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Interaction of Metal Ions with Nucleic Acids and Related Compounds. XVII. On the Mechanism of Degradation of Polyribonucleotides and Oligoribonucleotides by Zinc(II) Ions*

James J. Butzow and Gunther L. Eichhorn

ABSTRACT: Zinc(II) cleavage at neutral pH of the 3′–5′-phosphodiester bonds in homopolyribonucleotides and various oligoribonucleotides is found to occur with intermediate formation of 2′–3′-cyclic phosphate, whose subsequent opening to a mixture of the 2′ and 3′ forms is also promoted by zinc. In the degradation of trinucleoside diphosphates, there is a preference toward cleavage at the phosphodiester bond nearer the 5′ end. Susceptibility to zinc degradation increases with chain length to the tetramer level, where it becomes similar to that of the polymer. The course of degradation of homopolymers, the cleavage preference in a trinucleoside diphosphate, and the effect of chain length are all correlated with an accentuation of cleavage rate dependent on the existence, configuration, and charge of an adjacent phosphate group. The rates of cleavage of the internal phosphodiester bonds of adenosine dimers and ApApA fall in the order: ApA3′p ≫ ApApA > ApA2′p ≥ ApA2′–3′(cyclic)p ≥ ApApA ≥ ApA.

The degree of preference for cleavage at the phosphodiester bond nearer the 5′ end is greater for ApApA than ApApU, much greater for ApApG than ApApU, and much greater for UpUpG than UpUpU, reflecting the greater tendency to cleave next to U and the lesser tendency to cleave next to G found in the zinc degradation of RNA. Moreover, the presence of G at the 3′ end of these trinucleoside diphosphates leads to a marked increase in the actual cleavage rate of the phosphodiester bond nearer the 5′ end.

Zinc(II) depolymerization of polyribonucleotides can occur efficiently with a high nucleoside base specificity. At neutral pH and elevated temperature, and with a small excess of zinc ion over phosphate, poly(rI) is degraded much more slowly than poly(rA), poly(rC), or poly(rU) (Butzow and Eichhorn, 1965; Eichhorn *et al.*, 1971). With natural RNA there is very significantly reduced cleavage next to guanosine residues and increased cleavage next to uridine residues (Eichhorn *et al.*, 1971); different conditions of pH, temperature, and zinc concentration lead to a modified or accentuated cleavage pattern.

Although the zinc depolymerization is known (Butzow and Eichhorn, 1962) to operate by breakage of phosphodiester bonds with retention of phosphate at the 3′(2′) position of the ribose, the mechanism has not been well understood. The 2′ hydroxyl is required—DNA is not readily degraded by metal ions; thus, it was plausibly suggested (Bamann *et al.*, 1954; Eichhorn and Butzow, 1965) that degradation by metal ions proceeds through chelation of the metal between the phosphate and the 2′ hydroxyl.

Making use of simpler substrates, ribooligomers of defined nucleoside sequence, we now examine in some detail the breakage of the phosphodiester bond itself together with the influence of chain length and other phosphate groups in the chain, and of the nucleoside bases.

Experimental Section

Materials

Poly(rA), poly(rI), poly(rC), and poly(rU) (Na, K, or NH_4 salts) were obtained from Miles Laboratories, as were oligoadenylates $(Ap)_{1-5}A>p$[1] (Li salts).

ApApA, ApApG, ApApU, UpUpU, UpUpG, and CpCpC (Li salts) were from Miles Laboratories; another batch of UpUpG was kindly donated by Drs. Sober and Simpson.

IpIpI was prepared by KOH degradation of poly(rI), elimination of excess KOH as $KClO_4$ by neutralization with $HClO_4$, treatment with 0.1 M HCl (3 hr, 20–25°) as a precaution to open any residual 2′–3′-cyclic phosphates (see Heppel *et al.*, 1957; Simpkins and Richards, 1967a), phosphomonoesterase treatment, desalting on charcoal, and thin-layer chromatography on cellulose (30:70, 1 M aqueous NH_4OAc–95% ethanol) followed by extraction with H_2O and vacuum evaporation (30°) of the NH_4OAc from aqueous solutions. By two-dimensional thin-layer chromatography, the preparation contained 95.5% IpIpI, 0.3% IpI>p, and 4.1% IpI2′p plus IpI3′p.

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[1] Abbreviations used are: A, G, I, U, and C represent the ribonucleosides adenosine, guanosine, inosine, uridine, and cytidine; N represents any ribonucleoside. A diester phosphate group in 3′–5′ linkage is designated by NpN, a 3′-monoester phosphate by N3′p, a 2′-monoester phosphate by N2′p, and a 2′–3′-cyclic diester phosphate by N>p.

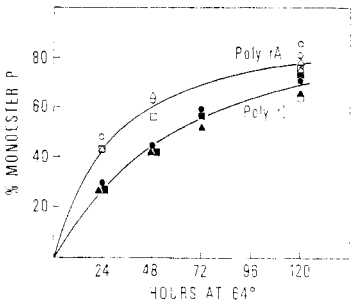


FIGURE 1: Comparison of the course of zinc degradation of poly(rA) and of poly(rI) at pH 7.0, 64°, 2 Zn/nucleoside residue, in the presence of 1.0×10^{-2} M $NaNO_3$ (△,▲), in the absence of added salt (○,●), and in the absence of added salt but with 1 Li/nucleoside residue (□,■) as $LiClO_4$. Extent of reaction is assayed as orthophosphate released by phosphomonoesterase.

A mixture of ApA2′p and ApA3′p was prepared by KOH degradation of poly(rA), elimination of excess KOH as $KClO_4$ by neutralization with $HClO_4$, and thin-layer chromatography on cellulose (80:18:2, saturated aqueous $(NH_4)_2SO_4$–1 M aqueous HOAc–2-propanol) followed by extraction with H_2O and desalting on charcoal (elution by aqueous ethanol–ammonia, then vacuum evaporation of solvent: the charcoal had been exhaustively extracted with ethanol–ammonia, then acid reactivated). By two-dimensional thin-layer chromatography the preparation contained 98.8% ApA2′p plus ApA3′p and 1.2% A>p.

ApA, ApG, ApU, and CpC were from Calbiochem, UpU was from Sigma, and IpI was from Miles Laboratories.

A>p (Na salt) was from Sigma; G>p and U>p (Ba salts, converted into Na by Dowex 50 (Na) treatment), and C>p (Na salt) were from Schwarz BioResearch. Various 3′- or 3′(2′)-mononucleotides and various nucleosides were from Sigma or Calbiochem-Boehringer.

