Negative Charge at the 3′ Terminus of Oligonucleotides and Resistance to Venom Exonuclease*

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ABSTRACT: Oligonucleotides bearing negatively charged groups at the ω (right) end have been tested between pH 4 and 9 for resistance to hydrolysis by venom exonuclease. Resistance conferred by the 3′-phosphoryl group is highly dependent on pH and chain length. The compounds dNpNp and dNpNpNp are hydrolyzed optimally near pH 6, with hydrolysis of dNpNp proceeding at a 30-fold slower rate. Compared with dNpN, which shows the usual pH optimum of 9, the hydrolysis of dNpNp is inhibited by a factor varying between 40 at pH 5 and 1700 at pH 9. The hydrolysis of ApA>p and ApApA>p occurs with a pH optimum of approximately 9. Compared with ApA, the hydrolysis of ApA>p is inhibited by a relatively constant factor varying between 7 at pH 6 and 2 at pH 8. The hydrolysis of CpC is inhibited 18 times more than that of dNpN by borate buffer at pH 9. These observations indicate that resistance to exonuclease attack is conferred largely by negative charge, particularly a double charge, at the ω end of an oligonucleotide. A practical result of these observations is that the complete hydrolysis of dNpNp can be carried out with less enzyme and with less danger of dephosphorylation at pH 5–6 than at the usual pH 9. With the presently available preparation of exonuclease (Richards, G. M., Tutas, D. J., Wechter, W. J., and Laskowski, M., Sr. (1967), *Biochemistry 6*, 2908), the complete hydrolysis of dNpNpNp can be carried out at any pH between 5 and 8 with no significant dephosphorylation.

The previous conclusion (Laskowski, M. (1967), *Advan. Enzymol. 29*, 165) that venom exonuclease hydrolyzes substrates bearing an ω-3′-monophosphoryl group by releasing an α nucleoside first is revoked. Trinucleotides $dN^{\alpha}pN^{\beta}pN^{\gamma}p$ are hydrolyzed by first releasing $dpN^{\gamma}p$ in agreement with the order established for all types of chains.

One of many uses of venom exonuclease is the determination of both termini and chain length in oligonucleotides bearing 3′-monophosphate.

$$N^{\alpha}pN^{\beta}.....pN^{\omega}p \longrightarrow N^{\alpha} + n(pN) + pN^{\omega}p^{1}$$

Since oligonucleotides of this type are rather resistant to the action of exonuclease, the amount of enzyme used must be quite large, and even small amounts of contaminating enzymes become intolerable. The approximate relative amounts of enzyme required to hydrolyze three classes of dinucleotide, based on estimates obtained in this and other laboratories, are quoted from a recent review (Laskowski, 1967): pNpN, 1; NpN, 10; and NpNp, 1000.

The measurements were performed at pH 9.0, which is optimal for exonuclease hydrolyzing chains of the type $pN^{\alpha}....pN^{\omega}$.

It occurred to us that chains of the type $N^{\alpha}...pN^{\omega}p$ may be resistant because of the double negative charge on the ω-phosphate at pH 9.0. This paper presents evidence that this indeed is the case. By shifting pH to lower values the charge is reduced and the substrate becomes more susceptible, although it never becomes as susceptible as the dephosphorylated analog. A result of practical value is that at pH 6.0 roughly 200 relative units of enzyme are required to digest $dN^{\alpha}pN^{\beta}p$, compared with 1000 required at pH 9.0.

Experimental Procedure

Materials. Exonuclease was prepared from the venom of *Crotalus adamanteus* by the method of Richards *et al.* (1967). Purified phosphatase from human prostate was the gift of Dr. W. Ostrowski, and was prepared by his recent method (1968). ApA, ApA>p, and ApApA>p were purchased from Miles Chemical Co. CpC (Zellstoffsfabrik Waldhof) was the gift of Dr. W. Wechter, and bis(*p*-nitrophenyl)phosphoric acid (Aldrich Chemical Co.) was the gift of Dr. Martin Schweizer. The substrates dNpNp and dNpNpNp, oligonucleotides of mixed-base content, were obtained by digesting calf thymus DNA with purified micrococcal nuclease and

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[1] A new system of abbreviations is now being used in our laboratory. It was suggested to us by Dr. Waldo Cohn, Director, NAS-NRC Office for Biochemical Nomenclature, to whom we express our gratitude. Unknown nucleosides are abbreviated as N, purine nucleosides as R, and pyrimidine nucleosides as Y. Where desirable, superscript Greek letters are used to indicate position within the oligonucleotide chain, starting with α at the left (5′) end. Where chain length is unspecified, ω is used to denote the right (3′) end. Internucleotide linkages are indicated either by a hyphen or by p, with a terminal phosphate group always indicated by p. Examples: $dpR^{\alpha}pN^{\beta}pN^{\gamma}$, A^{α}-G^{β}-R^{γ}-...$Y^{\omega}p$.

fractionating the digest (Tomlinson and Tener, 1963) on a DEAE-cellulose column in the presence of 7 M urea.[2] dNpN was prepared by treatment of dNpNp with human prostatic phosphatase. A stock solution of dNpNp (0.5 ml; A_{271} 448; pH 6) was mixed with 0.5 ml of 0.02 M ammonium acetate (pH 4.8), and 0.01 ml of enzyme solution containing 35 units of activity (Ostrowski and Tsugita, 1961) was added. The mixture was allowed to stand 24 hr at room temperature, at which time hydrolysis was determined to be 70% complete by paper electrophoresis (see below). Hydrolysis was completed in 8 hr at 37° with 0.02 ml more of enzyme solution.

Recovery of dNpN from the digest and purification of several other substrates were carried out by paper electrophoresis, using the general procedure described in the first paragraph under Methods. Prior to use, papers were washed with distilled water by downward chromatography, to remove colored matter. Material to be purified was applied as a band across a 7.5-in. wide paper, in the amount of 18 A_{271} units/in. Runs were made in 0.1 M ammonium carbonate (pH near 8.5) with an applied potential of 500 V (12 V/cm). Ultraviolet-absorbing bands on the paper were cut into 1-cm squares. The absorbing material was recovered by soaking the squares with water and centrifuging in 50-ml plastic tubes with perforated bottoms, supported by the rims in 250-ml plastic bottles. Over 99% of the material put on the paper was recovered in a small volume with three such elutions in sequence. The elutes were then concentrated under vacuum, initially in a rotary evaporator, and finally by lyophilization.

A single trinucleotide,[3] dTpCpCp, was isolated from the mixed dNpNpNp sample in the following manner. dNpNpNp (805 A_{271} units) was placed on a column of Dowex 1-X8 (0.75 × 105 cm). Elution of materials smaller than trinucleotides was carried out with a few hundred milliliters each of 0.1, 0.3, 0.6, 0.7, 0.8, and 0.9 M ammonium acetate (pH 4.5), after which elution was continued with 1.0 M ammonium acetate (pH 4.5). The first trinucleotide, dTpCpCp, emerged as a large symmetrical peak after 1.4 l. of 1 M eluent had been collected. Tubes containing less than one-third of the peak absorbance were discarded. The remaining material was freed from buffer by lyophilization and was subjected to preparative electrophoresis as described in the preceding paragraph.

