interconversion on ion permeability in artificial membrane systems.

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Use of Venom Exonuclease at Low pH for Preparation of Mononucleoside Diphosphates*

G. M. Richards† and M. Laskowski, Sr.

ABSTRACT: A general method for the preparation of three classes of nucleoside diphosphates is described. In each case all four nucleoside diphosphates are obtained in good yield. The method is based on hydrolytic degradation of nucleic acids: first with micrococcal nuclease under conditions assuring high yield of dNpNp (or NpNp), then with venom exonuclease, used in high doses at low pH to degrade the dinucleotides to dN + dpNp (or N + pNp). To obtain pN>p ribonucleic acid is hydrolyzed with alkali to the stage of maximal formation of cyclic termini, then treated with venom exonuclease (low dose, high pH). The presently available preparation of venom exonuclease, originating from the North American species *Crotalus adamanteus*, was found satisfactory for this purpose. With d- and rpNp, where a large amount of exonuclease is required, the success of the method depends upon the use of low pH. At low pH the susceptibility of the substrate to exonuclease is increased and the effect of contaminating monophosphatases is decreased. Because the substrates used to prepare pN>p are susceptible, the required amount of exonuclease is low, and the effect of contaminating monophosphatase is insignificant.

We have recently found (Richards and Laskowski, 1969) that the well-known resistance to venom exonuclease displayed by deoxyribooligonucleotides bearing a 3′-monophosphoryl group (for a review, see Laskowski, 1967) is caused by a double negative charge on this group at pH 9.0. At lower values of pH the charge is reduced and the substrate becomes more susceptible, although it never becomes as susceptible as the dephosphorylated analog. One consequence of this finding is that only one-fifth the amount of enzyme required to digest such oligonucleotides at pH 9 is required at pH 6.0. Obviously, the level of contaminating phosphatase that can be tolerated is also increased by at least the same factor of 5. In reality the number may be higher because at low pH the activity of monophosphates is reduced to about 10% of their maximal activity at pH 9. Under these conditions, the presently available preparation of exonuclease from venom of *Crotalus adamanteus* (Richards *et al.*, 1967) was found to be satisfactory for identification of termini in 3′-phosphoryl oligonucleotides (Richards and Laskowski, 1969).

A second application requiring equally pure enzyme is the preparation of 3′,5′-mononucleoside diphosphates. The principle of the method is simple: DNA is digested with micrococcal nuclease to the stage of highest accumulation of dinucleotides which in turn are digested with venom exonuclease to nucleosides and nucleoside diphosphates. The efficiency of the method depends upon purity of exonuclease.

At the time when *Bothrops atrox* venom was available, an experiment of this type was performed (Sulkowski *et al.*, 1963). The reaction was carried out at pH 9, the scale was small, and only the deoxyribonucleoside diphosphates were prepared.

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The preparation of exonuclease from venom of *Crotalus adamanteus* (Richards *et al.*, 1967) is known to contain some tenaciously bound contaminating monophosphatases. The level of contamination is of the order of magnitude of 10^{-4} unit/unit of exonuclease. It is not possible to predict from these values whether a satisfactory recovery of diphosphates can be assured. The actual experiment must be performed.

Work to be described was undertaken with these main goals in mind: (1) to decrease losses due to dephosphorylation by carrying out hydrolysis of the resistant substrates at low pH; (2) to extend the method to the derivatives of RNA; and (3) to increase the scale of preparation.

Enzymatic degradations which produce cyclic termination on the ω end are usually selective with respect to base. To assure a random distribution, alkaline hydrolysis (Bock, 1967) was used. Oligonucleotides terminated in cyclic phosphate are rather susceptible to exonuclease and dephosphorylation is not a problem in this case (Richards and Laskowski, 1969).

The present paper describes the preparation of the twelve nucleoside diphosphates in good yield, using exonuclease prepared from the venom of *C. adamanteus* according to Richards *et al.* (1967), in each case under optimal conditions.

Experimental Procedure

Materials. DNA was prepared from calf thymus by the method of Kay *et al.* (1952).[1] RNA from yeast either was purchased from Worthington Biochemical Corp. or was prepared from fresh yeast by the method of Crestfield *et al.* (1955).[2] Dinucleotides of mixed-base content (NpNp) were prepared by segregating a micrococcal nuclease digest of yeast RNA (Worthington) according to the procedure of Tomlinson and Tener (1963).[1] The preparation was additionally purified by paper electrophoresis in 0.1 M $(NH_4)_2CO_3$.

Micrococcal nuclease, prepared according to Sulkowski and Laskowski (1966), was generously supplied by Dr. Sulkowski.[3] Exonuclease was prepared from the venom of *C. adamanteus* by the method of Richards *et al.* (1967). Phosphatase from human semen (Kutscher and Pany, 1938) was purified as described in Figure 1. A brief study of its pH optimum and thermal stability (not described here) makes it seem likely that it is similar to or identical with the enzyme described by Ostrowski and Tsugita (1961). Potency of the final material (Figure 1) was slightly higher than that of a highly purified preparation from human prostate gland (Ostrowski, 1968), generously, supplied by Dr. Ostrowski. Other authors (Wittenberg and Kornberg, 1953; Scheffler *et al.*, 1968) have used human semen phosphatase in a less purified form.

