Hydrolysis of Ribonucleoside 3′-Diphosphates by Rye Grass 3′-Nucleotidase†

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ABSTRACT: Rye grass 3′-nucleotidase has been purified to apparent homogeneity on Sephadex A-25 and CM-cellulose columns and shown to hydrolyze 2′-*O*-methyladenosine 3′-monophohate and 2′-deoxyadenosine 3′-monophosphate 35.8 and 542 times more slowly than the normal substrate (3′-AMP), verifying the importance of the 2′-β-OH group of the substrate in the overall hydrolysis process. Although neither was hydrolyzed as rapidly as 3′-AMP, both the 2′-*O*-methyl and 2′-deoxy analogs acted as competitive inhibitors of the hydrolysis of 3′-AMP (K_m = 0.12 mM), with apparent K_i's of 0.39 and 0.51 mM, respectively. In order to determine the possible susceptibility of naturally occurring ribonucleoside 3′-diphosphates, such as guanosine tetraphosphate (ppGpp), to 3′-phosphohydrolase activities, the 3′-nucleotidase was also employed in the attempted pyrophosphorolysis of adenosine 3′-diphosphate and guanosine tetraphosphate. Neither adenosine 3′-diphosphate nor guanosine tetraphosphate was degraded at a significant rate by the nucleotidase, relative to the normal substrate.

At least two ribonucleoside 3′-diphosphates, guanosine 5′-diphosphate 3′-diphosphate (ppGpp), and guanosine 5′-triphosphate 3′-diphosphate (pppGpp), occur naturally in stringent strains of *Escherichia coli* and *Bacillus subtilus* (Cashel and Gallant, 1969; Cashel, 1969; Swanton and Edlin, 1972; Gallant and Margason, 1972). These ribonucleotides accumulate during amino acid starvation as a result of their rapid formation in a ribosomal "idling step" (Haseltine *et al.*, 1972) and disappear when nutritional sufficiency is reestablished. Substantial interest in these ribonucleotides has developed recently, based largely on the assumption that they are regulatory agents responsible for expression of the stringent response.

Although the biosynthesis of the ribonucleotides has been studied in some detail (Sy and Lipmann, 1973; Sy *et al.*, 1973; Haseltine and Block, 1973), much less information is available concerning, *e.g.*, the chemical stability of these species, the enzyme activities which may be responsible for their rapid degradation under conditions of nutritional sufficiency, or the structures of degradation products. The importance of ppGpp and pppGpp as potential regulatory agents suggests the need for studies of the chemistry and biochemistry of ribonucleoside 3′-diphosphates, particularly with respect to those chemical and enzymatic processes which may result in their destruction. Since the degradation of the guanosine ribonucleotides may well involve a net 3′-pyrophosphorolysis, which could be assisted by intervention of the 2′-β-OH group as an internal nucleophile in a chemically or enzymatically promoted breakdown, and because the actual stability of ribonucleoside 3′-diphosphates to 3′-phosphohydrolase activities is unknown, we have studied the interaction of adenosine 3′-diphosphate and guanosine 5′-diphosphate 3′-diphosphate with rye grass 3′-nucleotidase, the major activity of which has been shown to require a free 2′-β-OH group for the hydrolysis of 3′-AMP.

Materials and Methods

Adenosine, 2′-deoxyadenosine 3′-monophosphate, adenosine 3′-monophosphate, crude snake venom (*Crotalus adamanteus*), purified rye grass 3′-nucleotidase (lyophilized powder) and carboxymethylcellulose were purchased from Sigma Chemical Company. Guanosine 5′-monophosphate 3′-monophosphate was obtained from Boehringer Mannheim Corporation. Tritiated water (1.8 Ci/mol) was purchased from Schwarz/Mann and Sephadex A-25 from Pharmacia Fine Chemicals. Whatman No. 1 paper, DEAE-cellulose paper, and DEAE-cellulose were obtained from H. Reeve Angel and Co.

Ultraviolet and visible spectra were determined on a Cary 15 spectrophotometer. Radioactivity was determined on a Beckman LS-100C liquid scintillation spectrometer. Liquid samples were counted in a xylene-based scintillation fluid (Anderson and McClure, 1973).

Synthesis of Analogs

2′-Deoxyadenosine 3′-Monophosphate (**2**). This compound was prepared from 2′-deoxyadenosine by a procedure similar to that employed by Tener (1961) for the synthesis of nucleoside monophosphates. To 120 mg (0.40 mmol) of monobarium cyanoethyl phosphate, converted to the pyridinium salt, was added 50 mg (0.20 mmol) of 2′-deoxyadenosine. The mixture was rendered anhydrous by repeated evaporation of dry pyridine under diminished pressure. The anhydrous residue was dissolved in 2 ml of pyridine and treated with 330 mg (1.6 mmol) of *N,N′*-dicyclohexylcarbodiimide. The solution was permitted to stand at room temperature for 36 hr, then treated with 3 ml of water and heated on a steam bath for 30 min. The cooled solution was concentrated to dryness under diminished pressure, treated with 10 ml of 9 M ammonium hydroxide solution, and heated at 70° for 3 hr. The solution was concentrated to a small volume and filtered, and the solid was washed with portions of water. The combined filtrate was treated with 1 ml of 0.5 M barium acetate solution and the resulting precipitate was removed by centrifugation. The supernatant was applied to a DEAE-cellulose column (HCO_3^- form; 2.5 × 25 cm) and washed with 200 ml of water, which afforded 10 mg of un-

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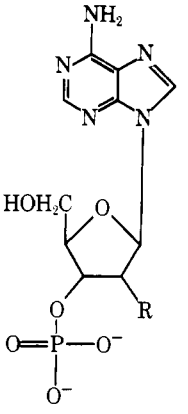
reacted 2′-deoxyadenosine. Elution with a linear gradient of ammonium bicarbonate (2 l. total volume 0–0.3 M; 12-ml fractions) at a flow rate of 175 ml/hr gave 30 mg (45%) of 2′-deoxyadenosine 5′-monophosphate, elution at 0.10 M ammonium bicarbonate, and 10 mg (16%) of 2′-deoxyadenosine 3′-monophosphate (**2**), elution at 0.18 M ammonium bicarbonate. The two monophosphates were specifically degraded to deoxyadenosine by the action of 5′- and 3′-nucleotidase activities, respectively, and each was shown by phosphate analysis (Allen, 1940) to contain 1 mol of phosphate/mol of deoxyadenosine.



