Shelton, K. R., and Clark, J. M., Jr. (1967), *Biochemistry 6,* 2735.
Shuster, L. (1957), *J. Biol. Chem. 229,* 289.
Shuster, L., and Kaplan, N. O. (1953), *J. Biol. Chem. 201,* 535.
Simoncsits, A., and Tomasz, J. (1974), *Biochim. Biophys. Acta 340,* 509.
Smith, M., Drummond, G. I., and Khorana, H. G. (1961), *J. Amer. Chem. Soc. 83,* 698.
Swanton, M., and Edlin, G. (1972), *Biochem. Biophys. Res. Commun. 46,* 583.
Sy, J., and Lipmann, F. (1973), *Proc. Nat. Acad. Sci. U.S. 70,* 306.
Sy, J., Ogawa, Y., and Lipmann, F. (1973), *Proc. Nat. Acad. Sci. U.S. 70,* 2145.
Tener, G. M. (1961), *J. Amer. Chem. Soc. 83,* 159.

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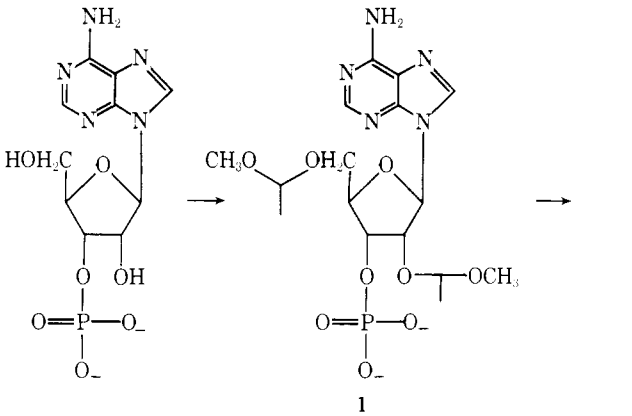
[1] After the synthetic work in this paper was completed, Simoncsits and Tomasz (1974) described the chemical synthesis of ppGpp in similar yield by another procedure.

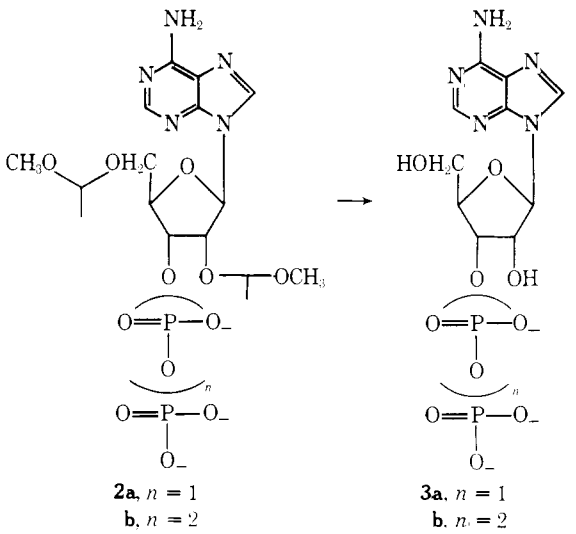
tion and polynucleotide phosphorylase (*Micrococcus luteus*) from P-L Biochemicals. Polyethyleneimine cellulose tlc plates were obtained from Brinkmann Instruments and Whatman DEAE-cellulose and GF/A glass fiber discs from H. Reeve Angel and Co. [$^3$H]Adenosine 5′-diphosphate, 0.19 Ci/mol, was prepared from [$^3$H]AMP (Shelton and Clark, 1964) *via* the phosphorimidazolidate intermediate (Kozarich *et al.*, 1973).