Hydrolysis of DNA with Micrococcal Nuclease. DNA (0.5 g) was dissolved in 600 ml of 0.01 M $CaCl_2$ and digested in the pH-Stat with 0.6 mg of crystalline micrococcal nuclease. Digestion was essentially complete in 1 hr (Sulkowski and Laskowski, 1968), but the reaction was continued for an additional 2 hr to ensure that only mononucleotides and dinucleotides remained. The digest was passed through a short phosphocellulose column (NH_4^+ form, pH 6.5) to adsorb the enzyme (which can be eluted later with 0.75 M ammonium acetate pH 6.5), and the nonadsorbed material was lyophilized to dryness. The residue, containing nucleotides and most of the $CaCl_2$ from the digest, was suspended in 10 ml of water and adjusted from pH 8 to 5.0 with 1 M acetic acid. Most of the material went into solution as the pH was lowered. The suspension was centrifuged and the supernatant liquid was recovered. This solution contained 8680 A_{271} units of ultraviolet-absorbing material and was about 0.5 M in $CaCl_2$. Fractionation of a similar digest on a Dowex 1-X2 column showed it to consist of dinucleotides (49% A_{271}) and mononucleotides (51%) (Sulkowski and Laskowski, 1968).

Hydrolysis of RNA with Micrococcal Nuclease. The degree of hydrolysis by micrococcal nuclease optimal for the subsequent preparation of pNp was determined in a pilot-type experiment in a pH-Stat. Starting conditions for hydrolysis were those of Sulkowski and Laskowski (1968), except for a 10-fold higher concentration of substrate. Samples (0.05 ml) were removed from the digest at times corresponding to hydrolysis of 50, 60, and 70% of the internucleotide bonds (estimated from the NaOH consumption). Times were approximately 20, 40, and 80 min. The samples were mixed with 1 μl of 10 M ammonium acetate pH 6.5 and 1 μl of venom

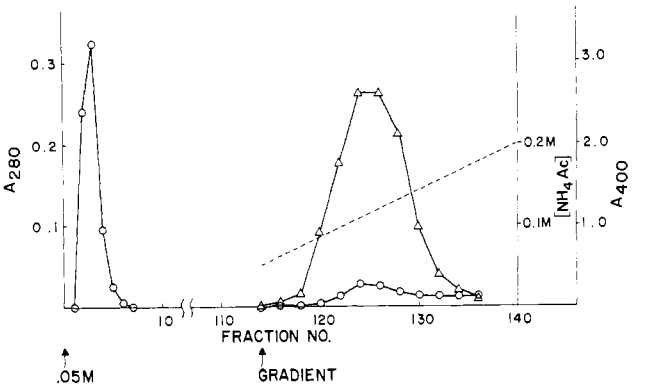

FIGURE 1: Purification of semen phosphatase on DE-52. Column, 0.9 × 21 cm, acetate form. (○) A_{280} of eluent; (△) A_{400} in phosphatase assay (see below). A sample of human semen was collected and immediately put on ice. The milky upper liquid was removed, diluted with four volumes of cold 0.1 M ammonium acetate, and centrifuged 0.5 hr at 48,000 *g*. The clear supernatant solution contained 538 units/ml of activity (Ostrowski, 1968), with a potency of 77. This solution (0.5 ml) was applied to the column. Elution was carried out overnight at room temperature with 0.05 M ammonium acetate (pH 6.0), followed by a gradient (dashed line) of 250 ml of 0.05 M buffer *vs.* 250 ml of 0.5 M buffer. Flow rate, 50 ml/hr; fraction volume, 5.1 ml. Fractions 118–134 were pooled and the enzyme was recovered by concentration dialysis. The final preparation contained 246 units/ml of activity, potency = 238. Recovery from the column was 64%. For phosphatase assay of column fractions (△), one drop of eluate was mixed with 1.0 ml of 1.0 mM sodium *p*-nitrophenylphosphate plus 1.0 ml of 0.01 M ammonium acetate (pH 5.0). After 10 min at room temperature, the reaction was stopped with 1.0 ml of NaOH and the A_{400} was read.

[1] The authors wish to thank Robert Vande Stouwe, summer participant of the National Science Foundation Research Participation Program in Science, Grant GY-4236, for the preparation of this material.

[2] At the time that pNp was being prepared, it was discovered that a sample of Worthington RNA released a substantial amount of pNp upon hydrolysis with exonuclease, and therefore, appeared to be highly degraded. It is not known whether Worthington samples used previously to prepare pN>p and NpNp were similarly degraded. For the preparation of pNp, therefore, a sample of Crestfield RNA was used that released no detectable pNp on hydrolysis with exonuclease alone.

[3] Dr. Sulkowski also provided many valuable suggestions in choosing conditions for the use of this enzyme.