Phosphomonoesterase (*Escherichia coli* alkaline phosphatase, EC 3.1.3.1, chromatographically purified) was obtained from Worthington Biochemical.

Methods

Initial reaction mixtures for zinc degradations were made by mixing $Zn(NO_3)_2$ and substrate to near final substrate concentration of 1.0×10^{-4} M nucleoside base, and with 2 Zn/nucleoside residue, with or without $NaNO_3$ (final concentration, 1.0×10^{-2} M), then the pH was adjusted to 7.0 by addition of dilute NaOH. No buffer was used; the pH fell during the course of the reactions to the extent of 0.3 or 0.4 pH unit.

Previously (Eichhorn and Butzow, 1965; Butzow and Eichhorn, 1965; Eichhorn *et al.*, 1971) we have included neutral salt (1×10^{-2} M $NaNO_3$) in our reaction mixtures. The salt interferes with the thin-layer chromatographic analysis we now employ, so that experiments conducted in the presence of salt may require a desalting step, a complication especially with the small amounts of oligomers we must use. Omission of the salt during polymer degradation, however, does not appear to affect significantly either the course of reaction, as determined by monoester phosphate (Figure 1), or the amounts of fragments produced after extensive reaction (Table I). The lithium contained as the counterion with most of the oligomers also does not appear to affect polymer degradation (Figure 1 and Table I). In view of these circumstances, all of the oligomer experiments we describe here have been conducted without added salt and without removal of lithium.

Reactions were terminated by lowering the temperature to 0–5° in the case of zinc, and neutralization in the case of KOH (with $HClO_4$, if excess salt was to be eliminated). Before chromatography, and generally before any other processing, zinc was removed using Dowex 50 (Na).

The techniques for dephosphorylation using phosphomonoesterase, and for desalting on charcoal are described elsewhere (Eichhorn *et al.*, 1971).

Separation of components of reaction mixtures was accomplished by ascending thin-layer chromatography on 20 × 20 cm glass plates coated with cellulose powder (Machery-Nagel, MN-300, applied as a slurry of 15 g/90 ml of H_2O), 0.25 mm (wet) for analytical and 1 mm (wet) for preparative plates. Before separation of fragments from polymer degradations, their terminal phosphates were eliminated by phosphomonoesterase treatment in order to simplify the chromatography; the reaction mixtures were then desalted on charcoal before chromatography. For most analyses, two-dimensional chromatography was used, with mixtures of 1 M aqueous NH_4OAc and 95% ethanol in the first dimension, and 80:18:2 saturated aqueous $(NH_4)_2SO_4$–1 M aqueous NaOAc (or HOAc, for A fragments)–2-propanol in the second. One-dimensional chromatography alone, with the first type of solvent, could suffice for phosphomonoesterase-treated polymer fragments; and was also conveniently employed for separation of ApA2′p plus ApA3′p degradation mixtures into A2′p plus A3′p, A>p, and ApA2′p plus ApA3′p (no separation of 2′- and 3′-phosphate isomers).

For quantitation of chromatograms the ultraviolet-absorbing zones, and adjacent blanks of equal areas, were scraped off the plates, eluted with 0.1 M HCl for 2 hr at 20–25° and the cellulose removed by filtration through acid-washed Millipore filters (GS, 0.22 μ), and spectra of the 0.1 M HCl solutions taken with a Cary 14 spectrophotometer. In calculating proportions of fragments no correction for different extinction coefficients was made when all (monomers, dimers, trimers) were of the same base, since the differences in extinction coefficients in 0.1 M HCl should be very small for them (see Table II of Toal *et al.*, 1968). Otherwise, the following molar (nucleoside residue) coefficients ($\times 10^{-3}$) were applied at 260 nm: ApApG, 13.0; ApApU, 12.4; UpUpG, 9.9; A dimers, 14.0; ApG, 12.5; ApU, 11.4; U dimers, 9.3; UpG, 10.0; A monomers, 14.0; G monomers, 11.8; U monomers, 9.5. In the cases of UpUpU and UpUpG fragments, UpU2′p, UpU-3′p, and UpU>p were not resolved from UpUpU, and not observed as resolved (except at high levels) from UpG, respectively. In both cases, amounts of UpU2′p plus UpU3′p plus UpU>p are calculated using

$$[\mathrm{NpN2'p} + \mathrm{NpN3'p} + \mathrm{NpN{>}p}] = [\mathrm{N}] - \tfrac{1}{2}([\mathrm{N2'p} + \mathrm{N3'p} + \mathrm{N{>}p}] - [\mathrm{NpN}])$$

which follows from the zinc reaction pathways starting with a trinucleoside diphosphate; these are deducted from the apparent amounts of UpUpU (proportionately) or UpG.

For determination of total breaks, used in finding reaction rates of NpN, orthophosphate was determined (Chen *et al.*, 1956) after treatment with 0.1 M HCl for 3 hr at 20–25° to open 2′–3′-cyclic phosphate (see Heppel *et al.*, 1957) and treatment with phosphomonoesterase. For determination of net phosphomonoester ends, in determining rates of opening N>p, and in assessing overall reactivity of $(Ap)_2A{>}p$, (Ap)-A>p, $(Ap)_4A{>}p$, $(Ap)_5A{>}p$, poly(rA), and poly(rI), this procedure was followed with omission of the HCl treatment. In all these cases, the maximum extent of reaction (100% monoes-

ter phosphate) was estimated from the absorbance of the initial sample: for NpN and N>p, in 0.1 M HCl using the extinction coefficients given in the preceeding paragraph; for $(Ap)_2A>p$, $(Ap)_3A>p$, $(Ap)_4A>p$, and $(Ap)_5A>p$, in 0.1 M HCl at the peak, 257 nm, using as molar (nucleoside residue) extinction coefficients 14.5×10^3, 14.2×10^3, 14.0×10^3, and 13.7×10^3, respectively (based on 15.1×10^3 for A3'p and a small decrement to correct for hypochromicity as noted in Table II of Toal *et al.*, 1968); and for poly(rA) and poly(rI), in 0.02 M NaOH using the extinction coefficients given by Eichhorn *et al.* (1971).

