis of the dimer frequencies with the same 3′-linked nucleoside.

Discussion

The eleven heteropolydeoxynucleotides and four homopolymers reported by Ratliff *et al.* (1967) have been resynthesized using one α-^{32}P-labeled deoxynucleoside 5′-triphosphate in each reaction. Each of the 32 products was digested and analyzed for its nearest neighbor frequencies according to Josse *et al.* (1961). The results of the micrococcal DNase step (Table I) as averaged for each labeled triphosphate agree with the previously reported observations (Roberts *et al.*, 1962) on calf thymus DNA in that the d(XpA) and d(XpT) types of bonds are preferentially cleaved over those of d(XpC) and d(XpG).

Copolymerization of dATP and dTTP with *Escherichia coli* DNA polymerase gives $d(A\text{-}T)_n$ with zero d(XpX) frequency; by using dCTP and dGTP with the same enzyme, $(dC)_n \cdot (dG)_n$ results with zero d(XpY) frequency (Josse *et al.*, 1961). The found nearest neighbor frequency data in Tables II–IV show by the significant values for d(XpY) sequences that when two or more different deoxynucleoside 5′-triphosphates were copolymerized every product was, for the most part, a copolymer and not a mixture of homopolymers.

TABLE V: Average Net Deviations from Randomness of Nearest Neighbor Frequencies.

3′-Linked Nucleoside	5′-Linked Nucleoside			
	dA	dC	dG	dT
dA	0	−10[a]	+14	−4
dC	−4	+12	−20	0
dG	+9	−17	+18	−24
dT	−8	+4	−25	+17

[a] All numbers in this table are parts per thousand.

TABLE VI: Per Cent Hydrolysis of Dimer Sequences with Micrococcal DNase.

3′-Linked Nucleoside	5′-Linked Nucleoside				
	dA	dC	dG	dT	Av
dA	78	13	21	96	52
dC	96	72	60	91	80
dG	100	77	46	100	81
dT	63	28	56	72	55
Av	84	48	46	90	

Likewise, the data for d(XpX) sequences show by their magnitude that strictly alternating structures are not significantly present in the heteropolymers, especially in $d(A,T)_n$ and $d(G,T)_n$ because of the great preponderance of incorporation of one nucleotide over the other.

The average deviation for each polymer, obtained by averaging the absolute values of the individual deviations, is assumed to have zero value for complete randomness. Among the two-nucleotide heteropolymers the least random are $d(C,G)_n$ with almost equal mole fractions and $d(A,T)_n$ and $d(G,T)_n$ with the most different mole fractions within a polymer. In all three cases, both the d(XpX) and d(YpY) sequences are in excess over the random prediction, while d(XpY) and d(YpX) are below. Extent of randomness is clearly dependent on more than nucleotide composition and may be to a large extent an intrinsic property of the sequences; this is further borne out by comparison of $d(C,G)_n$ and $d(C,T)_n$ with identical mole fractions but very different randomness. The most random polymers are $d(A,C)_n$, $d(C,T)_n$, and $d(A,C,T)_n$. However, *highly ordered* sequences such as in the hypothetical polymers $d(C\text{-}C\text{-}T\text{-}T)_n$ and $d(A\text{-}T\text{-}C\text{-}A\text{-}A\text{-}C\text{-}C\text{-}T\text{-}T)_n$ both have the "completely random" average deviation of zero. The nearest neighbor data do not distinguish between these sets of cases. Such ordered structures are thought to be highly unlikely, but proof of this must come from other experiments. In progress are studies of mono- and oligonucleotide products from chemical degradation of some of these polymers. Preliminary evidence on all-pyrimidine runs is that lengths ranging from monomer to greater than tetramer exist within $d(A,C)_n$, $d(A,T)_n$, $d(C,G)_n$, and $d(G,T)_n$. This supports the concept of extensive randomness of sequence in these heteropolymers.

A further use of the individual deviations was to consider each nearest neighbor sequence through all the polymers it appeared in and to average the deviations, conserving the sign. The results in Table V tend to be excessively negative or positive, depending on a great over-all lack or presence of that sequence; a value near zero shows that the sequence is generally present at about its random frequency. A geometric pattern of quantity and sign is present in Table V; the net deviation for d(XpY) is always near that of d(YpX). The sequences d(ApG) and d(GpA) were the most frequent of the d(XpY) type, and d(GpG) followed by d(TpT) were the most prominent of all. The least likely sequences were the pair d(TpG) and d(GpT).