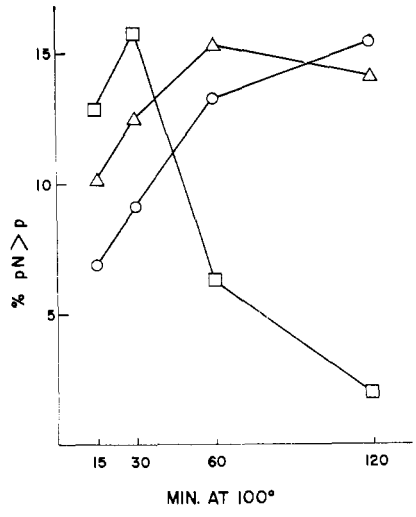

FIGURE 2: Yield of pN>p in exonuclease digests of RNA, as a function of prior hydrolysis in dilute ammonia. (○) 0.1 N NH_3, A_{271} = 100; (△) 0.1 N NH_3, A_{271} = 20; (□) 1.0 N NH_3, A_{271} = 100. Reaction mixtures consisting of NH_3 and 10 A_{271} units of RNA were heated in sealed glass tubes for different lengths of time. The tubes were cooled briefly on ice and opened, and acetic acid equivalent to the amount of ammonia used was added to reduce the pH to approximately 9. Exonuclease and $MgCl_2$ were then added to give approximate final concentrations of 1.0 mM and 0.14 unit/ml, respectively. (Sample △ was first reduced 10-fold in volume.) After incubation for 1 hr at 37°, 0.1-aliquots were electrophoresed on paper for 1.75 hr at pH 7, 23 V/cm. Amounts of pN>p were determined as per cent of total material (A_{271}) recovered from the paper.

exonuclease (0.133 unit). After incubation for 21.5 hr, 37°, 0.01-ml portions were electrophoresed for 2.0 hr at pH 5, 23 V/cm. Amounts of N and pNp were determined as per cent of total material (A_{271}) recovered from the paper. Table I shows the results. Clearly, the extent of hydrolysis with micrococcal nuclease is not critical.

Based on these results, a large-scale micrococcal nuclease digest was prepared as follows: 705 mg of RNA (Crestfield) was dissolved in 141 ml of 1.0 mM calcium acetate and brought to 36° and pH 9.0 in the pH-Stat with 0.1 N NaOH. Hydrolysis was carried out with 0.94 mg of crystalline micrococcal nuclease, pH being maintained with 0.1 N NaOH. The reaction was allowed to run for 30 min, at which time hydrolysis was 57.5% complete (estimated from NaOH consumption). The hydrolysate was adjusted to pH 6.5 with 0.1 N acetic acid, cooled to room temperature, passed through a short phosphocellulose column (NH_4^+ form, pH 6.5) to adsorb the enzyme, and concentrated in a rotary evaporator to 30 ml (A_{271} = 500).

Hydrolysis of RNA with Dilute Ammonia. Conditions optimal for the preparation of pN>p were determined as shown in Figure 2. A large digest of RNA was then prepared by heating 1 g of RNA (Worthington) in 600 ml of H_2O to 100° and adding 4 ml of concentrated NH_3. Final concentrations were A_{271} = 20 (determined in 0.1 N sodium phosphate, pH 6.7) and 0.1 N NH_3. Heating was continued for 1 hr with the flask tightly sealed, after which the mixture was rapidly cooled to room temperature in a −17° bath. The solution was adjusted to pH 6.5 with glacial acetic acid and concentrated to approximately 10 ml in a rotary evaporator. The material was then taken to dryness and ammonium acetate was removed by

TABLE I: Composition of Exonuclease Digests of RNA as a Function of Prior Hydrolysis with Micrococcal Nuclease.

% Hydrolysis by Micrococcal Nuclease[a]	% of Total A_{271} in Exonuclease Digest[b]	
	N	pNp
50	20.8	18.1
60	24.2	18.1
70	22.7	17.0

[a] Estimated from the amount of NaOH added, taking as 100% the amount added during complete hydrolysis of an identical RNA sample with venom exonuclease. [b] Remainder was mononucleotide plus a few per cent of resistant oligonucleotide.

two successive lyophilizations. The final residue was taken up in 23 ml of H_2O to give a solution of A_{271} = 670, pH 5.5.

Ion exchangers were obtained from Baker Chemical Co. (Dowex 1-X2, 50–100 mesh), from Reeve Angel (Whatman DE-52, microgranular preswollen), and from Schleicher & Schuell (Phosphocellulose type 20). Calcium bis(*p*-nitrophenyl)phosphate and sodium *p*-nitrophenylphosphate were purchased from Sigma Chemical Co.

Methods. Ion exchangers were pretreated according to manufacturer's instructions, and columns were packed under gravity flow from ¾ slurries in buffer (total volume/bed volume). Volatile buffers were prepared from solutions of acetic acid, formic acid, or ammonium carbonate of the desired molarity by adjusting pH with concentrated NH_3. Ammonium acetate buffer was removed in the preparation of dpNp by adjustment of the pH to 8.5 with triethylamine (Sulkowski and Laskowski, 1967) followed by lyophilization. In the preparation of pN>p and pNp, acetate and formate buffers were adjusted to pH 6.5 with 1 M NH_3, concentrated to 2–5 M in a rotary evaporator (bath temperature 30°), and finally lyophilized. Ammonium carbonate buffer was removed entirely in the rotary evaporator, except as noted for pN>p, under Results.