1. R = OH
2. R = H
3. R = OCH_3

2′-O-Methyladenosine 3′-Monophosphate (**3**). This compound was synthesized from the ribonucleoside by the method of Imai *et al.* (1969). To a cooled suspension of 87 mg (0.31 mmol) of 2′-*O*-methyladenosine (Robins and Naik, 1971) in 7.5 ml of *m*-cresol was added 0.25 ml of pyrophosphoryl chloride. The resulting solution was stirred at 0° for 2 hr, diluted with 5 ml of ice-water, and extracted with portions of ether. The aqueous layer was adjusted to pH 7 with sodium hydroxide solution and treated with 5 ml of 0.5 M barium acetate solution. The precipitate was removed by centrifugation and the supernatant was applied to a column of DEAE-cellulose (HCO_3^- form; 1.5 × 19 cm). The column was washed with water, effecting the elution of unreacted 2′-*O*-methyladenosine, then with a linear gradient of ammonium bicarbonate solution (2 l. total volume; 0–0.4 M; 12-ml fractions) at a flow rate of 150 ml/hr, which effected the elution of 92 mg (75%) of 2′-*O*-methyladenosine 5′-monophosphate and 8 mg (7%) of 2′-*O*-methyladenosine 3′-monophosphate (**3**). The identities of the monophosphates were verified by phosphate analysis (Allen, 1940) and by the specific degradation of each with the appropriate nucleotidase activity.

Ribonucleoside 3′-Diphosphates. The synthesis of adenosine 3′-diphosphate has been described (Mitchel *et al.*, 1967). A more efficient synthesis of this diphosphate, as well as the synthesis of adenosine 3′-triphosphate, will be reported elsewhere (Kozarich *et al.*, 1975) as will an additional synthesis of guanosine tetraphosphate (Simoncsits and Tomasz, 1974; Kozarich *et al.*, 1975).

[8-^{3}H]Adenosine 3′-Monophosphate. To 7.0 mg (20 μmol) of [8-^{3}H]adenosine cyclic 3′-5′-monophosphate, 0.89 Ci/mol, prepared from unlabeled adenosine cyclic 3′-5′-monophosphate by the general method of Shelton and Clark (1967), was added 2.0 ml of 0.1 M Tris-HCl solution (pH 8.7), 10 mg of crude snake venom (*Crotalus adamanteus*), and 1 drop of toluene (Smith *et al.*, 1961). The reaction mixture was incubated at 37° for 108 hr and then applied to a column of DEAE-cellulose (2 × 20 cm). Elution with a linear gradient of ammonium bicarbonate (1 l. total volume, 0–0.3 M) at a flow rate of 100 ml/hr afforded, after desalting by repeated evaporations of portions of water, 3.8 mg (50%) of adenosine 3′-monophosphate, 0.56 Ci/mol, as well as 0.7 mg (13%) of adenosine and 2.2 mg of unreacted adenosine cyclic 3′,5′-monophosphate. The homogeneity of the adenosine 3′-monophosphate was verified by paper chromatography in two solvent systems (Table I), which revealed a single ultraviolet-absorbing spot in each case. The chromatograms were cut into sections 1 cm in length and counted in 5 ml of toluene-based scintillation fluid. The single peak of radioactivity in each chromatogram was coincident with the ultraviolet-absorbing material.

[8-^{3}H]-2′-Deoxyadenosine 3′-monophosphate (**2**), 0.81 Ci/mol, and [8-^{3}H]-2′-*O*-methyladenosine 3′-monophosphate (**3**), 0.65 Ci/mol, were prepared according to the general method of Shelton and Clark (1967) by heating the unlabeled nucleoside 3′-monophosphates in T_2O, 1.8 Ci/mol. The products were purified by chromatography on DEAE-cellulose (as above) and the homogeneity of each sample was verified by paper chromatography (Table I).

Characterization of the Purified 3′-Nucleotidase

Stability of Purified 3′-Nucleotidase. A solution of 285 A_{258} units of adenosine 3′-monophosphate in 1.0 ml of 0.1 M Tris-HCl (pH 7.5) was equilibrated at 37° and then treated with 1.65×10^{-3} unit of 3′-nucleotidase. The combined solution was incubated at 37° for 12 hr and then assayed for released P_i as above, relative to standards which contained known concentrations of P_i. The results indicated that 16.5 A_{258} units of 3′-AMP had been hydrolyzed (*vs.* a theoretical value of 17.0 A_{258} units).

pH Profile of Purified 3′-Nucleotidase. To 57 A_{258} units of adenosine 3′-monophosphate was added 1.5 ml of 0.1 M Tris-HCl (pH 6.8, 7.2, 7.5, 7.8, 8.2, or 8.6) which had been preequilibrated at 37°. Each solution was treated with 24 μl of a solution of purified 3′-nucleotidase[1] (containing 0.01 unit of enzyme activity) and incubated at 37° for 20 min. Each reaction was quenched by the addition of 0.5 ml of 60% aqueous $HClO_4$ and assayed for released P_i (Allen, 1940). The resulting profile is shown in Figure 4.

Enzyme Assays

Hydrolysis of Adenosine 3′-Monophosphate in the Presence of Unfractionated 3′-Nucleotidase. A solution of 19.9 mg (52.4 μmol) of 3′-AMP in 1.0 ml of 0.1 M Tris-HCl (pH 7.5) was equilibrated at 37° and then treated with 0.10 unit of unfractionated 3′-nucleotidase. The solution was incubated at 37° and aliquots (50 μl) were removed at predetermined time intervals. Each was diluted to 1 ml with cold water and applied to a DEAE-cellulose column (12 ml; HCO_3^- form). Elution was effected with 50 ml of water to remove adenosine from the column and then with ammonium bicarbonate solution to remove unreacted adenosine 3′-monophosphate. The extent of reaction *vs.* time is shown in Figure 5.