Methods. Electrophoresis was carried out on strips of Whatman No. 1 chromatography paper 18.25 in. long (cross-machine direction) and up to 7.5 in. wide, using the apparatus of Markham and Smith (1952). A line 4 in. from the left (cathode) end of the paper marked the origin, and lines 1 in. from each end marked the points at which the paper left the vessel containing CCl_4.

The conditions for preparative electrophoresis have been described under Materials. For analytical work, four buffers of similar conductivity were used: 0.14 M ammonium acetate (pH 4.0), 0.05 M sodium acetate (pH 5.0), 0.033 M sodium phosphate (pH 6.0), and 0.02 M sodium phosphate (pH 7.0), at an applied potential of 1000 V (23 V/cm). Runs of the following duration could be made without danger of the fastest components (pNp) running off the paper: pH 4 and 5, 2.25 hr; pH 6, 2 hr; and pH 7, 1.75 hr. Electrophoresis was performed at pH 5 with hydrolysates of dNpNp, ApA>p, and ApApA>p; at pH 6 with dNpNpNp and dTpCpCp; and at pH 7 with dNpN, CpC, and ApA. Electrophoresis at pH 5 and 7 also was used to investigate the purity of several substrates.

Except where noted otherwise, the following uniform format was used in the experiments described under Results. Reaction mixtures were made up from 25 μl of stock substrate solutions (pH 6–7), 0.10 ml of 0.13 M buffer, 1 μl of 0.13 M $MgCl_2$, and 1–5 μl of enzyme solution containing between 1.3 and 132 units per ml. The stock substrate solution contained between 397 and 448 absorbance units per ml (A_{260} for ApA, ApA>p, and ApApA>p; A_{271} for all others). Final concentrations in the reaction mixtures therefore were as follows: CpC, dNpN, dNpNp, and bis(*p*-nitrophenyl) phosphate, 5 mM; ApA>p and dNpNpNp, 3 mM; ApApA>p, 2 mM; buffer, 0.1 M; Mg^{2+}, 1 mM; and enzyme, 0.01–5.3 units/ml. The buffers used were made up by mixing 0.13 M Tris base and 0.13 M succinic acid to give pH 4–9. In special cases, 0.65 M Tris-succinate pH 4, 6, and 9, and 0.13 M boric acid adjusted to pH 9 with 50% NaOH, were used.

The reaction mixtures were made up in 2-ml vials at 0°, incubated for 1 hr in a 37° water bath, and returned to the ice bath. The vials were centrifuged at 0° to strip condensate from the walls, and electrophoresis was carried out with 10-μl portions as described above. Whenever possible, all samples were run on a single piece of paper. After electrophoresis, the paper was marked into parallel lanes (including a blank lane) of equal width, and corresponding ultraviolet-absorbing areas were enclosed in rectangles of equal area. These areas, cut into small squares, were eluted batchwise with 2 or 3 ml of 0.1 M sodium phosphate (pH 6.7) and were centrifuged briefly before A_{271} of the supernatant solutions was read in a Gilford 2000 spectrophotometer (A_{260} in the case of ApA, ApA>p, and ApApA>p). Readings on duplicate electrophoretic runs usually were reproducible to within 0.002 or 0.003 absorbance numbers.

The experiment with bis(*p*-nitrophenyl) phosphate was run as above but without electrophoresis. Stock bis(*p*-nitrophenyl)phosphoric acid was first adjusted to pH 7 with 1.0 N NaOH. Reaction mixtures were made up and incubated 1 hr at 37°, after which they were transferred to an ice bath for 0.5 hr to simulate the time required to prepare the usual electrophoretogram. The reactions were then stopped with 3.0 ml of 0.1 N NaOH and A_{400} was read.

Because of limited amounts of material, experiments with dTpCpCp were run in a slightly different manner. Stock substrate solution (2.5 μl, A_{271} 383), 10 μl of 0.13 M buffer containing 1.3 mM $MgCl_2$, and 1 or 2.5 μl of

[2] This work was done in collaboration with Milton Schultz, Jr., to whom we express our gratitude. Mr. Schultz was a summer participant of the National Science Foundation Research Participation Program in Science, Grant NSF-GY-705.

[3] We are indebted to Mr. L. Stasiuk for assistance in preparing dTpCpCp.

TABLE I: Purification of Several Substrates by Paper Electrophoresis.

Material	Probable Contaminant	% Contaminant	
		Before Purifcn	After Purifcn
dNpNp	dNpN	0.5	*b*
	$dN(pN)_x$; $x = 2.3$	6.1	
dNpNpNp	? (at origin)	0.4	*b*
	$dN(pN)_x$; $x = 2.4$	5.6	
dTpCpCp	$dT(pN)_x$;[a] $x = 1.9$	3.6	*b*
ApA>p	ApAp, ApApAp	20	5.1
ApApA>p	ApAp, ApApAp	10	4.7

[a] Electrophoresis at pH 4 of an exonuclease digest revealed dT as the only nucleoside, and dpC, dpG, dpT, and dpA in the ratio 1.0:0.64:0.57:0.15. [b] None visible on electrophoresis.

enzyme solution containing 1.3–132 units/ml were mixed on a piece of parafilm. The mixture was drawn into a Pyrex melting point capillary and incubated after sealing the tube ends in a flame. After incubation, the entire contents of the tube was put on paper for electrophoresis.

Results

Figure 1 shows several types of electrophoretic separation encountered in this work. In general, it was found that the best separations between nucleotides of the same type but different chain length (*e.g.*, dNp, dNpNp, and dNpNpNp) were obtained at pH 5, while the best separations between nucleotides of different type (*e.g.*, dNpNpN and dNpNpNp, or ApA>p and ApAp) were obtained at pH 7.

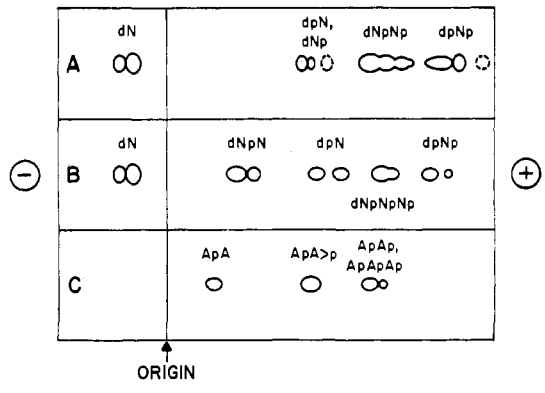

FIGURE 1: Composites of patterns obtained in paper electrophoresis of substrates and the hydrolytic products released by venom exonuclease. Electrophoresis was carried out as described under Methods. Identity of the components was confirmed by comparison with authentic samples of nucleosides, dinucleoside monophosphates, 3′- and 5′-mononucleotides, and deoxyribonucleoside 3′,5′-diphosphates. (A) Mixed deoxyribodinucleotides (dNpNp) and their hydrolytic products; electrophoresis for 2.25 hr, pH 5. (B) Mixed deoxyribotrinucleotides (dNpNpNp) and their hydrolytic products; electrophoresis for 2 hr, pH 6. (C) Unpurified, undigested ApA>p (Miles Chemical Co.); electrophoresis for 1.75 hr, pH 7.