Both paper electrophoresis and hydrolysis with exonuclease for analytical purposes were carried out using conditions and techniques described previously (Richards and Laskowski, 1969). Electrophoresis at pH 5 was used to determine the amount of dinucleotide remaining during exonuclease hydrolysis of micrococcal nuclease digests of DNA. This technique cannot be used with RNA because Up migrates with the dinucleotides.

A different assay was therefore developed, in which the RNA samples, already exposed successively to micrococcal nuclease and venom exonuclease, were treated with semen phosphatase and electrophoresed. The presence of any ultraviolet-absorbing material migrating toward the positive pole was taken to indicate that exonuclease action had not been complete that is, some phosphorus remained in the diester form and therefore could not be removed by monophosphatase.

Hydrolysis with semen phosphatase was carried out in sealed capillary tubes as described previously (Richards and

Laskowski, 1969). Reaction mixtures consisted of 2.0 μl of hydrolysate (0.24 A_{271} unit), 1.0 μl of 1.0 M ammonium acetate (pH 4.8), containing 0.1 M EDTA, and 5.0 μl of semen phosphatase (1.23 units). These mixtures were left for 1 hr at room temperature before electrophoresis (0.5 hr, pH 7, 23 V/cm). The conditions for complete hydrolysis with semen phosphatase were established in separate control experiments.

Semen phosphatase was also used to determine the amounts of noncyclic isomers present in preparations of pN>p. Both 3′,5′- and 2′,5′-diphosphates are converted into nucleosides, while pN>p gives rise to N>p. The procedure was similar to that described above.

Results

dpNp. The micrococcal nuclease digest of DNA at pH 5 (see Materials) was brought to 37° in a closed vial equipped with a magnetic stirrer, and 63.6 units of exonuclease was added (final concentration 5.05 units/ml). As hydrolysis proceeded, considerable precipitate formed, which was periodically scraped from the vessel walls where it tended to stick. Progress of the reaction was checked by electrophoresing small portions of the reaction mixture at pH 5. At 19 hr (about 70% hydrolysis), 63.6 more units of exonuclease was added (final concentration 9.56 units/ml). Hydrolysis was continued to 78.5 hr, at which time only a trace of dinucleotide was still visible on electrophoresis. The suspension was cooled to room temperature and the pH was raised to 7.7 with concentrated ammonia. After cooling for 0.5 hr at 0°, the suspension was centrifuged, the supernatant liquid was removed, and the precipitate was suspended in 25 ml of water. Analysis of these fractions by electrophoresis at pH 5 and 7 showed that the supernatant solution consisted entirely of N and Np (or pN), in roughly equal amounts.

The suspension of pH 7.7 precipitate contained approximately one-quarter of the total dN, one-half of the dNp, traces of dNpNp, and all of the dpNp.[4] This material containing 4730 A_{271} units (49.6% of total hydrolysate) was diluted to 500 ml with H_2O and applied to a Dowex 1-X2 column (2 × 53 cm). Nucleosides were removed with water and elution of nucleotides was carried out with ammonium acetate solutions (pH 4.5) giving a pattern similar to that published previously (Sulkowski *et al.*, 1963). Recovery from the column was 100%. Yields of diphosphates are given in Table II. A total of 2613 units of nucleoside was present in the hydrolysate, 2060 units in the supernatant solution (determined by electrophoresis of a sample at pH 7), and 553 units in the precipitate. Comparison with the total recovered dpNp (2041 units) shows a discrepancy of 572 units, dN − dpNp = 2613 − 2041 = 572. The maximal possible loss of dpNp by dephosphorylation is therefore 21.9%.

pNp. Figure 3 shows that with 3′-phosphorylated ribodinucleotides, the rate of hydrolysis by exonuclease at pH 6 is approximately 125% of the rate at pH 9. A similar (but larger) response to pH has been observed previously with 3′-phosphorylated deoxyribodinucleotides (Richards and Laskowski, 1969). (The lower curve of Figure 3 has been reproduced from this work for purposes of comparison.) NpNp is hydrolyzed slightly faster than dNpNp.

TABLE II: Yields of Nucleoside Diphosphates in the Fractionation of Hydrolyzed DNA on Dowex 1-X2.

Nucleotide	A_{271} Units	% of Fractionated Material	% of Total Digest
dpCp	431	9.1	4.5
dpTp	154	3.2	1.6
dpAp	346	7.3	3.6
dpGp	1110	23.4	11.7
Total	2041	43.0	21.4[a]

[a] This material contained 10–20% dinucleotide.

In addition to the slightly faster rate of hydrolysis at pH 6 than pH 9, there is the advantage in preparing pNp near pH 6 that the monophosphatases of venom have decreased activity at this pH (Sulkowski *et al.*, 1963). Such a procedure is illustrated in Figure 4. At 31 and 45 hr, 11% of the digest cannot be dephosphorylated to nucleoside by semen phosphatase, indicating the persistence of substrate, probably dinucleotide, which resists hydrolysis by exonuclease. Between 19 and 45 hr, the amount of pNp is constant, suggesting little or no dephosphorylation of this material. Both of these observations are in contrast to results obtained previously with DNA derivatives (Richards and Laskowski, 1969).