Of special interest from this study of heteropolydeoxynucleotide sequence using nearest neighbor frequency analysis was the determination of individual dimer sequence per cent hydrolysis effected by extensive digestion with micrococcal DNase. Combination of the nearest neighbor frequencies (Table II) with data from the polymer digestion (Table I) that was part of the nearest neighbor procedure gave the desired results (Table VI). These were used with the nearest neighbor frequencies of the three- and four-nucleotide heteropolymers to calculate their per cent hydrolyses by micrococcal DNase under all the conditions of ^{32}P labeling in Table I. The last two columns of data in Table VII show good agreement for the final results of the calculations.

TABLE VII: Prediction of Per Cent Hydrolysis of ^{32}P-Labeled Bonds in Three- and Four-Nucleotide Heteropolymers by Micrococcal DNase.

Polymer	[5′-^{32}P]-Nucleotide	% Hydrolysis	
		Calcd	Found[a]
$d(A,C,G)_n$	dpA	92	91
	dpC	56	60
	dpG	40	49
$d(A,C,T)_n$	dpA	78	82
	dpC	38	38
	dpT	86	82
$d(A,G,T)_n$	dpA	80	81
	dpG	40	41
	dpT	87	80
$d(C,G,T)_n$	dpC	62	63
	dpG	52	55
	dpT	86	89
$d(A,C,G,T)_n$	dpA	86	82
	dpC	51	53
	dpG	44	44
	dpT	88	88

[a] Table I.

The averages at the bottom of Table VI represent relative extents to which d(XpA), d(XpC), d(XpG), and d(XpT) were hydrolyzed and support the results of other work (Laskowski, 1967) that d(XpA) and d(XpT) bonds are preferentially cleaved over the d(XpC) and d(XpG) linkages. This is further substantiated by the results of DeMeuron-Landolt and Privat deGarilhe (1964) and Alexander *et al.* (1961), who reported that dinucleotides are not cleaved by micrococcal DNase. Therefore, the majority of the dinucleotides present after digestion of a heteropolymer with micrococcal DNase would have to be those containing nucleotide sequences which sometime during the degradation are the most difficult to cleave.

The averages at the right hand of Table VI are per cent hydrolyses for d(ApX), d(CpX), d(GpX), and d(TpX); of these, d(CpX) and d(GpX) were most hydrolyzed. Considering the sequences of groups of four according to the general base designations 6-keto, 6-amino, purine, or pyrimidine, the largest hydrolysis average was 72% for purine-*p*-pyrimidine. The nondescript group d[(C or G)-*p*-(A or T)] gave 97% as its average hydrolysis.

Experiments are in progress to ascertain which bonds in these heteropolymers are broken in the earliest stages of micrococcal nuclease digestion. The group in

Table VI that was hydrolyzed to the greatest extent should include those sequences most rapidly hydrolyzed.

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Localization of Sparsomycin Action to the Peptide-Bond-Forming Step*

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ABSTRACT: Sparsomycin, a sulfur-containing antibiotic, which inhibits the puromycin-induced release of peptide from ribosomes, is a highly effective inhibitor of the single addition of [^{14}C]lysine residues onto polylysyl transfer ribonucleic acid (tRNA) bound to ribosomes. This latter reaction is inhibited 50% by 10^{-7} M sparsomycin.

Antibiotics such as erythromycin, chlortetracycline, and puromycin are somewhat less effective inhibitors; chloramphenicol and gougerotin are much less effective. Inhibition can not be reversed by increasing the concentration of lysyl-tRNA, polylysyl-tRNA, or ribosomes. The degree of inhibition is constant throughout the course of the addition reaction. These results provide further evidence for the action of sparsomycin on peptide-bond formation itself.

Previous work from this laboratory (Goldberg and Mitsugi, 1966, 1967a,b) has shown that sparsomycin, a sulfur-containing antibiotic, is a potent inhibitor of *in vitro* polypeptide synthesis and that its inhibitory action is probably exerted at or close to the peptide-bond-forming step. Sparsomycin, at very low levels, blocks the puromycin-induced release of polylysine from polylysyl-tRNA bound to *Escherichia coli* ribosomes. The formation of polylsylpuromycin, which takes place at 0.01 M Mg^{2+} in the absence of added GTP[1] and other factors, serves as a model for the formation of a single peptide bond. Sparsomycin neither causes the deacylation of polylysyl-tRNA nor affects its binding to ribosomes but acts as a competitive inhibitor of puromycin in this reaction. In order to obtain further evidence in support of the contention that sparsomycin acts on peptide-bond formation and not on the other reactions involved in peptide chain elongation, we have studied its effect on the limited addition of lysine units to polylysyl-tRNA bound to ribosomes. This reaction, which has been characterized by Gottesman

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[1] Abbreviations used: GTP, guanosine triphosphate, TCA, trichloroacetic acid.