The various rate constants are computed from slopes within linearized data from the rate studies, or by a combination of rate constants from such slopes. The experimental uncertainty in a rate constant from an individual rate study will be expressed using the weighted mean deviation of the slope

$$\Sigma\left(\left|\frac{y_i}{x_i} - \text{slope}\right| x_i\right) \Big/ \Sigma x_i$$

where y is a concentration function, x is a time function, and the zero point is fixed at the origin. From duplicate rate studies, the rate constants are averaged weighting by their deviations, and the deviations are averaged weighting by the sums of experimental times. The quantitative repeatability of the trinucleoside diphosphate rate studies was tested by performing duplicate rate studies for two substrates having widely different behavior, UpUpU and UpUpG, as well as for ApApA: the paired rates differed from the mean by about ±6, ±7, and ±22%, respectively, while the paired ratios of the rate of cleavage at one site to that for cleavage at both sites differed from the mean by less than ±2% in all three cases.

Results and Discussion

Phosphodiester-Bond Breakage. COURSE OF HOMOPOLYMER DEGRADATION. Zinc degradation of ribopolymers (pH 7.0, 64°, 2 Zn/nucleoside residue) as measured by production of monoester phosphate ends appears to level off (after about 120 hr) at less than 100% of the possible extent of reaction (Eichhorn *et al.*, 1971). Various phosphodiester compounds can be detected in significant amounts after such a time in reaction mixtures from poly(rA), poly(rI), poly(rC), and poly(rU), notably N>p and NpN2'p and/or NpN3'p, and to lesser extents NpN>p and NpNpN2'p and/or NpNpN3'p. Table I gives the results of chromatographic separation (after removal, for technical reasons, of monoester phosphates). Although such data do not in themselves have kinetic significance, the relative amounts of components shown suggests that some of the phosphodiester-containing fragments may be relatively resistant to degradation.

The fact that 2'–3'-cyclic phosphodiesters, namely, N>p and NpN>p, are found in the reaction mixtures strongly suggests that the zinc degradation goes through a 2'–3'-cyclic phosphate form (as in acid and alkaline degradation; Brown and Todd, 1952), rather than through a chelate ring as previously postulated. Much of the following discussion is concerned with the elucidation of formation and breakdown of this cyclic phosphate.

In our previous investigation we had identified the 3'-mononucleotides from such reaction mixtures of poly(rA) and poly(rI), but not the 2'-mononucleotides (Butzow and Eichhorn, 1965); we have reinvestigated this for poly(rA) using more sensitive thin-layer chromatographic techniques, and actually detect both A3'p and A2'p. The ratio of amounts of A3'p to A2'p, however, is quite large after shorter times of reaction (10 after 2 days) and not small even after leveling off (5 after 5 days), which could explain our earlier failure to detect the 2'-mononucleotides; we shall come back to this later. The detection of both 3'- and 2'-mononucleotides, as well as 2'–3'-cyclic phosphodiesters, definitely points to the cyclic forms as intermediates.

TABLE I: Components from Extensive Zinc Degradation[a] of Homopolymers (after Phosphomonoesterase Treatment).

	% as Nucleoside Residues		
	+Na	−Na	−Na,+Li
Poly(rA)			
A	88	76	87, 67
A>p	6	20	5, 24
ApA	7	2	8, 3
ApA>p	–	2	–, 5
ApApA	–	–	–, 1
Poly(rI)			
I	72, 65	65	66
I>p	12, 10	12	14
IpI	11, 10	10	11
IpI>p	2, 2	3	2
IpIpI	1, 4	4	2
Higher oligos	1, 7	6	2
Poly(rU)			
U	45	55	
U>p	17	21	
UpU	16	18	
UpUpU	8	5	
Other	14	1	
Poly(rC)			
C	46	47	
C>p	20	26	
CpC	20	18	
CpCpC	5	6	
Other	9	2	

[a] Reaction conditions: pH 7.0, 64°, 2 Zn/nucleoside residue; 120 hr except 144 hr for poly(rU) "−Na" and poly(rC) "−Na." Na: 1.0×10^{-2} M $NaNO_3$. Li: 1 Li/nucleoside residue as $LiClO_4$ (or LiCl, first citation for poly(rA)).

OBLIGATORY FORMATION OF A 2'–3'-CYCLIC PHOSPHODIESTER. As simpler models for studying the zinc degradation we have chosen various trinucleoside diphosphates: ApApA, ApApG, ApApU, CpCpC, UpUpU, UpUpG, and IpIpI. These were selected because of the demonstrated differential cleavage next to U, G, and I, in polymers, as well as their commercial availability. Also, none of these is expected to form a double-stranded structure (Brahms *et al.*, 1969). Rate studies were performed for each of the trimers. As a control, the reaction for ApApA was attempted omitting zinc: no significant degradation of ApApA was found through 65 hr.

The course of disappearance of trimer and appearance of fragments is given in Figures 2 and 3. In all cases, just as in the polymer reaction mixtures, N>p is detected, and in some cases, net NpN>p as well. These figures demonstrate that N>p is indeed an intermediate, in general by its appearance