Table I lists the substrates purified by electrophoresis and the amounts of the contaminants. The nature of these contaminants was tentatively established (see Table I, column 2) by electrophoresis at pH 5 and 7 and in the case of dNpNp, dNpNpNp, and dTpCpCp, was confirmed by hydrolysis with exonuclease.

The time course of hydrolysis of dNpNp and CpC is shown in Figure 2 at several values of pH. (The obvious differences between the two substrates with respect to pH optimum have been studied more thoroughly and are presented below in Figures 3 and 4). The purpose of Figure 2 is to show that the pH optima do not depend greatly upon the extent of hydrolysis of substrate; that is, the curves do not cross. Nonetheless, in subsequent experiments the amounts of enzyme used were the minimum consistent with reliable detection of the products.

The effect of pH on the hydrolysis of several substrates bearing no negatively charged group at the ω end is shown in Figure 3. All of the curves have the "normal" response to pH commonly associated with exonuclease activity. Although these experiments were not carried to a pH higher than 9, the optimal values are known to be near 9. The substrate containing deoxyribose is digested at about half the rate of the two substrates containing ribose. The similarity of the results with ApA and CpC suggest that the relative resistance of dNpN probably is due to the sugar moiety.

In contrast, oligonucleotides bearing a 3′-monophosphoryl group, besides being much more resistant to exonuclease, are hydrolyzed with a markedly different response to pH. Figure 4 shows the pH 6 optimum for $dN^{\alpha}pN^{\beta}p$. Besides the major products dN^{α} and $dpN^{\beta}p$, a small and relatively constant amount of mononucleotide was recovered from the electrophoretogram. The amount of this material, as well as the slight excess of dN over dpNp, was very little changed after a second hour of hydrolysis.

A plot of the ionic strength of the buffers used in this experiment, *vs.* pH, also has a maximum near pH 6.

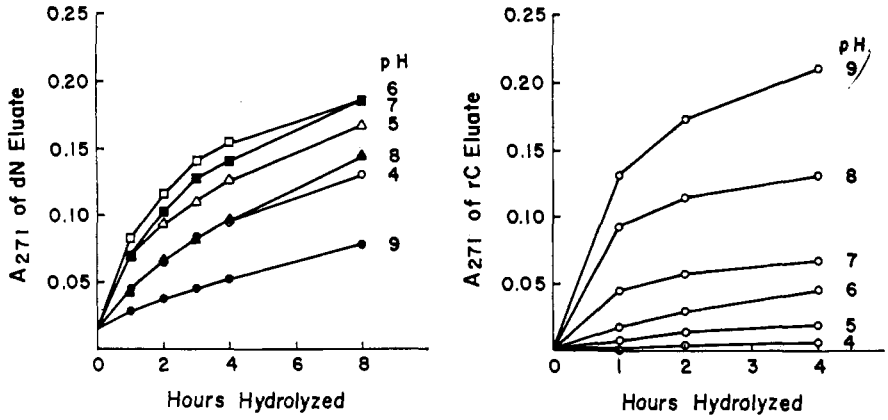

FIGURE 2: Time course of the hydrolysis of two substrates by venom exonuclease at different values of pH. Procedure was as described under Methods, except that electrophoresis was carried out only to separate dN^{α} from the remaining components (0.5–1 hr), and only dN^{α} was assayed. Left half: hydrolysis of unpurified dNpNp. (○—○) pH 4, (△—△) pH 5, (□—□) pH 6, (■—■) pH 7, (▲—▲) pH 8, and (●—●) pH 9. Substrate A_{271} 85, [enzyme] = 5.3 units/ml. The amounts of dN released in 1 hr at pH 4, 6, and 9 were reproduced with 7, 1, and 0% error in a similar experiment 7 weeks later. The curves start from a *y*-axis value of 0.015, which corresponds to dN released rapidly from contaminants such as dNpN and dNpNpN. This value was determined in a later experiment with purified dNpNp. A *y*-axis value of 0.22 approximates 100% hydrolysis. Right half: hydrolysis of CpC. Substrate A_{271} 88, [enzyme] = 0.0115 unit/ml. A *y*-axis value of 0.22 approximates 100% hydrolysis.

To check the possibility that the curve of Figure 4 represents a response to ionic strength as well as pH, experiments were run with unpurified dNpNp, in 0.1 and 0.5 M buffers. Table II shows that higher ionic strength gives decreased rates at all three pH values; thus, variation in ionic strength cannot explain the pH 6 optimum in Figure 4.

Trinucleotides bearing a 3′-monophosphoryl group, while less resistant to exonuclease than the corresponding dinucleotides, share the anomalous response to pH during hydrolysis. The rate of release of $dpN^{\gamma}p$ from $dN^{\alpha}pN^{\beta}pN^{\gamma}p$, shown in Figure 5, has the same general profile as seen in Figure 4, except that the optimal pH appears to be approximately 0.5 unit higher, and the increase in rate from pH 9 to pH 6–7 is somewhat less. On the other hand, the rate of release of dN^{α} from $dN^{\alpha}pN^{\beta}$, the other product of the first hydrolytic split, has the expected "normal" shape (compare with Figure 3), except for a drop at high pH, presumably due to low rate of formation of dNpN.

The amount of mononucleotide dpN^{β} is anomalously high, since, coming from $dN^{\alpha}pN^{\beta}$, it should be equal to dN^{α}. If no subsequent adjustment is made for the larger amounts of enzyme used at pH 4, 5, and 9, the resulting curve for dpN^{β} approximates the curve for dN^{α} displaced upwards by an average of 2.2% of the total A_{271}. In similar experiments performed with unpurified dNpNpNp (Table I), the curve for dpN^{β} was displaced upwards by an average of 6.1%.

The anomalous hydrolysis optima of 3′-phosphoryl oligonucleotides can be explained by assuming that the phosphoryl group inhibits exonucleolytic attack because of its negative charge, which is dependent upon pH. Figure 6 shows the relative increase in rate of hydrolysis upon removal of the 3′-phosphoryl group from dNpNp, as a function of pH. The ratio of rates at low pH values (4–5), where almost all molecules carry a single negative charge at the ω-3′ end, indicates that such a charge causes approximately 40-fold inhibition of hydrolysis. At pH 9, where almost all substrate molecules bear a double negative charge at the ω-3′ position, the ratio of rates is 1700, indicating an additional inhibition of at least 40 due to the second negative charge.

This explanation has been verified with oligonucleotides bearing an ω-2′,3′-cyclic phosphoryl group. The negative charge on this group does not change over the pH range 4–9, but in other respects it should resemble the 3′-phosphoryl group. Figure 7 shows the hydrolysis of ApA>p and ApApA>p as a function of pH. These curves resemble the "normal" curves of Figure 3. It also can be seen that the products from each substrate appear in the expected ratios, without the anomalously high mononucleotide levels found in Figure 5 and to a lesser extent in Figure 4.