*Synthesis of Ribonucleoside Phosphates*

*2′,5′-Di-O-(1-methoxyethyl)adenosine 3′-Monophosphate* (**1**). This procedure is based on the general method of Mackey and Gilham (1971). To 98 mg (0.25 mmol) of adenosine 3′-monophosphate ($Na^+$ salt) and 237 mg (1.25 mmol) of *p*-toluenesulfonic acid was added 3 ml of dimethyl sulfoxide. The solution was frozen in an ice bath and treated with 4 ml of methyl vinyl ether at 0°. The resulting solution was stirred at 0° for 10 min, treated with 0.5 ml of concentrated ammonium hydroxide, and allowed to warm to room temperature. The reaction mixture was treated with 50 ml of water and concentrated *in vacuo* to remove excess ammonia. Purification was effected on a column of DEAE-cellulose (2.4 × 30 cm), elution with a linear gradient of triethylammonium bicarbonate (2 l. total volume; 0–0.4 M; 15-ml fractions) at a flow rate of 225 ml/hr (Figure 1). The appropriate fractions (shaded area, Figure 1) were combined and desalted by repeated evaporations of portions of water under diminished pressure to afford **1** as a colorless glass, yield 135 mg (81%). This material gave a single peak when further analyzed by chromatography on DEAE-cellulose and was cleanly converted back to 3′-AMP by treatment with dilute acetic acid (pH 3.0) at 37° for 90 min (Mackey and Gilham, 1971) (Figure 2).



**1**



**2a**, $n = 1$
**b**, $n = 2$

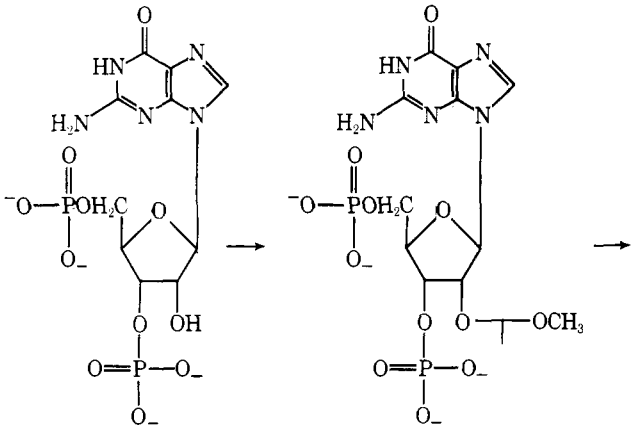
**3a**, $n = 1$
**b**, $n = 2$

*2′,5′-Di-O-(1-methoxyethyl)adenosine 3′-Diphosphate* (**2a**). To 67 mg (100 μmol) of 2′,5′-di-*O*-(1-methoxyethyl)-adenosine 3′-monophosphate was added 3.0 ml of pyridine and 0.5 ml of water. The solution was treated with 24 μl (100 μmol) of tri-*n*-butylamine, concentrated to dryness, and rendered anhydrous by repeated evaporations of portions of pyridine and then dimethylformamide. The anhydrous ribonucleotide was dissolved in 1.0 ml of dimethylformamide and then treated with 81 mg (500 μmol) of 1,1′-carbonyldiimidazole in 100 μl of dimethylformamide (Hoard and Ott, 1965; Kozarich *et al.*, 1973). The combined solution was maintained under anhydrous conditions for 12 hr and then treated with 33 μl (800 μmol) of methanol. After 30 min, the solution was treated with 500 μmol of mono(tri-*n*-butylammonium) phosphate in 1.0 ml of dimethylformamide and then maintained under anhydrous conditions for 24 hr. The precipitate was filtered and the solution was treated with methanol and concentrated to dryness under diminished pressure. The residue was purified by chromatography on a column of DEAE-cellulose (2.0 × 22 cm), elution with a linear gradient of triethylammonium bicarbonate (2 l. total volume; 0–0.4 M; 15-ml fractions) at a flow rate of 150 ml/hr. The appropriate fractions were pooled and desalted by repeated evaporations of portions of water under diminished pressure to afford **2a** as a colorless glass, yield 66 mg (75%).