Relative Rates of Substrate Hydrolysis. To a solution of 3.75–160 A_{258} units of tritiated nucleotide in 0.10 M Tris-HCl buffer (pH 7.8), preequilibrated at 37°, was added

[1] One unit of 3′-nucleotidase is defined as that amount which hydrolyzes 1.0 μmol of P_i from 3′-AMP per min at pH 7.5 and 37°.

Table I: Chromatographic Properties (R_F Values) of the Tritiated Nucleoside 3′-Monophosphates.[a]

Compound	Solvent[b] A	Solvent[b] B
[8-^{3}H]Adenosine 3′-monophosphate (**1**)[c]	0.29	0.23
[8-^{3}H]-2′-Deoxyadenosine 3′-monophosphate (**2**)	0.15	0.14
[8-^{3}H]-2′-*O*-Methyladenosine 3′-monophosphate (**3**)	0.18	0.11
Adenosine	0.69	0.64

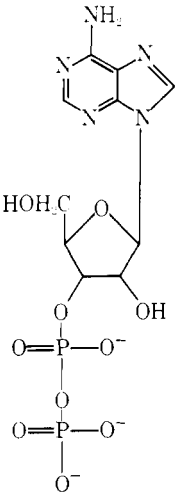
[a] Determined on Whatman No. 1 paper by descending chromatography. Each compound gave a single spot in each solvent system, detectable by ultraviolet visualization. The monophosphates all had radioactivity associated exclusively with the ultraviolet-absorbing material. [b] Solvent A, 5:2:3 1-butanol–acetic acid–water; solvent B, 7:1:2 2-propanol–ammonium hydroxide–water. [c] An authentic sample of adenosine 3′-monophosphate had the same R_F values.

0.01–0.43 unit of purified 3′-nucleotidase. The reaction mixture was incubated at 37° for 0.5–480 min and quenched by the addition of 200 μl of 0.6 M sodium acetate solution (pH 4.5) containing 1% sodium dodecyl sulfate and 30 A_{258} units of unlabeled nucleoside. Analysis of the extent of hydrolysis was effected by chromatographic separation of the respective nucleosides and nucleotides on a column of Sephadex LH-20 (3.5 × 45 cm), elution with water. The radioactivity of the nucleosides and nucleotides was determined by scintillation counting of concentrated solutions of each (Anderson and McClure, 1973). The relative rates of hydrolysis, which were determined from the linear portion of each substrate hydrolysis curve, are given in Table II.

Total Hydrolysis of [8-^{3}H]Adenosine 3′-Monophosphate. To a solution of 2.0 A_{258} units of [^{3}H]adenosine 3′-monophosphate, 0.56 Ci/mol, in 1.0 ml of 0.1 M Tris-HCl buffer (pH 7.8), which had been equilibrated at 37°, was added 0.038 unit of purified 3′-nucleotidase. The combined solution was incubated at 37° for 80 min and then analyzed for ribonucleotide content by chromatography on a 2-ml DEAE-cellulose column, elution with 20 ml of water and then 20 ml of 0.3 M ammonium bicarbonate solution. Each solution was concentrated to a small volume and counted in 10 ml of xylene-based scintillation fluid. All of the radioactivity was recovered and less than 1% was still present as adenosine 3′-monophosphate.

Hydrolysis of [8-^{3}H]-2′-Deoxyadenosine 3′-Monophosphate by 3′-Nucleotidase. To 1.0 ml of 0.1 M Tris-HCl (pH 7.8) containing 15 A_{258} units of [8-^{3}H]-2′-deoxyadenosine 3′-monophosphate, 0.81 Ci/mol, was added 0.2 unit of 3′-nucleotidase. The combined solution was incubated at 37° for 24 hr and then analyzed on a 2-ml DEAE-cellulose column as described above. All of radioactivity was recovered and 27% was found to have been hydrolyzed to nucleoside (after correction for a control run at the same time without added enzyme). Hydrolysis of the ribonucleotide under these conditions should theoretically have given 53% hydrolysis (based on a measured rate of 0.00182 μmol per enzyme unit per min), the difference presumably arising from product inhibition of the enzyme (see Figure 5; Table II).

Hydrolysis of Adenosine 3′-Diphosphate by 3′-Nucleotidase. A solution of 15 A_{258} units of adenosine 3′-diphosphate (**4**) in 1.0 ml of 0.1 M Tris-HCl (pH 7.5) was equilibrated at 37° and then treated with 0.2 unit of unfractionated 3′-nucleotidase. The combined solution was incubated at 37° for 5 hr and then applied to a DEAE-cellulose column (12 ml; HCO_3^-). Elution was effected with 20 ml of water and then with a linear gradient of ammonium bicarbonate (200 ml total volume; 0–0.8 M; 2-ml fractions). The water wash was found to contain 1.9 A_{258} units of adenosine (after correction for 0.2 A_{258} unit found in the water wash of a control reaction run without added enzyme), representing about 0.2% of the hydrolysis which would have been obtained with 3′-AMP.



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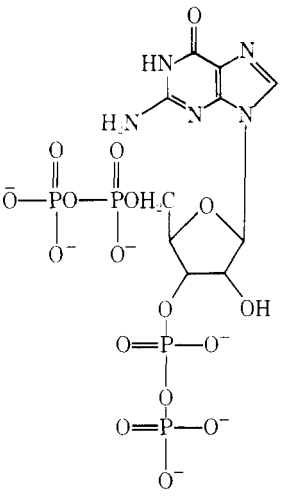

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Hydrolysis of Guanosine Tetraphosphate (ppGpp) by 3′-Nucleotidase. A solution of 1.37 A_{253} units of guanosine tetraphosphate (**5**), 0.95 Ci/mol, in 50 μl of 0.1 M Tris-HCl (pH 7.5) was equilibrated at 37° and then treated with 0.1 unit of unfractionated 3′-nucleotidase. The combined solution was incubated at 37° for 1 hr and the total solution was analyzed by chromatography on a polyethyleneiminecellulose tlc plate which had been pretreated with a solution of 50% aqueous methanol containing 0.01% Triton X-100 (Cashel, 1974). The plate was developed with 1.5 M aqueous KH_2PO_4 (pH 3.4) and analyzed by scraping the support from the plate at appropriate R_F values (Simoncsits and Tomasz, 1974) and removing the compounds from the support by washing with 1 ml of 1.5 M aqueous KH_2PO_4 (pH 3.4). The aqueous solutions were counted in a xylene-based scintillation fluid (Anderson and McClure, 1973). It was found that 0.57 A_{253} unit of ppGpp was converted to ppG under these conditions (after correction for degradation of ppGpp which occurred in a control reaction in the absence of added enzyme). This represented about 0.8% of the hydrolysis which would have been obtained if 3′-AMP had been the substrate.