Data from Figure 3 (ApA) and Figure 7 (ApA>p) are replotted in Figure 6 in the same manner as was done previously for dNpN and dNpNp. Between pH 5 and 9 there is approximately a threefold variation in the effect of the ω-2′,3′-cyclic group, in contrast to the marked change in effect over the same pH range found for the ω-3′-phosphoryl group (50-fold, in the opposite

TABLE II: The Effect of Buffer Concentration on the Rate of Release of dN^{α} from Unpurified $dN^{\alpha}pN^{\beta}p$ by Venom Exonuclease at Three Values of pH.

pH	Buffer (M)	A_{271} of dN^{α} Eluate
4	0.1	0.042
	0.5	0.029
6	0.1	0.082
	0.5	0.075
9	0.1	0.028
	0.5	0.016

TABLE III: The Effect of pH 9 Borate Buffer on the Rate of Hydrolysis of CpC and dNpN by Venom Exonuclease.

Substrate	Buffer	Enzyme (unit/ml)	% Hydrolysis in 1 hr	Rate in Tris/ Rate in Borate
CpC	Tris-succinate	0.011	64	46
	Sodium borate	0.011	1.4	
CpC	Tris-succinate	0.011	55	18
	Sodium borate	0.11	30	
dNpN	Tris-succinate	0.011	26	2.5
	Sodium borate	0.011	10.4	

direction). It should be noted, however, that a single ω-terminal negative charge is less inhibitory in the case of ApA/ApA>p than in the case of dNpN/dNpNp.

In the experiments with ApA>p and ApApA>p, blanks at pH 5 and 9 were run along with the enzyme hydrolysates to detect any spontaneous opening of the terminal ring on the substrates. No change was found at pH 5, but slight hydrolysis was observed at pH 9.0. Since the enzymatic digests were fractionated by electrophoresis at pH 5, where cyclic and noncyclic terminated nucleotides have the same mobility, this slight spontaneous hydrolysis was disregarded.

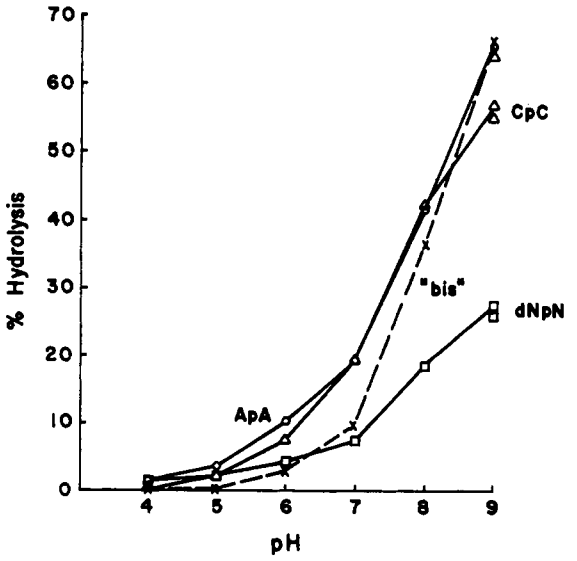

FIGURE 3: Effect of pH on the hydrolysis of some dinucleoside monophosphates and bis(*p*-nitrophenyl) phosphate by venom exonuclease. (O—O) ApA, (Δ—Δ) CpC, (□—□) dNpN, and (× - - - ×) bis(*p*-nitrophenyl) phosphate. The experiments were carried out as described under Methods. Substrate A_{271}: ApA, 48 (A_{260} 78); CpC, 88; dNpN, 86; bis-(*p*-nitrophenyl) phosphate, 78. [Enzyme]: with dinucleoside monophosphates, 0.0115 unit/ml; with bis(*p*-nitrophenyl) phosphate, 0.105 unit/ml. Electrophoresis of dinucleoside monophosphates: 1 hr at pH 7. Per cent hydrolysis of dinucleoside monophosphates was calculated as the sum of A_{271} or A_{260} of nucleosides and mononucleotides divided by the total A_{271} or A_{260} recovered from the electrophoretogram. Per cent hydrolysis of bis(*p*-nitrophenyl) phosphate was calculated by comparing the values of A_{400} with those of a blank (no enzyme) and a standard (tenfold amount of enzyme), both run at pH 9. The extra points at pH 9 for CpC and dNpN are from experiments performed several weeks later.

The effect of negative charge at the ω end has also been examined by hydrolyzing CpC and dNpN in pH 9 borate buffer, which forms a negatively charged complex at the ω end of the substrate containing ribose. Table III shows the results of the experiment. Borate inhibits the hydrolysis of CpC 50-fold, or 18 times more than it does the hydrolysis of dNpN. This inhibition can be partly overcome by using larger amounts of enzyme. Unfortunately, this experiment does not distinguish between the effect of charge and steric factors.

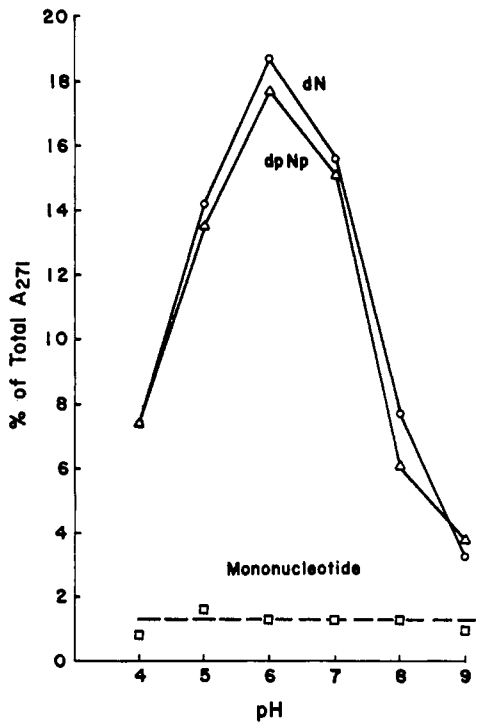

FIGURE 4: Effect of pH on the hydrolysis of purified mixed deoxyribodinucleotides ($dN^{\alpha}pN^{\beta}p$) by venom exonuclease. (O—O) dN^{α}, (Δ—Δ) $dpN^{\beta}p$, and (□ - - - □) dpN or dNp. Unhydrolyzed dNpNp is not shown, but is included in the total A_{271}. The experiments were carried out as described under Methods. Substrate A_{271} 83, [enzyme] = 5.1 units/ml; electrophoresis for 2.25 hr, pH 5.