The final hydrolysate contained a greater variety of partial digestion products than did the DNA hydrolysate[5] (besides mono- and dinucleotides, higher oligomers were present), and it was felt that an unworkably complex fractionation pattern would be obtained on Dowex 1-X2. In addition, the Dowex column is slow (9 days) and the recovery of material from large volumes of concentrated ammonium acetate is also cumbersome, and can lead to some dephosphorylation of products (Sulkowski and Laskowski, 1967). An alternative procedure was therefore used in which pyrimidine and purine nucleoside diphosphates, respectively, were segregated first from most other components (Figure 5, upper), after which pCp was separated from pUp, and pAp from pGp in separate column runs (Figure 5, middle and lower).

pN>p. A trial hydrolysis with exonuclease was carried out on a 0.2-ml portion of the NH_3 digest of RNA (see Materials). Conditions were adjusted to give complete liberation of pN>p, without substantial release of pNp from oligonucleotides terminated in 3′-phosphate.[6] Richards and Laskowski (1969) previously showed that chains terminated in cyclic 2′,3′-phosphate are hydrolyzed optimally at pH 9, and require only a low level of exonuclease. The hydrolysate was electro-

[4] It also contained essentially all of the $CaCl_2$ and exonuclease. Of the latter, 96 units, potency = 6.3, was recovered by concentration dialysis under vacuum (Schleicher & Schuell collodion membrane).

[5] As in the preparation of dpNp, exonuclease was recovered from the hydrolysate by concentration dialysis under vacuum (141 units, potency = 4.0. The low potency is probably due to protein from the RNA).

[6] A_{271} = 103, [exonuclease] = 0.1 unit/ml, 0.1 M ammonium acetate pH 9; 1-hr hydrolysis at 37°. The same considerations apply to the experiment of Figure 2.

TABLE III: Distribution of Products During Fractionation of an Ammonia–Exonuclease Digest of RNA on DE-52 Columns.

Column Run	A_{271} Units Charged	% Charge Recovd	Pooled Fractions: Principal Component and % of Total Fraction[a]
1, pH 5	15,140	101	N, 15; C>p + U>p, 11; A>p + G>p, 20: Np + pN, 12; pC>p + pU>p, 11; pA>p + pG>p, 15
2, pH 4	1,710	98.6	pC>p, 34; pU>p, 36
3, pH 4	2,160	98.4	pA>p, 29; pG>p, 23

[a] Listed in order of elution.

phoresed for 4 hr in 0.1 M $(NH_4)_2CO_3$, 12 V/cm. Nucleoside diphosphates corresponding to 16.5% of the total A_{271} were recovered by elution. Treatment of a small portion of this material with semen phosphatase yielded 84% N>p, indicating that 14% of the exonuclease hydrolysate was pN>p, and 2.5% was pNp.

Of the remaining NH_3 digest, 20 ml was diluted with 114 ml of water and brought to 36° and pH 9.0 with 1.0 N NaOH. Hydrolysis with exonuclease was carried out in a pH-Stat under conditions similar to those used in the trial experiment[6] except that buffer was absent and 0.1 N NaOH was used to maintain pH. After 2 hr, when the net reaction slope had fallen to about 10% of its initial value, the reaction was stopped by adding EDTA to a final concentration of 1.0 mM. The blank values of NaOH consumption recorded before addition of enzyme and after addition of EDTA were equal.

Fractionation of the hydrolysate was carried out in essentially the same manner as was used for pNp (see Figure 5). An initial concentration of 0.01 M ammonium acetate and a total volume of 8 l. was used in the gradient for the first of these column runs, in order to obtain the 2′,3′-cyclic pyrimidine and purine nucleotides, respectively, as separate peaks. In the second and third column runs, it was observed that each pNp was eluted slightly ahead of the corresponding pN>p. Table III summarizes the results of these column runs.

Final Purification. The twelve preparations of nucleoside diphosphate were freed of buffer and subjected to electrophoresis. In the case of dpNp, electrophoresis was carried out at pH 5 (Sulkowski and Laskowski, 1967). Because of its high conductivity and ease of removal, ammonium carbonate buffer was used for the electrophoresis of ribonucleoside diphosphates (Figure 6). To avoid complications from charges on the bases pGp and pUp, pH 8.5 was used. Table IV shows the extent of purification during electrophoresis of pNp. A similar degree of purification was obtained in the case of dpNp and pN>p (Table V). Between 10 and 30% of contaminants, principally dinucleotides, were removed in each case. Total recoveries during electrophoresis were 95–100%.