Kinetics of Adenosine 3′-Monophosphate Hydrolysis. (a) DETERMINATION OF THE K_m OF 3′-AMP. To 49.5 μg (0.13 μmol) of [8-^{3}H]adenosine 3′-monophosphate, 0.56 Ci/mol, was added sufficient nonradiolabeled adenosine 3′-monophosphate to give final substrate concentrations of 0.097, 0.210, 0.532, or 1.01 mM in 1.0–1.33 ml of 0.10 M Tris-HCl (pH 7.8). The solution was equilibrated at 37° and reaction was initiated by the addition of enzyme to a final concentration of 0.001 unit/ml of reaction mixture. At the appropriate times (4–30 min), the reactions were

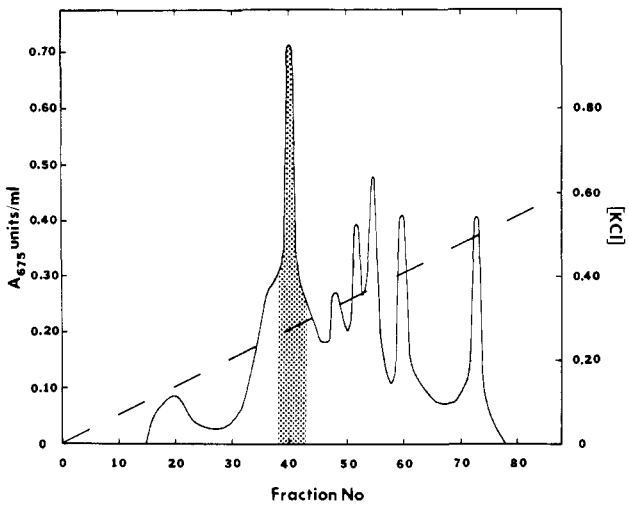


FIGURE 1: Initial fractionation of commercial rye grass 3′-nucleotidase. Two units (145 μg of protein) of the enzyme was applied to a column of Sephadex A-25 (2 × 26 cm) which had been preequilibrated in 20 mM Tris (pH 9.0) at 4°. The column was washed with a linear gradient of KCl in 20 mM Tris (pH 9.0) (400 ml total volume; 0–0.67 M; 4.1-ml fractions) at a flow rate of 75 ml/hr. An aliquot of each fraction was treated with an equal volume of 0.5 M Tris-HCl (pH 7.5) containing 33 A_{258} units of adenosine 3′-monophosphate. The aliquots were incubated at 37° for 3 hr and then assayed for inorganic phosphate (Allen, 1940). Recovered activity was 81% of that applied to the column. The major activity (fractions 38–43; shaded area) was further purified by additional chromatography on Sephadex A-25 (see Figure 2).

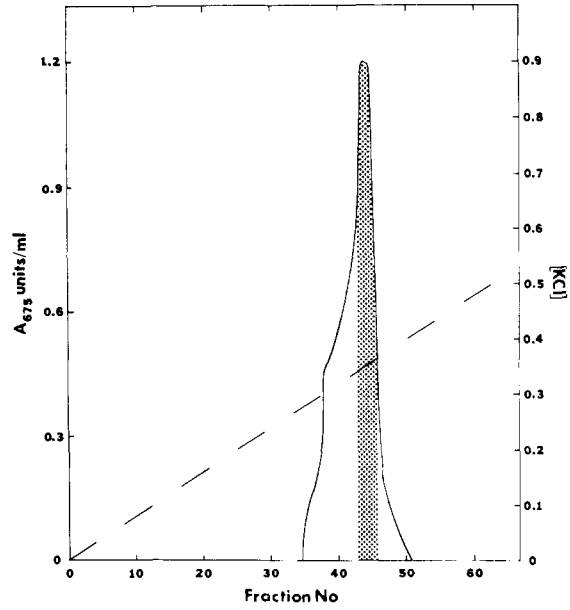


FIGURE 2: Chromatography of partially purified rye grass 3′-nucleotidase. The appropriate fractions from the first Sephadex A-25 chromatography (Figure 1; fractions 38–43; 25% of recovered activity) were combined, concentrated, and applied to a Sephadex A-25 column (2 × 16 cm) which had been preequilibrated in 20 mM Tris (pH 9.0) at 4°. The column was washed with a linear gradient of KCl in 20 mM Tris (pH 9.0) (300 ml total volume; 0–1.0 M; 2.4-ml fractions), at a flow rate of 35 ml/hr. Aliquots were assayed for released P_i (Allen, 1940) after incubation with 3′-AMP. Recovered activity represented 83% of that applied to the column. Fractions 43–46 (shaded area) were combined, adjusted to pH 7.5, and desalted. This solution was concentrated and utilized as a purified 3′-nucleotidase activity.

quenched by the addition of 200 μl of 0.6 M sodium acetate solution (pH 4.5) containing 1% sodium dodecyl sulfate and 0.36 mg of adenosine. The reaction mixtures were then frozen until each was analyzed by chromatography on a column of Sephadex LH-20 (2.8 × 32 cm), elution with water. Each sample was diluted with 0.5 mg of unlabeled 3′-AMP immediately prior to the chromatography to permit location of this material by ultraviolet absorbance. The fractions containing adenosine and 3′-AMP were each pooled and the radioactivity of each was determined by scintillation counting of concentrated aqueous solutions (Anderson and McClure, 1973). After correction for chemical hydrolysis, determined under the same conditions in the absence of enzyme, these values were used to determine per cent hydrolysis. The K_m value was determined from the X intercept of a Lineweaver–Burk plot and found to be 0.12 mM (Table III).