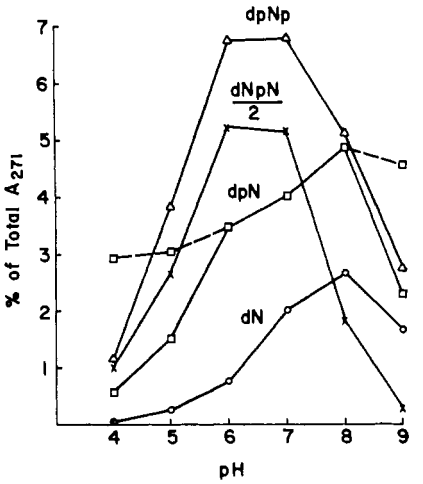

FIGURE 5: Effect of pH on the hydrolysis of purified mixed deoxyribotrinucleotides ($dN^{\alpha}pN^{\beta}pN^{\gamma}p$) by venom exonuclease. (Δ—Δ) $dpN^{\gamma}p$, (×—×) $dN^{\alpha}pN^{\beta}/2$, (□—□) dpN^{β}, and (○—○) dN^{α}. Unhydrolyzed dNpNpNp is not shown, but is included in the total A_{271}. The experiments were carried out as described under Methods. Substrate A_{271} 83, [enzyme] = 0.525 unit/ml for pH 4, 0.210 unit/ml for pH 5 and 9, and 0.105 unit/ml for pH 6–8. Electrophoresis for 2 hr, pH 6. Observed values for (per cent of total A_{271}) of the separated products were divided by 5, 2, or 1 to compensate for the different amounts of enzyme used. Dashed lines show values for mononucleotide without such correction. Values of per cent of total A_{271} for dNpN were divided by 2 to facilitate comparison with other products on a molar basis.

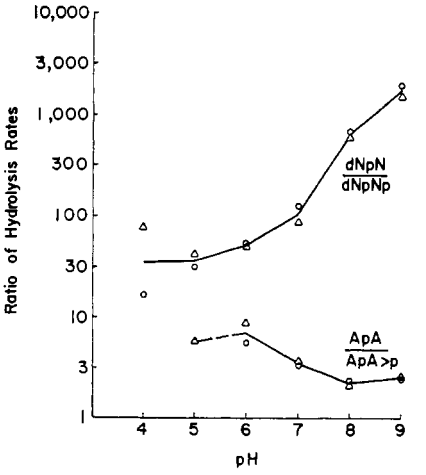

FIGURE 6: Effect of removing negatively charged groups from the ω end of an oligonucleotide, at several values of pH. The data were obtained from the experiments of Figures 3, 4, and 7. (○—○) Ratio of hydrolytic rates expressed as (dN^{α} from $dN^{\alpha}pN^{\beta}$)/(dN^{α} from $dN^{\alpha}pN^{\beta}p$), and as (A^{α} from $A^{\alpha}pA^{\beta}$)/(A^{α} from $A^{\alpha}pA^{\beta}>p$); (Δ—Δ) ratio expressed as (dpN^{β} from $dN^{\alpha}pN^{\beta}$)/($dpN^{\beta}p$ from $dN^{\alpha}pN^{\beta}p$) and as (pA^{β} from $A^{\alpha}pA^{\beta}$)/($pA^{\beta}>p$ from $A^{\alpha}pA^{\beta}>p$). *y*-axis values are plotted on a logarithmic scale.

Figure 8 directly compares a number of substrates, illustrating several points. First, up to pH 8 or 9, the rates of hydrolysis of the "normal" substrates are roughly linear functions of pH when plotted on a logarithmic scale. Second, trinucleotides are hydrolyzed more rapidly than dinucleotides, although this difference is less in the .... $A^{\omega}>p$ substrates than in the d.... $N^{\omega}p$ substrates, especially at high pH. Third, the cyclic 2′, 3′-phosphoryl group is less inhibitory than the 3′-phosphoryl group with one charge. This has already been seen with dinucleotides (Figure 6). Finally, because the complete hydrolysis of $dN^{\alpha}pN^{\beta}pN^{\gamma}p$ depends upon hydrolysis of $dN^{\alpha}pN^{\beta}$, the over-all reaction is completed most rapidly at pH 8 in spite of the optimum for the first step at pH 6–7.

The results up to this point have been concerned with initial hydrolysis rates, or an approximation to these. Figure 9 shows the composition of the hydrolysates near the end point in the hydrolysis of $dN^{\alpha}pN^{\beta}p$. The values of dN^{α} do not exceed the theoretical value of 50%, and mononucleotide appears largely at the expense of the theoretical 50% value for $dpN^{\beta}p$. Dephosphorylation therefore seems to be fairly specific for dpNp, and occurs maximally near pH 8. Complete hydrolysis appears to occur with minimal dephosphorylation in the region of pH 5–6.

The composition of complete hydrolysates of $dN^{\alpha}pN^{\beta}pN^{\gamma}p$ is shown in Figure 10. Because the amounts of enzyme used in this experiment varied with pH, hydrolysis was complete at aproximately the same time at all pH values, in contrast to the experiments of Figure 9. Taking into account the different amounts of enzyme and lengths of time required for complete hydrolysis in the experiments of Figures 9 and 10, cleavage of $dN^{\alpha}pN^{\beta}pN^{\gamma}p$ to $dN^{\alpha}pN^{\beta}$ and $dpN^{\gamma}p$ at pH 6–8 requires roughly 0.025 as much enzyme as complete hydrolysis of dNpNp, and subsequent cleavage of $dN^{\alpha}pN^{\beta}$ to dN^{α} and dpN^{β} requires about 0.065 as much enzyme. Levels of both dN^{α} and $dpN^{\gamma}p$ are below theoretical, while excessive dpN^{β} is present. This state of imbalance does not change substantially between 8 and 26 hr: the average excess of dpN^{β} over $dpN^{\gamma}p$ increases from 5.5% to 5.7% of the total A_{271}, while the average excess of dpN^{β} over dN^{α} increases from 4.0 to 5.4%. These latter figures can be compared to the somewhat smaller excess of 2.2% present near the start of hydrolysis (Figure 5).

The imbalance among the three hydrolytic products of dNpNpNp makes it impossible to determine whether dephosphorylation is limited to $dpN^{\gamma}p$, or whether dephosphorylation of dpN^{β} also occurs. Since this imbalance could be due to impurities yielding excessive mononucleotides, a number of experiments were performed with purified dTpCpCp. Figure 11 illustrates the results from two partial hydrolysates. The optimal pH for the initial hydrolytic cleavage is approximately 1 pH unit higher than that found with mixed dNpNpNp. As with dNpNpNp, an excess of mononucleotide over nucleoside is present from very early in the reaction, averaging 2.6% of the total A_{271} in Figure 11, left, and 2.7% in Figure 11, right.