Table IV also gives the final yields of pNp. Comparing the

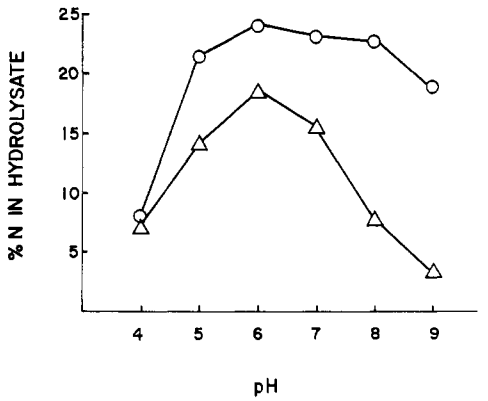

FIGURE 3: Effect of pH on the hydrolysis of mixed-base 3′-phosphorylated ribodinucleotides (NpNp) by venom exonuclease. (○) Reaction mixtures (total volume 0.063 ml) were incubated for 1 hr at 37° in sealed capillary tubes. Substrate A_{271} = 65, [enzyme] = 4.2 units/ml, [buffer] = 0.1 M (Tris-succinate), $[MgCl_2]$ = 1.0 mM. After electrophoresis on paper for 0.5 hr, 23 V/cm, pH 7.0, amounts of N were determined as per cent of total material (A_{271}) recovered from the paper. (△) Results obtained previously with deoxyribodinucleotides by Richards and Laskowski (1969). Substrate A_{271} = 83, [enzyme] = 5.1 units/ml; [buffer] and $[MgCl_2]$ as above.

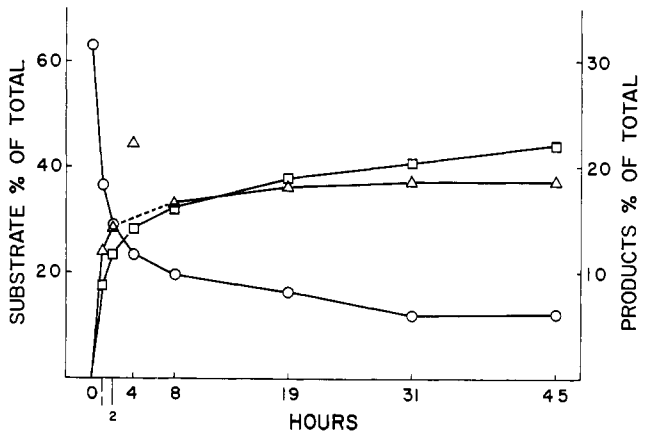

FIGURE 4: Exonuclease hydrolysis of a micrococcal nuclease digest of RNA. (○) Material not completely hydrolyzed by exonuclease (see below); (△) pNp and (□) N. The micrococcal nuclease digest (see Materials) was brought to 37° and exonuclease was added. A_{271} = 476, [exonuclease] = 5.5 units/ml, pH 6.2. Periodically, 2.0-μl portions were electrophoresed on paper for 0.5 hr, 23 V/cm, pH 7.0. Amounts of N and pNp were determined as per cent of total material (A_{271}) recovered from the paper. In addition, 2.0-μl portions were treated with semen phosphatase, as described in Methods, prior to electrophoresis. From this the per cent of material not hydrolyzed to N was calculated (○). Interpretation of this number is discussed in the text.