(b) K_i DETERMINATIONS. The apparent K_i's of 2′-*O*-methyladenosine 3′-monophosphate, 2′-deoxyadenosine 3′-monophosphate, and adenosine were determined in the same fashion indicated above for the K_m determination, except that three inhibitor concentrations (0.373–4.05 mM) were utilized at each concentration of substrate and the reactions were run for 4–60 min. The K_i values for each compound were determined from Lineweaver–Burk plots of the data from an average of the three determinations involved and are given in Table III.

Results

Samples of 2′-deoxyadenosine 3′-monophosphate (**2**) and 2′-*O*-methyladenosine 3′-monophosphate (**3**) were prepared by phosphorylation of 2′-deoxyadenosine and 2′-*O*-methyladenosine, respectively. The desired products were separated from nucleoside 5′-monophosphates and unreacted nucleosides by chromatography on DEAE-cellulose and shown to be homogeneous when assayed by paper chromatography in two different solvent systems. Radiolabeled samples were prepared by the general method of Shelton and Clark (1967). Their homogeneity on paper chromatography was also verified (Table I). A sample of [8-^{3}H]adenosine 3′-monophosphate was obtained from [8-^{3}H]adenosine cyclic 3′,5′-monophosphate by the action of crude snake venom, according to the method of Smith *et al.* (1961). The product was purified by chromatography on DEAE-cellulose and shown to afford a single spot when analyzed by paper chromatography.

A commercially available sample of "purified" rye grass 3′-nucleotidase was applied to a column of Sephadex A-25 which had been preequilibrated in 20 mM Tris (pH 9.0). The column was washed with a linear gradient of KCl and subsequent assay of individual fractions for protein content (Lowry *et al.*, 1951) revealed several peaks of material, only one of which was coincident with a broad peak of 3′-phosphohydrolase activity, as judged by the ability of individual fractions to effect the release of P_i from adenosine 3′-monophosphate. Purification of the nucleotidase was effected by chromatography on a larger column of Sephadex A-25 at 4° (Figure 1). Those fractions containing the major peak of 3′-nucleotidase activity (fractions 38–43, representing 25% of the recovered activity) were combined, desalted, and further purified by chromatography on Sephadex A-25 at 4° (pH 9.0), and 83% of the activity applied to the column was recovered (Figure 2). Fractions 43–46 were combined and desalted, and aliquots of this material were found to afford single, symmetrical peaks of nucleotidase activity when subjected to further chromatography on Sephadex A-25 (pH 9.0; 84% recovery of activity) or CM-cellulose (pH 5.5, 93% recovery of activity) (Figure 3).

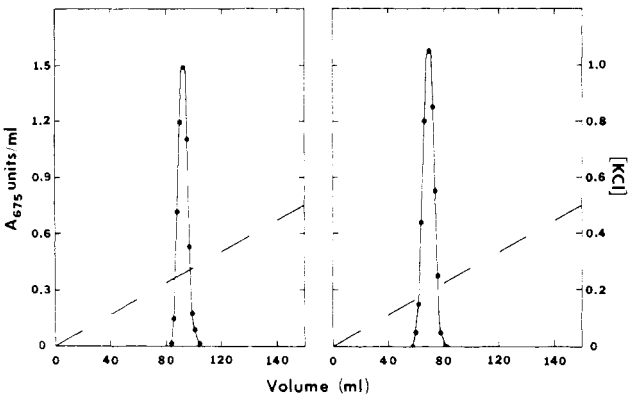


FIGURE 3: (a) (Left panel) Analysis of the purified 3′-nucleotidase activity by further chromatography on Sephadex A-25. A portion of the purified 3′-nucleotidase was applied to a Sephadex A-25 column (2 × 12 cm) which had been preequilibrated in 20 mM Tris (pH 9.0) at 4°. The column was washed with a linear gradient of KCl in 20 mM Tris (pH 9.0) (300 ml total volume; 0–1.0 M; 2.2-ml fractions), at a flow rate of 45 ml/hr. Aliquots of each fraction were assayed for released P_i (Allen, 1940) after incubation with 3′-AMP. Recovered activity represented 84% of that applied to the column. (b) (Right panel) Analysis of the purified 3′-nucleotidase activity by chromatography on carboxymethylcellulose. A portion of the purified 3′-nucleotidase was applied to a column of CM-cellulose (2 × 12.5 cm) which had been equilibrated in 20 mM sodium acetate (pH 5.5) at 4°. The column was washed with a linear gradient of KCl in 20 mM sodium acetate (pH 5.5) (300 ml total volume; 0–1.0 M; 2.3-ml fractions), at a flow rate of 80 ml/hr. Assay of individual fractions for nucleotidase activity (as above) revealed that 93% of the activity applied to the column was recovered.

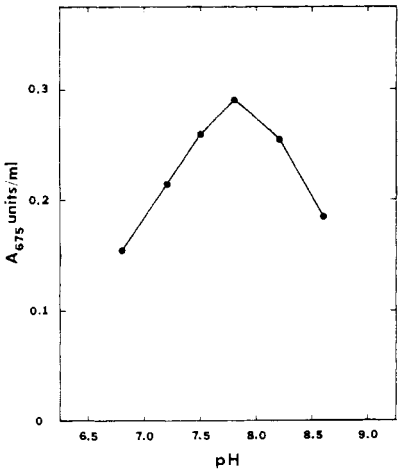


FIGURE 4: pH profile of the purified 3′-nucleotidase activity as determined in 0.1 M Tris-HCl at pH values between 7.5 and 8.6, based on the ability of the enzyme to release P_i from 3′-AMP.

Although the pH optimum of rye grass 3′-nucleotidase has been reported to be at 7.5 (Shuster and Kaplan, 1953; Shuster, 1957), measurement of the purified activity revealed that the activity was greatest at pH 7.8 in 0.1 M Tris-HCl buffer (Figure 4). The stability of the purified 3′-nucleotidase was assayed over a period of 12 hr, utilizing 1.65×10^{-3} unit of enzyme and 285 A_{258} units of adenosine 3′-monophosphate. The results indicated that at least 97% of the theoretical amount of P_i was released during the 12-hr period. In spite of the stability of the enzyme for at least 12 hr, and the fact that P_i release was linear for the first 10% of adenosine 3′-monophosphate hydrolysis, incubation of 52 μmol of 3′-AMP with 0.1 unit of 3′-nucleotidase for 24 hr resulted in only 62% release of adenosine (Figure 5). As documented by kinetic measurements (Table III), this was undoubtedly due to inhibition of P_i release by adenosine.