The composition of two total hydrolysates is given in Table IV. Exposure of enzyme (*i.e.*, concentration of enzyme times length of incubation) in the 1-hr digest is roughly equal to that in the 8-hr digest of dNpNpNp (Figure 10, left). Similarly, exposure in the 21-hr digest is roughly equal to that in the 48-hr digest of dNpNp (Figure 9, right) and represents drastic overdigestion for

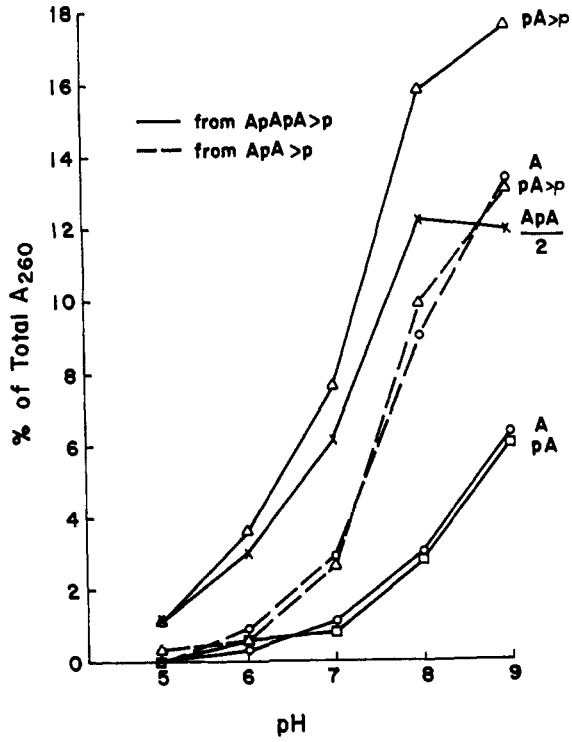

FIGURE 7: Effect of pH on the hydrolysis of ApA>p and ApApA>p by venom exonuclease. Dashed lines, products from $A^{\alpha}pA^{\beta}$>p: (O---O) A^{α} and (Δ---Δ) pA^{β}>p. Solid lines, products from $A^{\alpha}pA^{\beta}pA^{\gamma}$>p: (Δ—Δ) pA^{γ}>p; (×—×) $A^{\alpha}pA^{\beta}/2$, (O—O) A^{α}, and (□—□) pA^{β}. Unhydrolyzed ApA>p and ApApA>p are not shown, but are included in the totals of A_{260}. Values of (per cent of total A_{260}) for ApA have been divided by 2 to facilitate comparison with other products on a molar basis. The experiments were carried out as described under Methods. Substrate A_{260} 79, [enzyme] = 0.0105 unit/ml. Electrophoresis for 2.25 hr, pH 5.

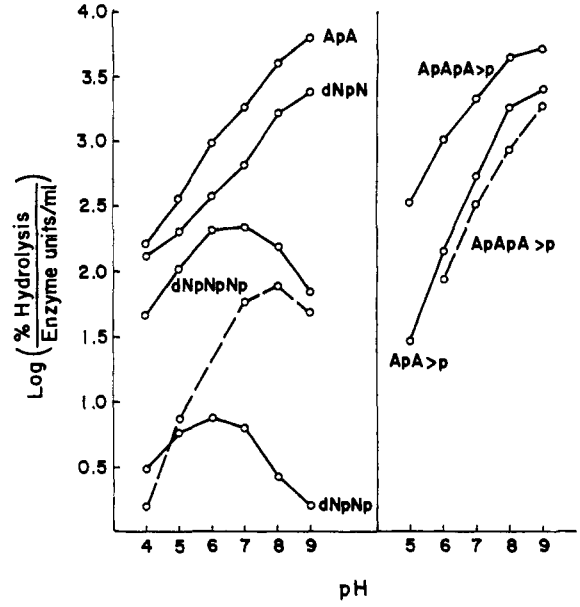

FIGURE 8: Effect of pH on the hydrolysis of several substrates by venom exonuclease, with rates of hydrolysis adjusted for differences in enzyme concentration. Per cent hydrolysis for dinucleoside monophosphates and for the first step in the hydrolysis of trinucleotides (solid lines) is calculated as 100% less per cent of total absorbance due to unhydrolyzed substrate. Per cent hydrolysis for the second step in the hydrolysis of trinucleotides (dashed lines) is calculated as three times the per cent of total absorbance due to released nucleoside.

a trinucleotide. It is apparent from Table IV that the major products are in close balance at the completion of hydrolysis. Further exposure to enzyme results in the disappearance of some $dpC^{\gamma}p$, along with appearance of a nearly equivalent amount of mononucleotide. A small increase in nucleoside is also apparent, indicating a dephosphorylation of dpC^{β} about sevenfold less than that of $dpC^{\gamma}p$. Loss of $dpC^{\gamma}p$ is clearly most severe at high pH values.

Discussion

Four main issues in this paper require discussion: (1) negative charge at the ω terminus and resistance to exonuclease, (2) evaluation of the exonuclease preparation from *Crotalus adamanteus* as a reagent for determining oligonucleotide chain length and terminal residues, (3) importance of substrate purity in making this evaluation, and (4) order of cleavages in trinucleotides.

(1) The experimental evidence (Results) has been presented in such a manner as to emphasize the first issue. All experiments lead to the conclusion that negative charge at the ω terminus is principally responsible for the resistance of a substrate to exonuclease. This is seen in the experiments in which the effect of ionization (pH dependence) was studied (Figures 2, 3, 4, 5, 7, 8, and 11), but is most clearly demonstrated in Figure 6, which compares phosphorylated substrates with their dephosphorylated analogs. The large pH effect observed with 3′-monophosphoryl termini and the lack of pH effect observed with 2′,3′-cyclic phosphoryl termini both lead to the same conclusion. Less conclusive but still suggestive is the greater effect of borate on ribo-CpC than on deoxyribo-NpN (Table III). Unexpected and difficult to explain is the observation that trinucleotides dNpNpNp are 30 times more susceptible than dinucleotides dNpNp. Oligonucleotides longer than trinucleotide were not studied, but one might expect them to be even more susceptible, by analogy to the results of Dolopchiev (1969) obtained with members of the series $U(pU)_n$.

An important practical result of these observations is that less enzyme can be used for the determination of oligonucleotide chain length and termini. Previously, such determinations were made at pH 9, and large amounts of very pure enzyme were required. It is apparent that the most resistant substrates of all, dinucleotides dNpNp, can be hydrolyzed with less than one-fifth of the amount of enzyme previously required simply by lowering the pH from 9 to 6. The hydrolysis of trinucleotides dNpNpNp can be improved in a similar manner, although to a lesser extent. Besides reducing the requirement for quantity of enzyme, reducing the pH would be expected to decrease the danger from contaminating monophosphatases, which are most active at pH 9. This point is discussed below.

TABLE IV: Composition of Complete Hydrolysates of dTpCpCp.

Time (hr)	pH	Enzyme (units/ml)	% Deviation from Theoretical 33.3% of Hydrolysate dT	dpC	dpCp
1	5	22.0	+0.1[a]	0[a]	0[a]
	6	9.8	0	0	0
	7	9.8	−0.5	+0.3	+0.3
	8	9.8	−0.3	+0.3	+0.1
	9	22.0	+0.3[a]	0[a]	−0.3[a]
21	5	22.0	+0.7[a]	+3.0[a]	−3.6[a]
	6	9.8	+1.0	+2.3	−3.2
	7	9.8	+0.1	+2.9	−3.0
	8	9.8	+0.2	+5.8	−5.9
	9	22.0	+1.5[a]	+6.3[a]	−7.7[a]

[a] The observed deviations have been divided by 2.25 to adjust for the higher [enzyme] in these samples.