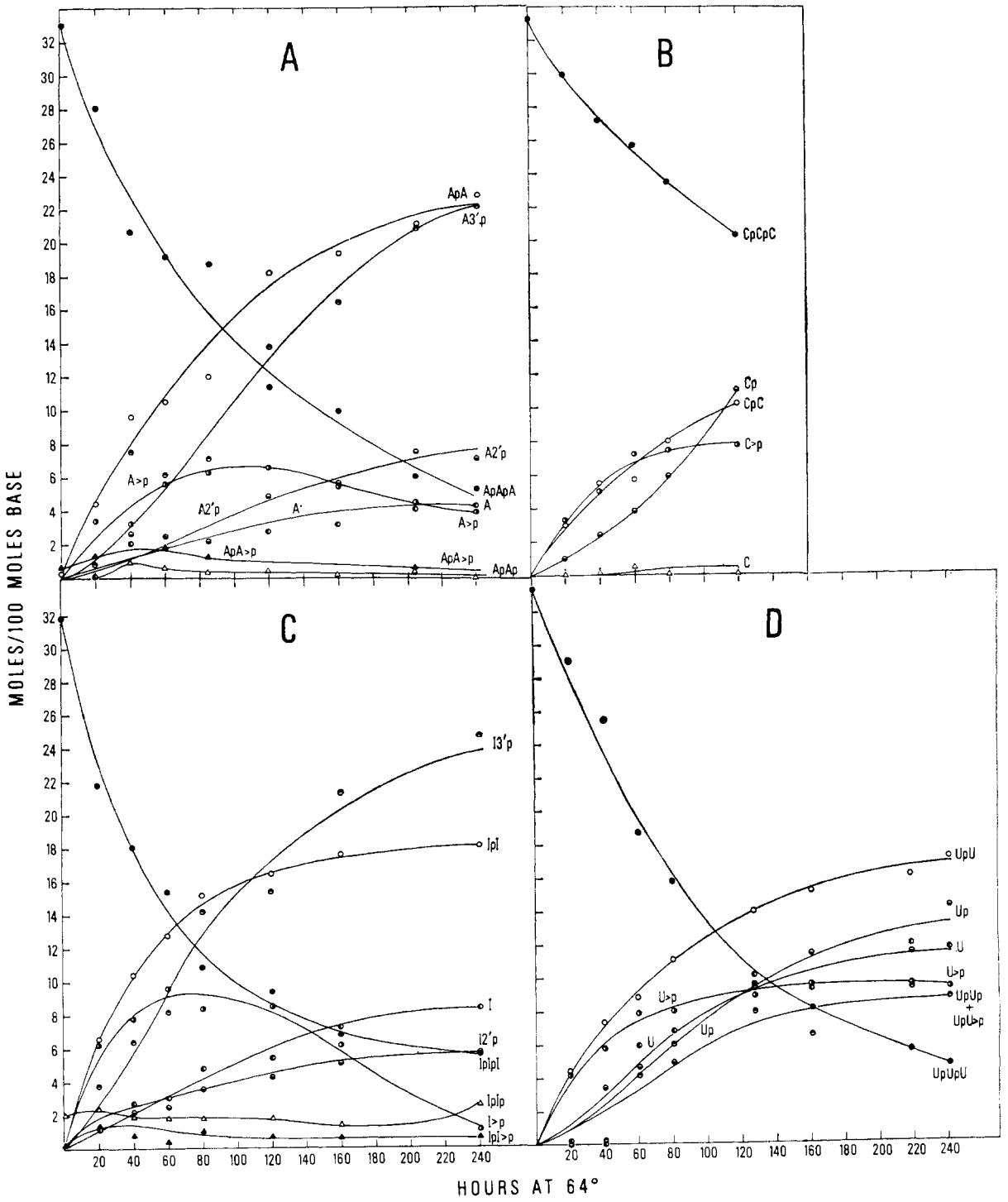


FIGURE 2: Course of zinc degradation of homotrimers (trinucleoside diphosphates): disappearance of trimer and appearance of fragments. (A) ApApA, (B) CpCpC, (C) IpIpI, and (D) UpUpU. Reaction conditions: pH 7.0, 64°, 2 Zn/nucleoside residue. NpNp means both NpN3'p and NpN2'p, and Np means both N3'p and N2'p. Neither UpUp nor UpU>p was separated from UpUpU, but the sum of their amounts is calculated and the amount of UpUpU corrected (see Experimental Section).

before N3'p or N2'p and in the initial convex shape of the N>p curve compared to the concave shape of the N3'p and N2'p curve(s), by the leveling off of N>p while N3'p and N2'p rise, and even by the eventual decline of N>p after sufficiently long intervals (see especially the longer-time data in Figure 2A,C).

That the zinc cleavage of a 3'–5'-phosphodiester bond operates exclusively through formation of a 2'–3'-cyclic phosphate is suggested from the trimer studies, in that the ratio of amounts of N3'p to N2'p remains fairly constant over the course of degradation (as will be discussed, there is no interconversion of N3'p and N2'p with either zinc or alkali). For ApApA this ratio remains approximately 3.0, for ApApU it remains approximately 3.4, and for IpIpI it remains near 4.0 (except initially, when some extra IpI2'p plus IpI3'p was present). In the KOH degradation for ApApA, the ratio remains reasonably constant, at approximately 1.2, over the course of the reaction—and alkaline hydrolysis of 3'-phos-