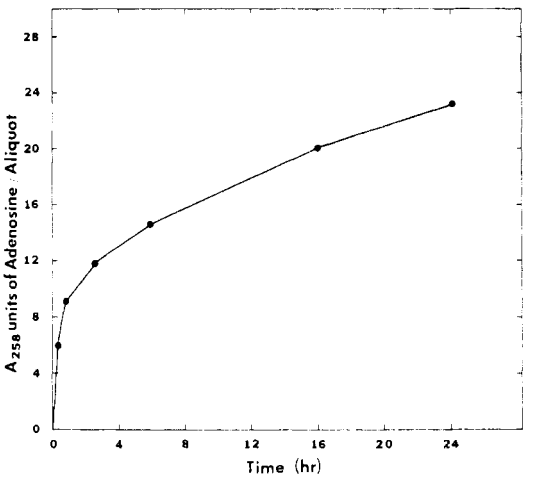


FIGURE 5: Hydrolysis of 3′-AMP by unfractionated 3′-nucleotidase. The nonlinear behavior is due to product inhibition by adenosine (see Table III), rather than loss of enzyme activity. Complete experimental details are given under Materials and Methods.

The relative rates of hydrolysis of adenosine 3′-monophosphate (**1**), 2′-deoxyadenosine 3′-monophosphate (**2**), and 2′-*O*-methyladenosine 3′-monophosphate (**3**) were determined at saturating substrate levels from measurement of the first 2.0–4.7% hydrolysis of these nucleotides, *i.e.*, from the linear portion of the individual hydrolysis curves (see, *e.g.*, Figure 5). As shown in Table II, the relative rates of hydrolysis of **1**/**3**/**2** were 542:15.2:1. The activity of the enzyme toward compounds **2** and **3** was unaffected by the addition of dimethyl sulfoxide up to concentrations of 50%. To verify that the [^{3}H]-3′-AMP utilized in the experiment consisted entirely of this single phosphorylated species, a sample of the nucleotide was subjected to enzymatic hydrolysis under conditions which should have resulted in complete conversion to adenosine. Analysis of the product mixture indicated that hydrolysis had proceeded at least 99% to completion. A similar experiment was carried out on [^{3}H]-2′-deoxyadenosine to show that the observed hydrolysis was not due to contamination of the nucleotide with 3′-AMP. When **2** was treated with sufficient 3′-nucleotidase to effect 53% hydrolysis of the nucleotide, assuming linear release of P_i at the rate given in Table II, 27% hydrolysis was actually observed. The difference was undoubtedly due to product inhibition of the enzyme, as shown for adenosine (Table III; Figure 5).

Initial velocity measurements were carried out on 3′-AMP, with and without **2**, **3**, and adenosine as added inhibitors. Individual experiments were analyzed for hydrolysis of 3′-AMP by chromatographic separation of 3′-AMP and adenosine on Sephadex LH-20, followed by counting of concentrated solutions of each product. The K_m for 3′-AMP was thus measured as 0.12 mM. Compounds **2** and **3**, as well as adenosine, were found to be competitive inhibitors of 3′-AMP utilization by the nucleotidase, with apparent K_i's of 0.51, 0.39, and 1.30 mM, respectively.

Hydrolyses of 3′-ADP and ppGpp were carried out for 1–5 hr with 0.01–0.20 unit of both fractionated and unfractionated 3′-nucleotidase. The extent of 3′-ADP hydrolysis was estimated by chromatography of the product mixtures on DEAE-cellulose columns, elution with a linear gradient of aqueous ammonium bicarbonate, and assay of the columns by ultraviolet absorbance. Hydrolysis of [^{3}H]gua-

Table II: Relative Rates of Hydrolysis of Ribonucleotides.

Substrate	Substrate Concn (mM)	Reaction Volume (ml)	3′-Nucleotidase (units)	Time (min)	Hydrolysis (%)	Rate of Hydrolysis (μmol per enzyme unit per min)[a]
Adenosine 3′-monophosphate (**1**)	10.1	0.024	0.01	0.5	2.03	0.988[b]
2′-Deoxyadenosine 3′-monophosphate (**2**)	10.1	1.03	0.43	480	3.61	0.00182
2′-*O*-Methyladenosine 3′-monophosphate (**3**)	9.80	0.50	0.20	60	4.72	0.0276

[a] The relative rates of hydrolysis of 3′-AMP–**3**–**2** were 542:15.2:1. [b] Theoretical value, 1.000.

Table III: Kinetics of Adenosine 3′-Monophosphate Hydrolysis.[a]

Compound	K_m(mM)	K_i(mM)
Adenosine 3′-monophosphate (**1**)	0.12	
2′-Deoxyadenosine 3′-monophosphate (**2**)		0.51
2′-*O*-Methyladenosine 3′-monophosphate (**3**)		0.39
Adenosine		1.30

[a] Experimental procedure given under Materials and Methods section.

nosine tetraphosphate was monitored by chromatography of the product mixtures on polyethyleneiminecellulose tlc plates and subsequent scintillation counting of the individual compounds recovered from appropriate positions on the plates after development with 1.5 M aqueous KH_2PO_4 (pH 3.4) (Simoncsits and Tomasz, 1974). Hydrolysis to adenosine or guanosine 5′-diphosphate proceeded very slowly in each case, affording less than 1% of the hydrolysis that would have been obtained with 3′-AMP. The incubation mixtures containing the diphosphates were also noted to contain quantities of the respective cyclic 2′,3′-phosphates as products (*i.e.*, adenosine cyclic 2′,3′-monophosphate and guanosine 5′-diphosphate, cyclic 2′,3′-monophosphate), but the possible enzymatic origin of these compounds is uncertain since incubation of **4** and **5** in the same buffer without added enzyme afforded similar quantities of the cyclic nucleotides.