(2) In earlier work, Richards *et al.* (1967) described a method of preparing exonuclease from the venom of *C. adamanteus*. Levels of the two monophosphatases and the endonuclease known to be present in this venom were followed through the course of purification. The final material was found still to be slightly contaminated with endonuclease (Georgatsos and Laskowski, 1962) and with the nonspecific phosphatase (as detected with *p*-nitrophenyl phosphate by Sulkowski *et al.*, 1963). Contamination with 5′-nucleotidase was reduced to the level of bare detectability. This preparation of exonuclease from *Crotalus* venom has not been tested by actual determination of termini. The present work is the first attempt to do so.

With a pure exonuclease acting on dinucleotides, no mononucleotides should be formed and dN^{α} should be equal to $dpN^{\beta}p$. Figure 9 shows that under conditions approaching 95% hydrolysis (24 hr) more than 5% of the total hydrolysate is mononucleotide. After 48-hr hydrolysis the amount is higher and at pH 8 reaches 15% (loss of 30% of $dpN^{\beta}p$). However, there is little evidence in Figure 9 of further dephosphorylation to nucleoside. Several authors (Sulkowski *et al.*, 1963; McLennan and Lane, 1968) have suspected that venom contains a phosphatase which is either specific for or highly preferential to nucleoside diphosphates. Present results support this contention. The nature of the mononucleotides released (dpN *vs.* dNp) has not been investigated. Sulkowski *et al.* (1963) showed that purified nonspecific phosphatase from venom attacks dpNp to produce both the 3′- and the 5′-monophosphates. Figure 9 shows that with dinucleotides as substrates the error introduced by dephosphorylation at slightly alkaline pH is quite high; the best results were obtained at pH 5, where almost complete digestion occurred with a minimum of dephosphorylation.

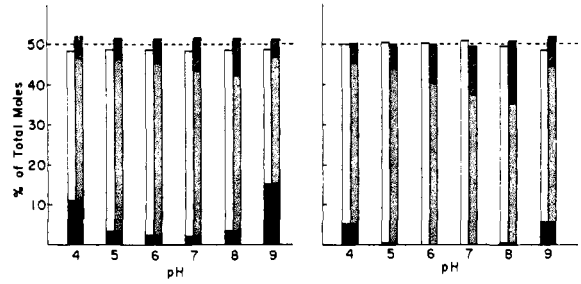

FIGURE 9: Composition of the hydrolysates of Figure 4 after further incubation at 37°. Left half: 24 hr; right half: 48 hr. Stippled areas, $dpN^{\beta}p$; striped areas, mononucleotide of undetermined nature; clear areas, dN^{α}; black areas, $dN^{\alpha}pN^{\beta}p$. Lengths of the bars represent moles, areas represent A_{271} units. The placement of some bars on top of others (*i.e.*, not starting at the base line) indicates the reaction scheme: $dN^{\alpha}pN^{\beta}p \rightarrow dN^{\alpha} + dpN^{\beta}p$; $dpN^{\beta}p \rightarrow dpN^{\beta}$ or $dN^{\beta}p$. The dashed line at 50% represents the theoretical amounts of products at complete hydrolysis.

Less exposure to enzyme is required for the hydrolysis of trinucleotides, and the danger of dephosphorylation should therefore be less. With a carefully purified single trinucleotide, dTpCpCp, and with proper exposure to enzyme, the three hydrolytic products are found in the expected amounts (Table IV, 1 hr). Further exposure to enzyme, equivalent to that needed to digest dinucleotides, results in a loss of $dpC^{\gamma}p$ (23% at pH 9) and a corresponding increase in mononucleotide.

The general evaluation of this enzyme preparation, therefore, is that it may be used successfully for the determination of chain length and termini, provided the substrate is pure (see part 3, below) and the exposure to enzyme is not excessive.

(3) A specific danger in the use of exonuclease in characterizing 3′-phosphoryl oligonucleotides is the presence of dephosphorylated contaminants. Material that has been segregated by the method of Tomlinson and Tener (1963) shows such contaminants on electro-

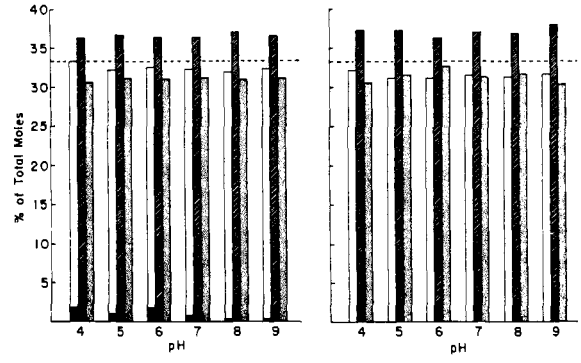

FIGURE 10: Composition of the hydrolysates of Figure 5 after addition of approximately tenfold more enzyme and further incubation at 37°. Left half: 8 hr; right half: 26 hr. Stippled areas, $dpN^{\gamma}p$; black areas, $dN^{\alpha}pN^{\beta}$; clear areas, dN^{α}; striped areas, dpN^{β}. No dNpNpNp remained. Lengths of the bars represent moles, areas represent A_{271} units. The placement of some bars on top of others (*i.e.*, not starting at the base line) indicates the reaction scheme $dN^{\alpha}pN^{\beta}pN^{\gamma}p \rightarrow dN^{\alpha}pN^{\beta} + dpN^{\gamma}p$; $dN^{\alpha}pN^{\beta} \rightarrow dN^{\alpha} + dpN^{\beta}$. The dashed line at 33.3% represents the theoretical amounts of products at complete hydrolysis.