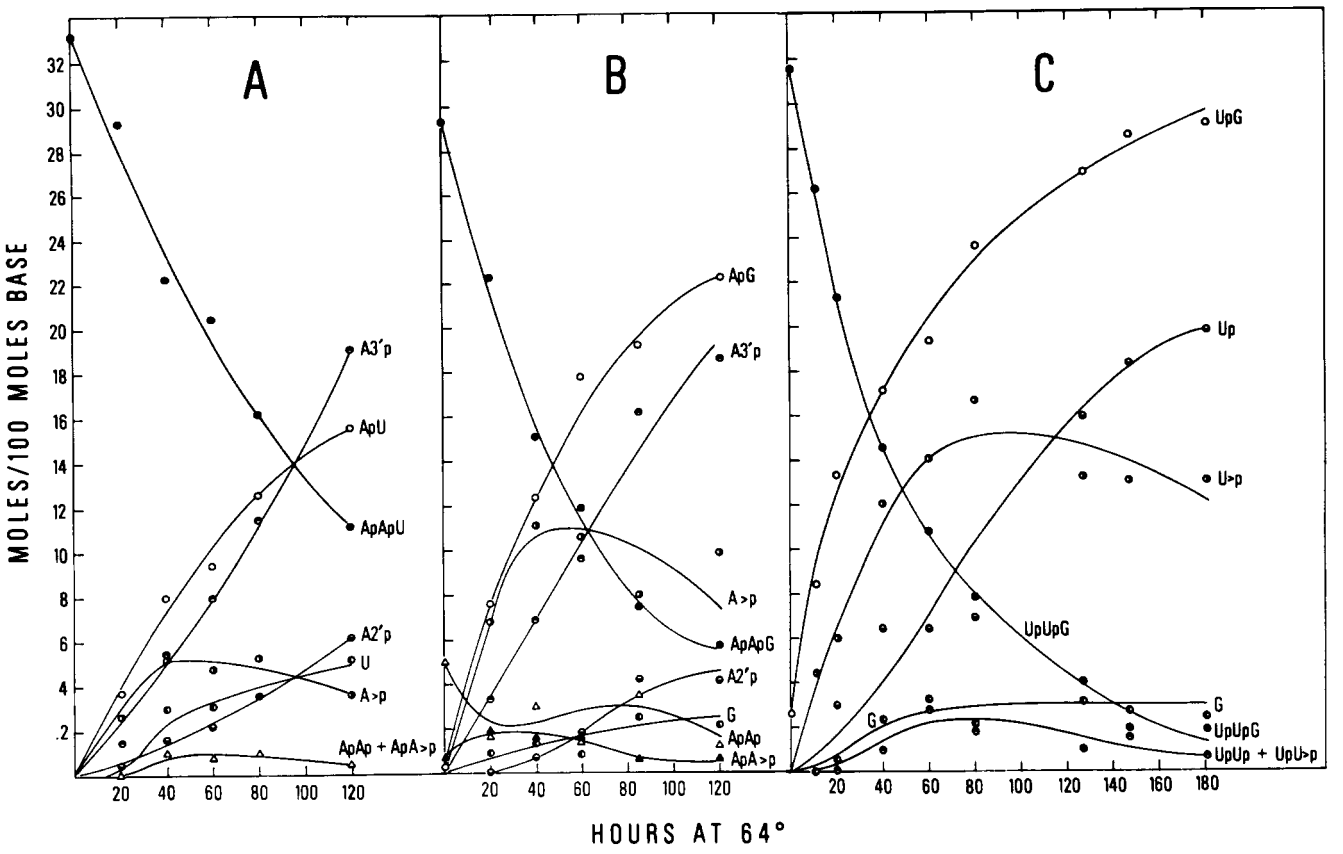


FIGURE 3: Course of zinc degradation of heterotrimers (trinucleoside diphosphates): disappearance of trimer and appearance of fragments. (A) ApApU, (B) ApApG, and (C) UpUpG. Reaction conditions: pH 7.0, 64°, 2 Zn/nucleoside residue. NpNp means both NpN3'p and NpN2'p, and Np means both N3'p and N2'p. Neither UpUp nor UpU>p was separated from UpG, but the sum of their amounts is calculated and the amount of UpG corrected (see Experimental Section).

phodiesters of ribonucleosides is known to proceed with formation of a 2'–3'-cyclic intermediate (Brown and Todd, 1952). But, in cases where there is a high degree of preference for cleavage of the trinucleoside diphosphate nearer the 5' end (see below) it is simple to prove from relevant kinetic equations that the ratio of amounts of N3'p to N2'p cannot remain constant if, for example, N3'p is produced directly by an alternate pathway.

Consequently, it is not to be expected that the 2' hydroxyl would effectively function in the cleavage reaction by participating (with the phosphate) in a zinc chelate, since *a priori* such chelation should favor the direct cleavage. The mechanism of the zinc ion cleavage, instead, must be similar to that of acid or alkaline hydrolysis, in which the 2'–3'-cyclic phosphodiester is the initial product. Considering a realistic role for the zinc, the mechanism must be more like that of the acid hydrolysis: with the polarizing assistance of the zinc ion bound to the phosphate as an electron-withdrawing agent analogous to the proton in acid hydrolysis, the 2'-oxygen would attack the phosphodiester phorphorus resulting in an unstable pentacovalent phosphorus intermediate from which the 5'-linked group is expeled, leaving a 2'–3'-cyclic phosphate group at the 3' side of the cleavage. Such a mechanism has been previously suggested by Dimroth and Witzel (1959) on the basis of degradations of RNA by metal hydroxides, in which both 3'- and 2'-mononucleotides could be detected, and under special conditions 2'–3'-cyclic mononucleotides also could be detected.

FACILITATED OPENING OF 2'–3'-PHOSPHODIESTERS. The zinc can also function as an electron-withdrawing agent in the opening of 2'–3'-cyclic phosphodiesters, since we find the opening of the 2'–3'-cyclic mononucleotides to 2'- plus 3'-mononucleotides to be considerably accelerated. The apparent first-order rates (hr^{-1}, $\times 10^3$) were approximately: for A>p, 13 with zinc *vs.* 2 without; for G>p, 9 *vs.* 2; for U>p, 8 *vs.* 1; for C>p, 4 *vs.* 1 (at pH 7.0, 64°, 2 Zn/nucleoside residue).

LACK OF INTERCONVERSION OF PHOSPHOMONOESTERS. Under alkaline conditions, N3'p and N2'p are stable; but in acid they are known to be readily interconverted, *via* N>p (Brown and Todd, 1952). Whether such interconversion occurs with zinc was tested for A3'p and A2'p after a long reaction period (120 hr): only a few per cent of the other phosphomonoester or the cyclic phosphomonoester were detected, with or without

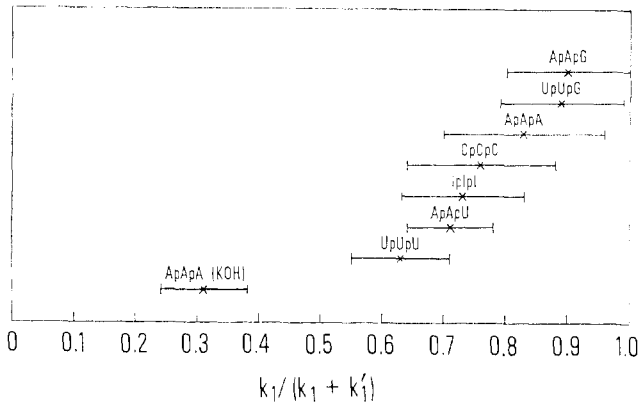


FIGURE 4: Ratios of apparent first-order rate constants for cleavage in trinucleoside diphosphates at the phosphodiester bond near the 5′ end (k_1) to cleavage at both bonds ($k_1 + k_1'$), during zinc degradation (pH 7, 64°, 2 Zn/nucleoside residue), and during the degradation of ApApA by alkali (40°, 0.3 M KOH). The horizontal lines refer to mean deviations (+ and −) within experimental points in individual rate studies. In the case of zinc degradation, the ratios are calculated from the data of Table II. In the case of the alkaline degradation of ApApA, the following apparent first-order rate constants were found, and the ratio calculated using them: $k_1 + k_1' = 0.81 \pm 0.06\ hr^{-1}$, k_2 (cleavage of ApA) $= 0.36 \pm 0.03\ hr^{-1}$, $k_1 = 0.32 \pm 0.01\ hr^{-1}$.

zinc. In this respect, degradation by zinc is not analogous to degradation by acid.

PREFERENTIAL CLEAVAGE DEPENDENT ON LOCATION OF PHOSPHODIESTER GROUP. Besides demonstrating the intermediate formation of the 2′-3′-cyclic phosphate, Figures 2 and 3 also suggest another important aspect of the zinc cleavage mechanism, namely that the susceptibility to cleavage of a phosphodiester bond in an oligomer (even a homooligomer) depends on its location in the oligomer. It can be inferred from the curves of Figures 2 and 3 that zinc cleavage of (at least some of) these trinucleoside diphosphates occurs preferentially at the phosphodiester linkage near the 5′ end, as shown by the preponderant production of dinucleoside monophosphate relative to the removal of trimer (for example, Figures 2A,C and 3B,C); an analysis of the data makes this clear.