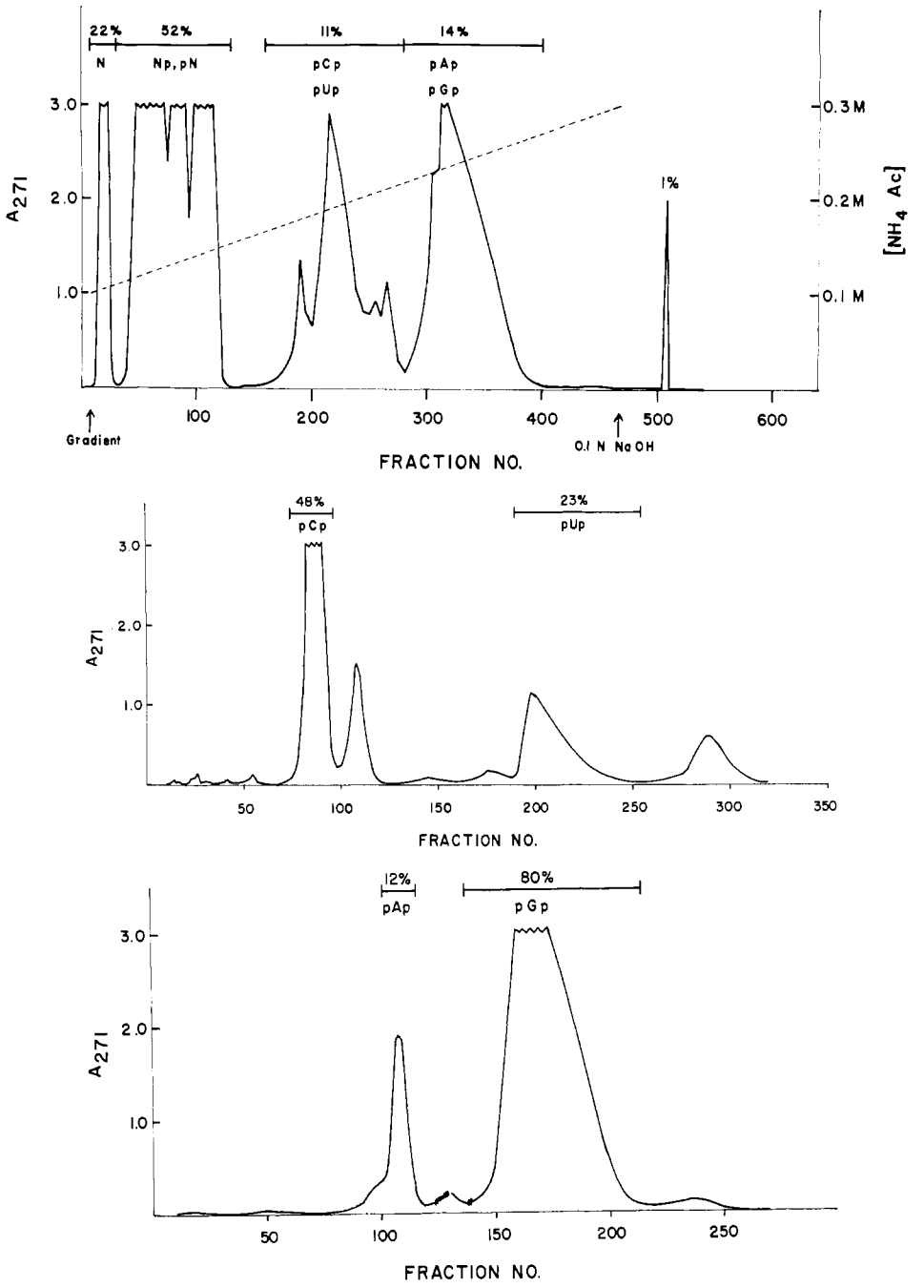

FIGURE 5: Fractionation of a micrococcal nuclease–venom exonuclease digest of RNA in the preparation of pNp. Upper figure: separation of pPyp from pPup. Dialysate of digest (Figure 4) adjusted to pH 5.0 with 1.0 N acetic acid and applied to column of DE-52, pH 5, acetate form, 2.5 × 40 cm. Eluted with a gradient (dashed line) of 3.0 l. of 0.1 M ammonium acetate (pH 5.0) *vs.* 3.0 l. of 0.3 M buffer, followed by 1.0 l. of 0.1 N NaOH. Flow rate 108 ml/hr, fraction volume 12.6 ml. Applied to column, 16,600 A_{271} units (pH 5); recovered in fractions, 92.6% of charge. Center figure: separation of pCp from pUp. Pooled fractions of pPyp from upper figure freed of ammonium acetate buffer (see Methods). Residue dissolved in 0.1 M ammonium formate (pH 4.0) to give A_{271} = 100, and pH adjusted to 4.0 with 1.0 N formic acid. Sample applied to column of DE-52, pH 4, formate form, 2.5 × 46 cm. Eluted with 0.1 M ammonium formate (pH 4.0). Flow rate 107 ml/hr, fraction volume 12.5 ml. Applied to column, 1650 A_{271} units (pH 4); recovered in all fractions, 94.8% of charge. Lower figure: separation of pAp from pGp. Pooled fractions of pPup from upper figure freed of ammonium acetate buffer (see Methods). Residue dissolved in 0.15 M ammonium formate pH 4.0 to give A_{271} = 100, and pH adjusted to 4.0 with 1.0 N formic acid. Column from center figure washed with 300 ml of 0.15 M ammonium formate (pH 4.0) and sample was applied. Eluted with same buffer. Flow rate 101 ml/hr, fraction volume 12.4 ml. Applied to column, 2100 A_{271} units (pH 4); recovered in all fractions, 99.8% of charge. All three figures: A_{271} was read on selected fractions, based on continuous monitoring at 280 mμ. Fractions pooled as indicated. Percentage figures are based on total A_{271} units recovered = 100%. Principal components of each pooled fraction identified by paper electrophoresis at pH 7 and 4.

total of 3064 A_{271} units of pNp with the 3640 A_{271} units of nucleoside found in the hydrosylate (Figure 5, upper) shows that a maximum of 15.8% dephosphorylation of pNp could have occurred. The actual amount of dephosphorylation may be

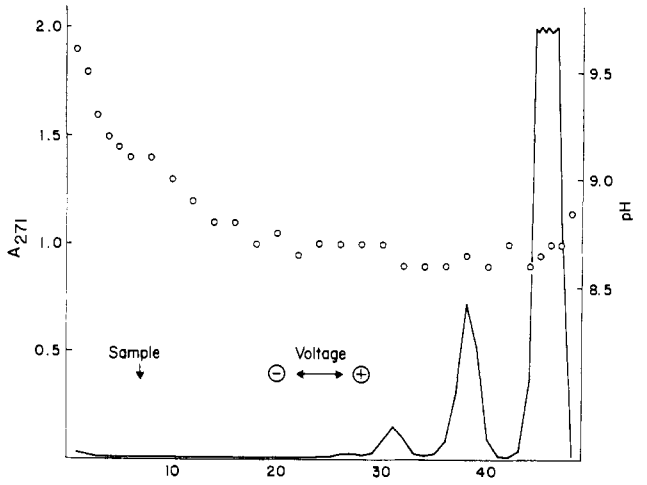

FIGURE 6: Final purification of pG>p by continuous free-flow electrophoresis in the Brinkman FF electrophoretic separator. All samples were dissolved in separating buffer at A_{271} = 30, and pH was readjusted to 8.5. Samples were injected at the chamber position indicated. Maximum sample volume for 1-day run was 5 ml. (—) A_{271} and (○) pH. Separating buffer 0.01 M ammonium carbonate adjusted to pH 8.5 with concentrated NH_3, electrode buffer 0.03 M, buffer pump 7 rpm, sample pump 3.5 rpm, separation at 1800 V and 75 mA. Other pN>p, pNp, and dpNp gave similar separation patterns under appropriate conditions. Other pN>p: as above. pNp: as above, but at 1600 V and 69 mA. dpNp: separating buffer 0.05 M ammonium acetate (pH 5.0), electrode buffer (0.15 M), buffer and sample pumps, 3.5 rpm, separation at 1500 V and 160 mA.

less, since some pNp was lost in the leading and trailing edges of peaks during pooling of fractions in the experiments of Figure 5.