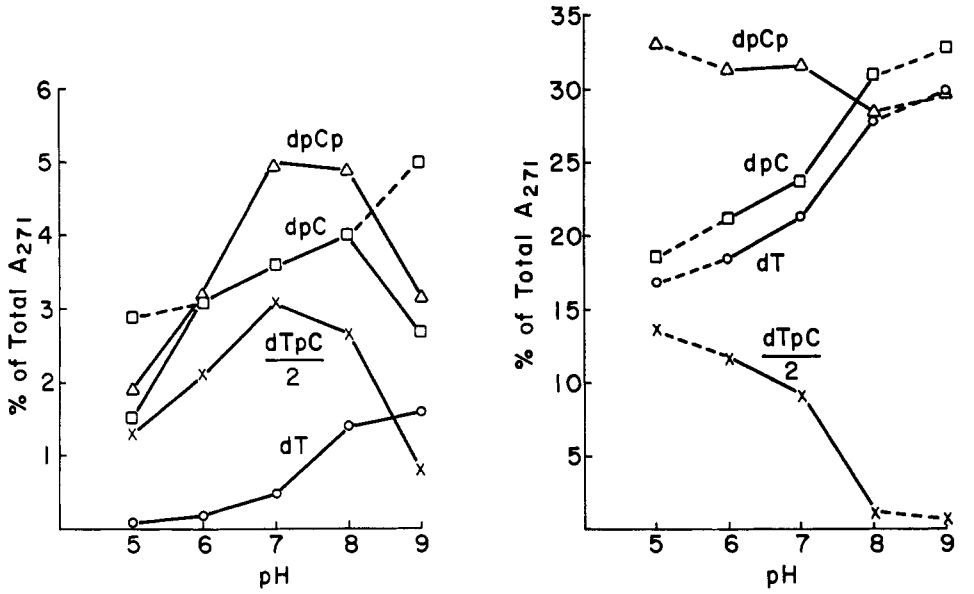

FIGURE 11: Effect of pH on the hydrolysis of purified $dT^{\alpha}pC^{\beta}pC^{\gamma}p$ by venom exonuclease. (△—△) $dpC^{\gamma}p$; (□—□) dpC^{β}, (×—×) $dT^{\alpha}pC^{\beta}/2$, and (○—○) dT^{α}. Unhydrolyzed dTpCpCp is not shown, but is included in the total A_{271}. The experiments were carried out as described under Methods. Left half: substrate A_{271} 66–71, [enzyme] = 0.182 unit/ml for pH 5 and 9, and 0.098 unit/ml for 6, 7, and 8. Electrophoresis for 2 hr, pH 6. Observed values for per cent of total A_{271} of the separated products were divided by 1.86 at pH 5 and 9 to compensate for the larger amounts of enzyme used. Dashed lines for dpC^{β} indicates values without such correction. Values of per cent of total A_{271} for dTpC were divided by 2 to facilitate comparison with other products on a molar basis. Right half: same as left half except for tenfold higher [enzyme]. No corrections were made for differences in enzyme level at pH 5 and 9 (dashed lines) and 6–8 (solid lines).

phoresis (Table I), even after further purification on a Dowex 1 column. It also has been shown by Sulkowski *et al.* (1963) and Ohsaka *et al.* (1964) that dephosphorylation of as much as 10% of the material can occur during recovery of fractions obtained from chromatographic columns. Such contaminants in 3′-phosphoryl oligonucleotides are hydrolyzed sufficiently rapidly by exonuclease that the extra nucleoside and mononucleotide appear to be released in a manner rather independent of pH, length of incubation, and amount of enzyme. Examples in the present work are the appearance of extra nucleoside in the hydrolysis of unpurified dNpNp (left, Figure 2) and the low, even level of mononucleotide across the pH spectrum in Figure 4. Most illustrative is a comparison of the hydrolysis of dTpCpCp (Table IV, 1 hr) and dNpNpNp (Figures 5 and 10). The significant excess of mononucleotide in the latter case cannot be due in any great extent to dephosphorylation of $dpN^{\gamma}p$ for several reasons, but principally because most of the excess is present before the hydrolysis of substrate is complete. Experiments with dNpNpNp which had not been purified by electrophoresis (Table I) gave a threefold greater excess of mononucleotide. We therefore conclude that the excess arose principally from dephosphorylated contaminants of the type dNpNpNpN.

This explanation would not seem to apply to the excess mononucleotide found in the two partial hydrolysates of dTpCpCp (Figure 11), in view of the results of Table IV. For this apparent discrepancy we can offer no explanation.

(4) Results of experiments presented in Figures 5 and 11 show that with trinucleotides of the type $dN^{\alpha}pN^{\beta}pN^{\gamma}p$ the first split is to $dN^{\alpha}pN^{\beta} + dpN^{\gamma}p$ This finding agrees with the established mechanism of action of venom exonuclease, but does not agree with the previous conclusions of Felix *et al.* (1960). These authors used as substrate a fraction of the digest of DNA by splenic DNase II that was eluted from a Dowex 1 column by 1 M ammonium acetate. The fraction was heterogeneous with respect to length (from di- to pentanucleotides), but was assumed to be homogeneous in respect to termination in 3′-phosphate. This assumption was incorrect. Later work in this laboratory (see above, point 3) showed that unless very specific precautions are taken (Sulkowski and Laskowski, 1967) dephosphorylation occurs. In the experiments of Felix *et al.* (1960) nucleoside and nucleotide appeared simultaneously and much earlier than 3′,5′-diphosphate. Such a result would be expected if the substrate used by Felix *et al.* was contaminated by dephosphorylated substrate (see point 3 above).

With a substrate composed of trinucleotides only two alternative first splits are possible.

$$dN^{\alpha}pN^{\beta}pN^{\gamma}p \longrightarrow dN^{\alpha}pN^{\beta} + dpN^{\gamma}p \quad (1)$$

$$dN^{\alpha}pN^{\beta}pN^{\gamma}p \longrightarrow dN^{\alpha} + dpN^{\beta}pN^{\gamma}p \quad (2)$$

In the present work, using purified substrate, no early formation of dN^{α} was observed at any of the pH values studied. At all pH values $dN^{\alpha}pN^{\beta}$ was found in amounts appropriate for a major product. In view of this finding all previous statements concerning the order of degradation of chains terminating in 3′-monophos-

phate, starting with the report of Felix *et al.* (1960) and extending to the last review of Laskowski (1967), are being revoked.

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On the Mechanism of Erythropoietin-Induced Differentiation. V. Characterization of the Ribonucleic Acid Formed as a Result of Erythropoietin Action*

Martin Gross† and Eugene Goldwasser‡

ABSTRACT: Erythropoietin induces the process by which primitive hematopoietic cells eventually become mature erythrocytes. The earliest action of erythropoietin so far determined is the stimulated synthesis of ribonucleic acid by rat marrow cells *in vitro*. The nature of this ribonucleic acid was reexamined. Within a few minutes of its addition to the cells erythropoietin causes the formation of a very large ribonucleic acid (150 S) that is not detectable in the unstimulated cells. This ribonucleic acid appears to be a discrete component, and not a complex of ribonucleic acid with ribonucleic acid, deoxyribonucleic acid, or protein. At later times, increased synthesis of components with sedimentation coefficients in the range 55–65, 45, 9, 6, and 4 S occurs. The 150S ribonucleic acid has a half-life of about 6 min while the other components are longer lived. Some of the properties and possible functional roles of the ribonucleic acid components are discussed.

The induction of erythrocyte formation from primitive precursor cells of the blood-forming tissues appears to be initiated by erythropoietin (Goldwasser, 1966). Even though the purification of erythropoietin is not yet completed, its availability in a partially purified form and its ability to initiate cytodifferentiation provide an attractive system for studying the biochemical mechanisms underlying this process. Previous work from this laboratory has demonstrated that erythropoietin stimulates heme synthesis (Krantz *et al.*, 1963), hemoglobin synthesis (Gallien-Lartigue and Goldwasser, 1964), glucosamine incorporation into stroma (Dukes *et al.*, 1964; Dukes and Goldwasser, 1965), and iron uptake (Hrinda and Goldwasser, 1966) by rat bone marrow cells in tissue culture, and that these effects are inhibited by actinomycin D. We have also shown that erythropoietin increases the rate of RNA synthesis in this system as early as 15 min after its addition (Krantz and Goldwasser, 1965). These findings indicate that the stimulation of RNA synthesis plays an important early role in the process of erythroid differentiation induced by erythropoietin.

Recent studies of RNA metabolism in animal cells have demonstrated two different classes of rapidly labeled, quickly sedimenting RNA (Scherrer *et al.*, 1963, 1966; Yoshikawa-Fukada *et al.*, 1965; Attardi *et al.*, 1966). One of these, sedimenting at 45 S, is the precursor of rRNA. The other component is heterodisperse, in-

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