We designate the two possible initial cleavages of a trinucleoside diphosphate (with retention of phosphate at the 3′ side of the cleavage site) as follows

$$\text{NpNpN} \xrightarrow{k_1} \text{"Np"} + \text{NpN}$$
$$\text{NpNpN} \xrightarrow{k_1'} \text{"NpNp"} + \text{N}$$

where "Np" refers to N2′p plus N3′p plus N>p and "NpNp" refers to NpN2′p plus NpN3′p plus NpN>p. The first-order disappearance of NpNpN is given by

$$[\text{NpNpN}]/[\text{NpNpN}]_0 = e^{-(k_1+k_1')t}$$

and the amount of NpN, subject to cleavage according to k_2 in a first-order process

$$\text{NpN} \xrightarrow{k_2} \text{"Np"} + \text{N}$$

is given by

$$[\text{NpN}]/[\text{NpNpN}]_0 = \frac{k_1}{k_1 + k_1' - k_2}(e^{-k_2 t} - e^{-(k_1+k_1')t})$$

The k_2 values have been determined from separate NpN degradation experiments. The sum $k_1 + k_1'$ was found from disappearance of NpNpN; using this value and the separate value for k_2, k_1 was found from net production of NpN.

Values of $k_1 + k_1'$ and k_1 from zinc degradations are collected in Table II. The k_2 values were determined to be approximately (hr^{-1}, $\times 10^3$) 0.7 for ApA, 0.5 for ApG, 0.4 for ApU, 0.6 for UpU, 0.6 for UpG, 0.3 for IpI, and 0.5 for CpC (since these are much smaller than $k_1 + k_1'$ or k_1, they are applied essentially as correction factors). The ratio $k_1/(k_1 + k_1')$ for zinc degradation of each of the trimers (Figure 4) turns out to be above 0.5 (it ranges from about 0.6 to 0.9), meaning that the majority of the reaction goes through cleavage at the phosphodiester bond near the 5′ end (k_1) rather than near the 3′ end (k_1'). For comparison, ApApA was degraded using 0.3 M KOH at 40° (common alkaline hydrolysis conditions); the alkaline degradation behaves (Figure 4) quite differently from the zinc degradation, perhaps in an opposite fashion, with $k_1/(k_1 + k_1')$ approximately 0.3.

ACCENTUATION OF CLEAVAGE BY ADJACENT PHOSPHATE GROUP. An obvious possible cause of the zinc cleavage rate near the 3′ end (k_1') of a trinucleoside diphosphate being lower than that near the 5′ end (k_1), is an effect of an adjacent phosphate group—there is no phosphate group at the 3′ end—which difference correlates with the rate of zinc cleavage of dinucleoside monophosphates (k_2) being lower than k_1 for trinucleoside diphosphates. We have further investigated this possibility by performing rate studies for adenosine dimers having each of the different end-phosphate configurations which could occur in reaction mixtures from polymer degradation: ApA>p, ApA2′p, and ApA3′p.

It proved impracticable to separate ApA2′p and ApA3′p preparatively, so a mixture of the two isomers was subjected to simultaneous zinc degradation. This degradation was not satisfied by a single rate (Figure 5), and led to early produc-

TABLE II: Apparent First-Order Rate Constants for Zinc Cleavage[a] of the Phosphodiester Bonds in Trinucleoside Diphosphates.

	hr^{-1} ($\times 10^3$)	
	$k_1 + k_1'$[b]	k_1[c]
IpIpI	13.4 ± 1.3	9.7 ± 0.4
ApApA	9.4 ± 0.8	7.3 ± 0.5
UpUpU	7.8 ± 0.5	4.8 ± 0.3
CpCpC	4.3 ± 0.2	3.3 ± 0.3
UpUpG	16.2 ± 1.0	14.4 ± 0.6
ApApG	15.0 ± 1.1	13.6 ± 0.5
ApApU	9.0 ± 0.5	6.4 ± 0.3

[a] Reaction conditions: pH 7.0, 64°, 2 Zn/nucleoside residue. The deviations shown refer to mean deviations within experimental points in individual rate studies. [b] For combined cleavage of either phosphodiester bond. [c] For cleavage of the phosphodiester bond near the 5′ end.

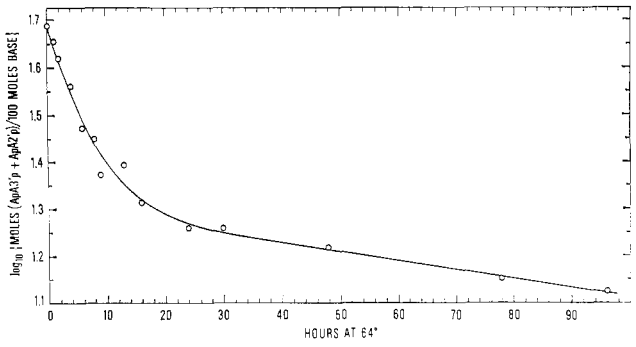


FIGURE 5: Course of zinc degradation of a mixture of ApA3'p and ApA2'p: disappearance of substrate. Reaction conditions: pH 7.0, 64°, 2 Zn/nucleoside residue. The *calculated curve* is fit by a search procedure to the experimental points, resulting in an initial ratio of ApA3'p to ApA2'p of 1.44 and first-order disappearance rates of 155×10^{-3} and 4.2×10^{-3} hr^{-1}, respectively, with a standard deviation of points to line of approximately 1.1 (concentration units; the concentration value in this plot ranges from about 50 down to about 14 moles/100 moles base).

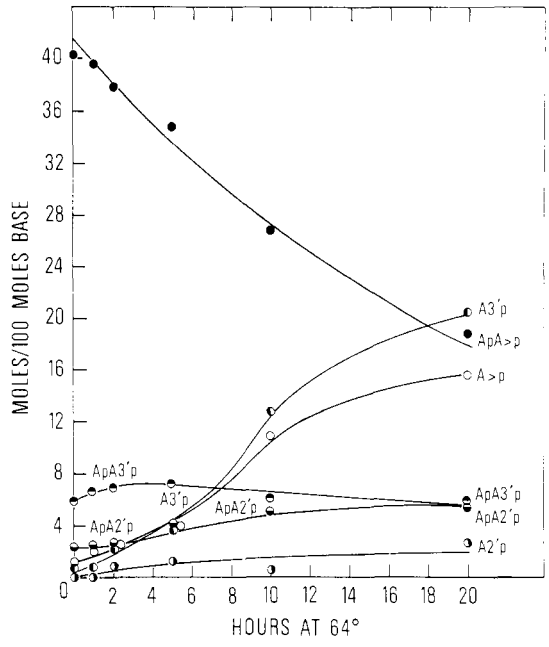
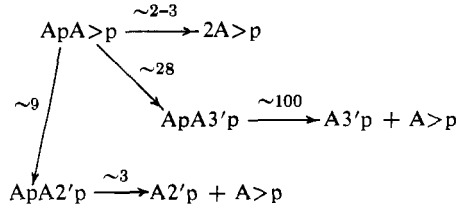


FIGURE 6: Course of zinc degradation of ApA>p: disappearance of ApA>p and appearance of fragments. Reaction conditions: pH 7.0, 64°, 2 Zn/nucleoside residue.

tion of a disproportionate amount of A3'p, indicating that ApA3'p was cleaved faster than ApA2'p. The data for the combined degradation were fit (Figure 5) as two simultaneous first-order processes with rate constants of 155×10^{-3} hr^{-1} (ApA3'p) and 4.2×10^{-3} hr^{-1} (ApA2'p), using a search procedure in which the two rate constants and the initial isomer ratio were varied.