The preparations of pN>p obtained from the DE-52 columns (Table III, runs 2 and 3) contained between 3.1% (pC>p) and 7.4% (pU>p) of noncyclic isomers, as determined with semen phosphatase. Electrophoresis in ammonium carbonate buffer effectively removed contaminants such as dinucleotides (Table V), but no useful separation between pN>p (expected charge −3) and pNp (expected charge −4) was observed. Occasionally, decyclization was found to occur during evaporative removal of the ammonium carbonate buffer. To avoid this, pH was first adjusted to 6.5 with dilute acetic acid and recovery was carried out as with acetate buffers.

TABLE IV: Yields of Ribonucleoside 3′,5′-Diphosphates.

Nucleotide	% of Recovd Material	Total A_{271} Units[a]	% of Starting Hydrolysate
pCp	76.0	614	3.6
pUp	83.5	349	2.1
pAp	86.6	236	1.4
pGp	87.3	1865	9.8
Total		3064	16.9

[a] Calculated value based on electrophoresis of portions containing 150 A_{271} units.

TABLE V: Yields of Ribonucleoside (Cyclic 2′,3′),5′-Diphosphates.

Nucleotide	% of Recovd Material	Total A_{271} Units[a]	% of Starting Hydrolysate
pC>p	87.6	495	3.3
pU>p	86.1	513	3.4
pA>p	68.6	440	2.9
pG>p	80.8	384	2.5
Total		1832	12.1

[a] Calculated value based on average values in several electrophoretic runs.

Discussion

The first of the two major issues in this paper, the effect of low pH on the susceptibility of resistant substrates, has been discussed at length in the previous paper of this series (Richards and Laskowski, 1969). At that time only deoxyribodi- and trinucleotides were studied. Figure 3 shows that the inhibitory effect of negative charge at the ω end of oligonucleotides is a general one, occurring with ribose as well as deoxyribose derivatives.

While with rNpNp the increase in rate at pH 6 over that at pH 9 is only 125% compared with 500% with dNpNp, it should be remembered that with susceptible substrates such as NpN or bis(*p*-nitrophenyl)phosphate, the rate at pH 6 is between 5 and 15% of that at pH 9. Thus second negative charge on 3′-phosphate group of rNpNp still results in approximately 10-fold inhibition.

Recently Mikulski *et al.* (1969) showed that a smaller but essentially similar phenomenon occurs with micrococcal nuclease. In this case, however, the inhibitory effect of the phosphoryl group occurs at the 5′ terminus. The phenomenon may be common to all exonucleases attacking substrates with an unnatural termination with respect to phosphate.

The second issue to be discussed is the quality of the exonuclease preparation. When the venom of the Brazilian snake *B. atrox* could no longer be obtained in this country, the readily available venoms of three North American species were examined as possible sources for the preparation of exonuclease (Richards *et al.*, 1965). The venom of *C. adamanteus* was selected because its composition was most similar to that of *B. atrox*.

A four-step procedure (Richards *et al.*, 1967) leads to the present preparation. Using as criteria the activity toward 5′-AMP and the phosphomono- and phosphodiesters of *p*-nitrophenol, this preparation closely resembles the formerly used one from *B. atrox* (Björk, 1963) in potency and contamination with monophosphatases.

A far more rigorous and meaningful criterion, however, is the loss by dephosphorylation encountered in the preparation of pNp. Unfortunately, an accurate comparison of the two preparations is not possible. The only figures available for the preparation from *Bothrops* (Sulkowski *et al.*, 1963) are for digestion at pH 9. Less than 15% dephosphorylation was

found with d(ApGp) as substrate, when the experiment was performed on a small scale and the end point was carefully controlled. With mixed dinucleotides and the scale increased, dephosphorylation was about 40%. In the present work, at three times the scale and with exonuclease used at pH 6, dephosphorylation was 22% with dpNp and 16% with pNp (see Results). It can be seen that the end results are roughly comparable.

The principal limitation on the scale of the method appears to be the amount of exonuclease required, which is very expensive. As described in Results, approximately 80% of the exonuclease can be recovered at the completion of hydrolysis. On a pilot scale, the same exonuclease sample has been used in three different experiments with no noticeable deterioration.

Finally, it seems quite possible that the method could be applied to the preparation of diphosphates of the rare ribonucleotides. Two possible difficulties could be that (1) RNA were attacked by micrococcal nuclease in such a way that all rare nucleosides appear either as mononucleotides or in the α position of dinucleotides, and (2) dinucleotides with rare nucleosides in the β position were resistant to exonuclease. At present there seems to be no firm basis for either assumption.

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