Discussion

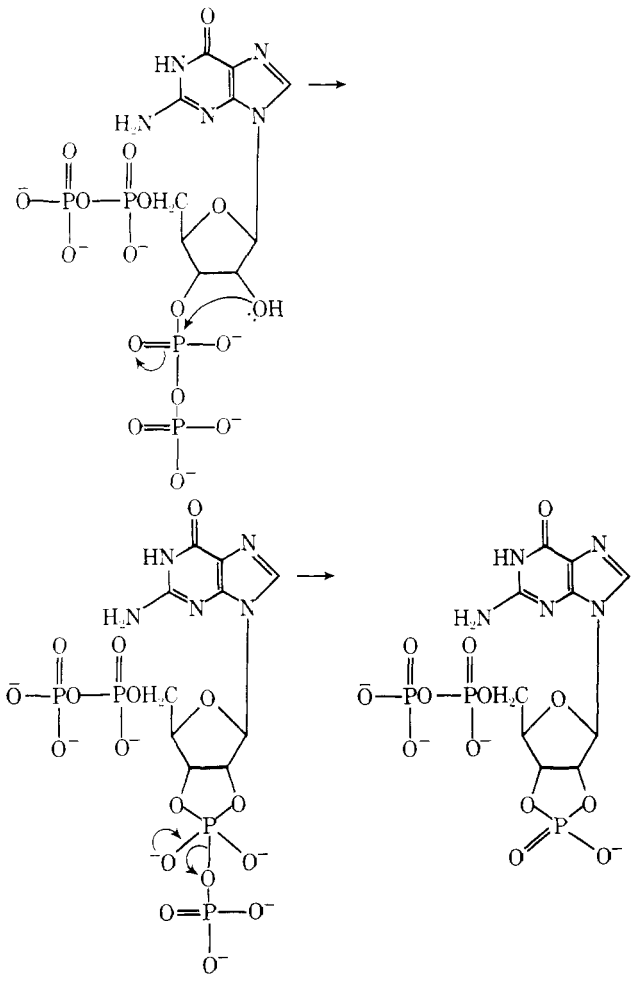
Amino acid deprivation of stringent (RC^+) strains of *E. coli* is known to result in a cessation of nucleotide and RNA synthesis (Gallant and Cashel, 1967; Cashel and Gallant, 1968; Cashel, 1969; Gallant and Harada, 1969; Gallant *et al.*, 1970, 1971) and the simultaneous synthesis of substantial quantities of two ribonucleotides during a ribosomal "idling" step (Cashel and Gallant, 1969; Cashel, 1969; Haseltine *et al.*, 1972). On the basis of systematic chemical and enzymatic degradation (Cashel and Kalbacher, 1970; Sy and Lipmann, 1973) as well as natural abundance ^{13}C nmr spectroscopy (Que *et al.*, 1973), the two ribonucleotides have been reported to be guanosine 5′-diphosphate 3′-diphosphate (ppGpp; **5**), and guanosine 5′-triphosphate 3′-diphosphate (pppGpp), formed from adenosine 5′-triphosphate and guanosine 5′-diphosphate or guanosine 5′-triphosphate, respectively (Sy and Lipmann, 1973; Sy *et al.*, 1973; Haseltine and Block, 1973). Because of the rapid accumulation of ppGpp and pppGpp during amino acid starvation, and the equally rapid disappearance of these ribonucleotides after reestablishment of nutritionally sufficient conditions and normal cellular metabolism, the two ribonucleotides have been of interest as possible regulatory agents produced by the RC^+ allele to mediate the expression of the stringent response to amino acid deprivation.

In contrast to the studies which have elucidated the biosynthetic pathways leading to ppGpp and pppGpp, relatively little is known about the degradation of these nucleotides. Since the rapid decline in ppGpp and pppGpp concentrations under nutritionally sufficient conditions must involve their conversion to other ribonucleotides, the elucidation of specific degradative pathways for ppGpp and pppGpp is of interest in understanding the mechanics of ribonucleotide function. An additional problem which has not been considered is the stability of ppGpp and pppGpp to chemical and enzymatic degradative processes ostensibly unrelated to the normal degradative pathway. Both ribonucleotides, *e.g.*, might be expected to be susceptible to degradation *via* chemically or enzymatically promoted nucleophilic attack of the 2′-hydroxyl group on the 3′-α-phosphate moiety, by analogy with the degradation of RNA (Scheme I). To facilitate an understanding of the inherent stability of ribonucleoside 3′-diphosphates to such chemical and enzymatic processes, we have studied the interaction of two ribonucleoside phosphates with rye grass 3′-nucleotidase, an enzyme reported to be specific for substrates with free 2′-β-OH groups.

Rye grass 3′-nucleotidase (EC 3.1.3.6) was first isolated in partially purified form by Shuster and Kaplan (1953). Subsequent work resulted in the fractionation of the 3′-nucleotidase activity from separate ribonuclease and deoxyribonuclease activities (Shuster, 1957), affording an enzyme specific for ribonucleoside 3′-monophosphates, as compared with ribonucleoside 5′-monophosphates and 2′-deoxyribonucleoside 3′- or 5′-monophosphates (Cunningham, 1958). Baddiley and his coworkers (1958) reported that the enzyme utilized adenosine 5′-monophosphate 3′-monophosphate as a substrate for 3′-phosphorolysis, but not adenosine 5′-monophosphate 2′-monophosphate. Finally, Barker and Lund (1962) demonstrated that 1-β-D-arabinofuranosyluracil 5′-monophosphate 3′-monophosphate was not hydrolyzed by the 3′-nucleotidase.

Isolation of the major 3′-nucleotidase activity from the complex mixture of such activities in the "purified" com-

SCHEME I



mercial preparation was accomplished by two successive chromatographies on Sephadex A-25, using 0.02 M Tris-HCl buffer (pH 9.0) at 4°. The relative rates of hydrolysis of adenosine 3′-monophosphate (**1**), 2′-deoxyadenosine 3′-monophosphate (**2**), and 2′-*O*-methyladenosine 3′-monophosphate (**3**) by this enzyme were determined by measurement of the initial velocity of hydrolysis of each substrate. As indicated in Table II, the relative rates for 3′-AMP, **3**, and **2** were 542:15.2:1. Although neither **2** nor **3** was hydrolyzed at a significant rate relative to 3′-AMP, verifying the reported requirement for a free 2′-β-OH group, both species acted as competitive inhibitors of 3′-AMP utilization, with apparent K_i's of 0.51 and 0.39 mM, respectively. Adenosine was also a competitive inhibitor and had an apparent K_i of 1.30 mM.