The course of disappearance of ApA>p and appearance of fragments is shown in Figure 6. Again, there was disproportionate production of A3'p over A2'p. The lag seen in production of A>p and of A3'p means that they must both be at least partly generated from products rather than directly from ApA>p; the similarity in the lag for A>p and for A3'p would be consistent with only a relatively low rate of internal cleavage of ApA>p since internal cleavage of ApA>p produces A>p directly but not A3'p. The rate of internal cleavage of ApA>p and the rates of opening of its cyclic phosphate to the 2' or to the 3' from, as well as the rates of cleavage of the open dimers, were assigned by an analysis of the A>p data; they are given in the scheme below. The A>p data were fit using a search procedure in which the first-order rates for opening of ApA>p to ApA3'p and to ApA2'p and the first-order rate for cleavage of ApA3'p were varied, holding constant the combined first-order disappearance rate (38.6×10^{-3} hr^{-1}) found for ApA>p, the ratio of rates (36.9) of ApA3'p cleavage to ApA2'p cleavage found in the separate study on ApA3'p plus ApA2'p, and the first-order rate (13×10^{-3} hr^{-1}) for opening of A>p found separately. The resulting set of first-order rate constants (hr^{-1}, $\times 10^3$)



is defined by a standard deviation of the A>p concentration data (compare Figure 6) of 1.7 units; note that the cleavage rates of ApA3'p and ApA2'p are in fairly good agreement with those found in the separate degradation of ApA3'p plus ApA2'p.

Arranging the rates found from degradation of the various adenosine dimers, and of ApApA (k_1, k_1'; Table II), the order of zinc cleavage rates (hr^{-1}, $\times 10^3$) for the adenosine series would be

ApA3'p,	ApApA,	ApA2'p,	ApA>p,	ApApA,	ApA
100–150	~7	~3	~2–3	~2	~1

This order shows that the rate of cleavage of an internucleoside phosphodiester bond is influenced by the existence, configuration, and charge of a neighboring phosphate group. By charge, since under the reaction conditions (pH 7) this phosphate group in ApApA on ApA>p would bear a charge of −1 (as a diester phosphate) while in ApA3'p or ApA2'p it would bear a charge between −1 and −2 (pK_2 of A3'p is 6.2 and of A2'p is 5.9; Michelson, 1963). It is tempting to explain this order of zinc cleavage in the A dimers and trimers in terms of (presumably) repulsive forces between the neighboring phosphate and the phosphodiester bond being cleaved, which are relaxed on cleavage of the bond, although the phosphate charge must be at least partially neutralized through zinc binding. Interactions between phosphate, base, and zinc also could be involved.

The ratio of the rates of opening of ApA>p to ApA3'p and to ApA2'p is approximately 4, similar to the ratio of amounts of A3'p to A2'p produced in ApApA degradation (3.0), mostly through A>p, suggesting a general bias toward opening to the 3' isomer.

Regardless of the explanation, these phenomena may be applicable in general, especially in considering zinc homopolymer and homooligomer degradation.

INCREASED REACTIVITY WITH CHAIN LENGTH. Since 2'–3'-cyclic phosphodiester ended oligomers can be considered realistic intermediate products in the zinc degradation of

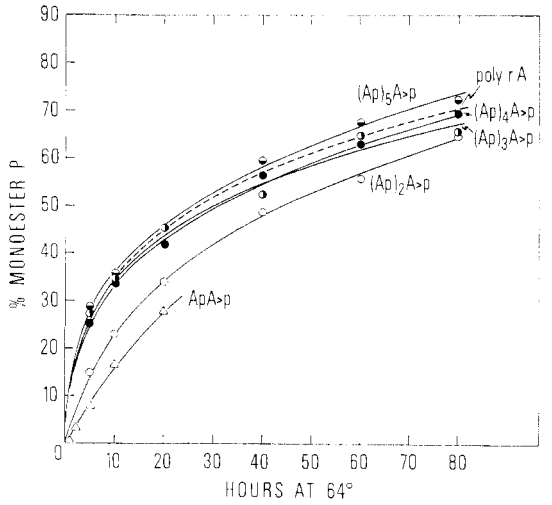


FIGURE 7: Comparison of the course of zinc degradation among adenylate oligomers and polyadenylate, the extent of reaction (except for ApA>p) assayed as orthophosphate released by phosphomonoesterase: △, ApA>p; ○, $(Ap)_2A>p$; ◑, $(Ap)_3A>p$; ●, $(Ap)_4A>p$; ◒, $(Ap)_5A>p$; - - -, poly(rA). Reaction conditions: pH 7.0, 64°, 2 Zn/nucleoside residue. The points for ApA>p are calculated from the data of Figure 6. The poly(rA) curve is from Eichhorn *et al.* (1971).

polymers, we have used such oligomers of 3′-adenylate in an evaluation of the effects of chain length on the zinc degradation. A set of studies of net open breaks (monoester phosphate) in $(Ap)_n$-A>p, from $n = 1$ to 5, show (Figure 7) that susceptibility to degradation increases gradually from the dimer to the tetramer level, at which it becomes similar to that of the polymer.

This result can be explained in terms of the accentuation of cleavage of a phosphodiester bond by an adjacent (3′) phosphate group. In accordance with the order of zinc cleavage rates among various adenosine dimers and ApApA discussed above, we consider the differences in susceptibility to degradation among the 2′–3′-cyclic ended oligomers to be due to cleavages next to nucleosides bearing a 3′-phosphate. We need only note, then, that the fraction of such cleavage sites among possible internal cleavage sites in the original substrates increases with chain length, but in smaller and smaller increments.

CORRELATION WITH COURSE OF HOMOPOLYMER DEGRADATION. Together with the intermediate formation of 2′–3′-cyclic phosphodiesters and the bias in opening of the cyclic phosphodiesters, the dependence of the cleavage rate on the adjacent phosphate group now makes understandable the peculiarities of homopolymer degradation by zinc which we have described: (1) only a small proportion of the mononucleotide is 2′ although this becomes larger as the reaction proceeds further; (2) the reaction appears to level off before complete degradation (to open mononucleotide) is reached; and (3) just after leveling off, the reaction mixtures consist mostly of mononucleotide and dinucleotide, some in the 2′–3′-cyclic phosphate form. (1) More mononucleotide and oligonucleotide is always produced in the 3′- than the 2′-ended form because of the tendency of the 2′–3′-cyclic form to open more to the 3′. The tendency toward cleavage at a phosphodiester bond adjacent to a 3′-phosphate group, especially if terminal, rather than one adjacent to a 2′–3′-cyclic phosphate or a 2′-(monoester) phosphate means that cleavage in an oligomer at the phosphodiester bond next to an end residue is most likely to produce N3′p, adding to the production of N3′p from opening of N>p. As the average chain length of the fragments becomes smaller with the progress of the reaction, however, less favorable cleavages producing N>p and N2′p are more and more likely to occur, with the result that the relative preponderance of N3′p will be lessened. (2) The reaction, as measured by phosphomonoester end production, could appear to level off solely due to the opening of N>p. But the lower rates of cleaving a phosphodiester adjacent to a 2′-monoester phosphate or a 2′–3′-cyclic phosphate, also, would tend to reduce the overall rate of phosphomonoester end production as the average chain length of the fragments becomes small enough. (3) Eventually, certainly at the dimer level, the effect of the adjacent phosphate group will allow the accumulation of oligomers terminating in N>p and N2′p. This accounts for the presence, in addition to N>p, of 2′–3′-cyclic and monoester phosphate ended dimer, despite the high reactivity of NpN3′p, after the reaction has leveled off.

Nucleoside Sequence Specificity. That zinc cleavage within the various trimers is influenced by the nucleoside sequence is quite evident from comparison of the apparent first-order rate constants (Table II) for cleavage (k_1, $k_1 + k_1'$), and the degree of preference of cleavage at the two possible sites (Figure 4) measured by $k_1/(k_1 + k_1')$.

CORRELATION AMONG HOMOTRIMERS AND HOMOPOLYMERS. We find that UpUpU has a significantly lesser degree of preference for cleavage near the 5′ end ($k_1/(k_1 + k_1')$) than ApApA, IpIpI, or CpCpC (Figure 4). The small degree of preference for UpUpU may be related to the fact that uracil participates in stacking interactions to the smallest extent among various bases (Warshaw and Tinoco, 1966; Brahms *et al.*, 1967; Simpkins and Richards, 1967a,b), but also may be related to the fact that it has the poorest ability to bind metal ion (Fiskin and Beer, 1965).

The order of the actual cleavage rates (k_1, $k_1 + k_1'$) among the homotrimers appears not to be consistent with the order of reactivity among homopolymers. IpIpI is not cleaved more slowly than ApApA, CpCpC, or UpUpU (Table II), while poly(rI) is degraded much more slowly than poly(rA), poly(rC), or poly(rU) (Butzow and Eichhorn, 1965; Eichhorn *et al.*, 1971). We cannot explain why the relative cleavage rates of the homotrimers are so different from those of the homopolymers. In particular, these results cannot be reconciled with the increase in rate with chain length (Figure 7). It may be noted that reactivity relationships for the zinc reaction in polymers are not always as predicted, the increased reactivity next to U in a heteropolymer, for instance, not being predicted from depolymerization of homopolymers conducted under the same conditions (Eichhorn *et al.*, 1971); also, that reversals in the order of production of phosphomonoester ends at different nucleosides in a heteropolymer can be effected by changing the reaction conditions (Eichhorn *et al.*, 1971).

DIFFERENTIAL EFFECTS IN TRIMERS—CORRELATION WITH HETEROPOLYMERS. Although the preference of cleavage at the two phosphodiester bonds in a trunucleoside diphosphate is not much different among homotrimers, changing the 3′-end nucleoside can markedly affect the preference of cleavage (Figure 4). Changing from ApApA to ApApU decreases $k_1/(k_1 + k_1')$ somewhat, changing from ApApU to ApApG increases it considerably, and changing from UpUpU to UpUpG greatly increases $k_1(k_1 + k_1')$. There is a strong resemblance between the cleavage preference in the various trimers and the end-group studies on heteropolymers (Eichhorn *et al.*, 1971) conducted under the same conditions, in terms of the tendency found for the heteropolymers of cleav-

age next to U and not next to G. Moreover, as shown in Table II, the presence of G at the 3′ end of these trinucleoside diphosphates leads to a marked increase in the actual cleavage rate of the phosphodiester bond near the 5′ end (k_1). The opposite seems to occur with U, although the change shown in Table II (ApApU *vs.* ApApA) is much less significant.

Can these sequence-specific differences be accounted for by differing stacking interactions between the bases? (The trimers studied are not expected to display any hydrogen-bonded base pairing.) A small degree of stacking does remain under the reaction conditions (64°) for ApApA, ApApG, and ApApU: the fractional increase in absorbance at 260 nm on raising the temperature from 30 to 70° is approximately 7%, in good agreement with the triadenylate data analyzed by Applequist and Damle (1966). No stacking, however, is expected under the reaction conditions for UpUpU (Simpkins and Richards, 1967a) or UpUpG (Brahms *et al.*, 1969)—the two heterotrimers which differ the most in $k_1/(k_1 + k_1')$—and no hypochromicity is detected on raising the temperature from 30 to 70°. No base stacking is expected in UpUpG (and other situations in which U and G are neighboring nucleosides), according to Brahms *et al.* (1969), due to a facile reorientation of guanine about the glycosidic bond with respect to the ribose, between syn and anti states, together with the nonstacking tendency of uracil. From the point of view of base interactions, then, it is necessary to consider interactions involving base orientation in addition to stacking; such interactions might be introduced or modified by metal binding.

Base binding of zinc ions, in itself, may play a very important role in the cleavage pattern of an oligomer or polymer, since, corresponding to some of our results in the present studies and in studies on the sites of zinc cleavage in heteropolymers (Eichhorn *et al.*, 1971), it has been found (Fiskin and Beer, 1965) that the binding of transition metal ions to bases is greatest for guanine and poorest for uracil.

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