Since 3′-AMP is the preferred substrate for rye grass 3′-nucleotidase, as compared with IMP, GMP, UMP, or CMP (Shuster and Kaplan, 1953), 3′-ADP was the diphosphate chosen for initial assay. Treatment of adenosine 3′-diphosphate with the 3′-nucleotidase under conditions routinely employed for the hydrolysis of 3′-AMP (15 A_{258} units of 3′-ADP; 0.01 unit of enzyme) did not result in the release of a detectable level of adenosine. Treatment at higher enzyme concentration (0.2 unit) indicated the rate of P_i release from the diphosphate to be less than 1% of that obtained with 3′-AMP. The commercial mixture of 3′-nucleotidase activities gave a similar result. Participation of the 2′-OH group as a nucleophile in the degradation might be expected to afford adenosine cyclic 2′,3′-monophosphate as a reaction product. Although a significant amount of this species was present in the reaction mixture, it also formed in similar quantities in the absence of 3′-nucleotidase, and its possible enzymatic origin is therefore uncertain. The net 3′-pyrophosphorolysis of ppGpp was also studied. The tetraphosphate was treated with fractionated and unfractionated samples of 3′-nucleotidase at concentrations ranging from 0.2 to 4.0 unit/ml. It was shown to be degraded at a rate less than 1% of that obtained with 3′-AMP.[2] The stability of these nucleotides to enzymatic degradation by 3′-nucleotidase parallels the surprising stability of adenosine 3′-diphosphate to degradation *via* internal nucleophilic attack by the 2′-hydroxyl group (Mitchel *et al.*, 1967; Kozarich *et al.*, 1975), in contrast with the relative chemical instability of adenosine 3′-triphosphate (Kozarich *et al.*, 1975).

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[2] The same qualitative result has been reported by Sy and Lipmann (1973).

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Ribonucleoside 3′-Di- and -Triphosphates. Synthesis of Guanosine Tetraphosphate (ppGpp)†

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ABSTRACT: A procedure has been outlined for the synthesis of ribonucleoside 3′-di- and -triphosphates. The synthetic scheme involves the conversion of a ribonucleoside 3′-monophosphate to its 2′-(5′-di)-*O*-(1-methoxyethyl) derivative, followed by successive treatments of the blocked ribonucleotide with 1,1′-carbonyldiimidazole and mono(tri-*n*-butylammonium) phosphate or pyrophosphate. The resulting ribonucleoside 3′-di- and -triphosphate derivatives are then deblocked by treatment with dilute aqueous acetic acid, pH 3.0. The use of this procedure is illustrated for adenosine 3′-monophosphate, which has been converted to its corresponding 3′-di- and -triphosphates in 61% overall yield. The decomposition of adenosine 3′-di- and -triphosphates to adenosine 2′-monophosphate, adenosine 3′-monophosphate, and adenosine cyclic 2′,3′-monophosphate as a function of pH at 100° has been studied as has the attempted polymerization of adenosine 3′-diphosphate with polynucleotide phosphorylase. Also prepared was guanosine 5′-diphosphate 3′-diphosphate (guanosine tetraphosphate; ppGpp), which was accessible *via* treatment of 2′-*O*-(1-methoxyethyl)guanosine 5′-monophosphate 3′-monophosphate with the phosphorimidazolidate of mono(tri-*n*-butyl ammonium) phosphate. The resulting blocked tetraphosphate was deblocked in dilute aqueous acetic acid to afford ppGpp in an overall yield of 18%.

Nucleoside 3′-di- and -triphosphates have received relatively little attention in spite of two lines of evidence which suggest their potential metabolic importance. Coutsogeorgopoulos *et al.* (1965, 1966), *e.g.*, showed that cell-free extracts from rat liver could mediate the conversion of thymidine 3′-monophosphate to the corresponding 3′-di- and -triphosphates and postulated the possible role of these deoxyribonucleotides as intermediates or end products on some metabolic pathway. A similar result was obtained by Canellakis *et al.* (1965), using cell extracts from *Bacillus subtilis*. The natural occurrence of ribonucleoside 3′-diphosphates was established by the isolation of guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and guanosine 5′-triphosphate 3′-diphosphate (pppGpp) from stringent (RC^+) strains of *Escherichia coli* (Cashel and Gallant, 1969; Cashel, 1969) and by the subsequent determination of the structure (Cashel and Kalbacher, 1970; Sy and Lipmann, 1973; Que *et al.*, 1973) and mode of biosynthesis (Sy and Lipmann, 1973; Sy *et al.*, 1973; Haseltine and Block, 1973) of these ribonucleotides.

Reports have appeared concerning the chemical synthesis of certain deoxyribonucleoside 3′-di- and -triphosphates (Josse and Moffatt, 1965) as well as ribonucleoside 2′- and 3′-diphosphates (Mitchel *et al.*, 1967). The latter report details the construction of the labile adenosine 3′-diphosphate in low yield by direct condensation of adenosine 3′-monophosphate with phosphoramidic acid, a procedure which is probably not applicable to the synthesis of ribonucleoside 3′-triphosphates. This report describes an additional approach for the elaboration of ribonucleoside 3′-di- and -triphosphates, utilizing phosphorimidazolidate intermediates. The syntheses of adenosine 3′-diphosphate and adenosine 3′-triphosphate in 61% overall yield are reported, as are data concerning the chemical stability of each and attempted utilization of the diphosphate as a substrate for polynucleotide phosphorylase. Also described is the chemical synthesis and characterization of guanosine 5′-diphosphate 3′-diphosphate (ppGpp).[1]

Materials and Methods

Adenosine 3′-monophosphate, 1,1′-carbonyldiimidazole, inorganic pyrophosphatase (baker's yeast), and rye grass 3′-nucleotidase were purchased from Sigma Chemical Company. Methyl vinyl ether was obtained from Matheson Gas Products. Guanosine 5′-monophosphate 3′-monophosphate was purchased from Boehringer Mannheim Corpora-

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[1] After the synthetic work in this paper was completed, Simoncsits and Tomasz (1974) described the chemical synthesis of ppGpp in similar yield by another procedure.