*2′,5′-Di-O-(1-methoxyethyl)adenosine 3′-Triphosphate* (**2b**). Compound **2b** was obtained from 2′,5′-di-*O*-(1-methoxyethyl)adenosine 3′-monophosphate by treatment of the intermediate phosphorimidazolidate with mono(tri-*n*-butylammonium) pyrophosphate (Hoard and Ott, 1965; Kozarich *et al.* 1973). Ribonucleoside triphosphate **2b** was isolated as the triethylammonium salt in 75% yield after purification by chromatography on DEAE-cellulose.

*Adenosine 3′-Diphosphate* (**3a**). A solution of 8.5 mg (10 μmol) of 2′,5′-di-*O*-(1-methoxyethyl)adenosine 3′-diphosphate in 10 ml of water was adjusted to pH 3.0 with acetic acid. The solution was maintained at 37° for 90 min and then concentrated to dryness under diminished pressure. Traces of acetic acid were removed by repeated evaporations of portions of water and the product was analyzed by chromatography on DEAE-cellulose (0.9 × 25 cm), elution with a linear gradient of ammonium bicarbonate (200 ml total volume; 0–0.8 M; 2-ml fractions). The effluent was assayed for absorbance at 258 nm and the elution profile is given in Figure 3. The results of a phosphate analysis of **3a** are given in Table I. [8-$^3$H]Adenosine 3′-diphosphate, 0.21 Ci/mol, was prepared in the same fashion starting from [$^3$H]-2′,5′-di-*O*-(1-methoxyethyl)adenosine 3′-monophosphate, which was accessible from the unlabeled compound by the general method of Shelton and Clark (1964).

*Adenosine 3′-Triphosphate* (**3b**). Compound **3b** was obtained from 2′,5′-di-*O*-(1-methoxyethyl)adenosine 3′-triphosphate (**2b**) by treatment with dilute acetic acid solution, as outlined above for the conversion **2a**→**3a.** The product was analyzed by chromatography on a DEAE-cellulose column and shown to give a single peak of material with absorbance at 258 nm. The elution profiles of 3′-AMP, 3′-ADP, and 3′-ATP on DEAE-cellulose are compared in Figure 3. 3′-ATP was also analyzed for phosphate content and shown to have 3 mol of phosphate/mol of adenosine (Table I).



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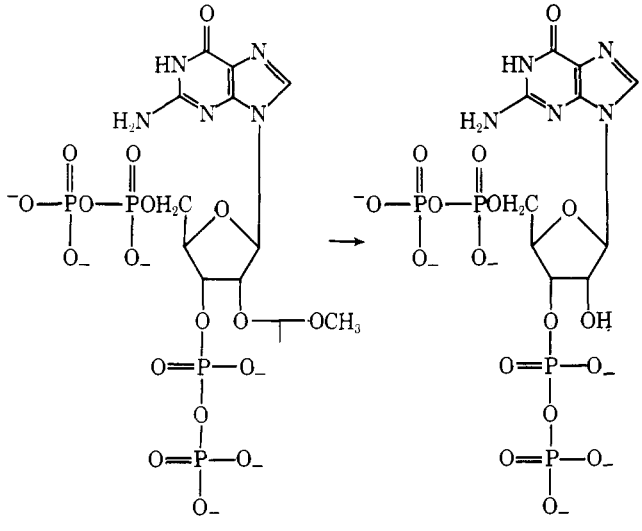


5 6

*2′-O-(1-Methoxyethyl)guanosine 5′-Monophosphate 3′-Monophosphate* (**4**). To 9 mg (20 μmol) of guanosine 5′-monophosphate 3′-monophosphate ($Li^+$ salt) was added 19 mg (100 μmol) of *p*-toluenesulfonic acid and 300 μl of dimethyl sulfoxide.The solution was frozen in an ice bath and treated with 400 μl of methyl vinyl ether at 0°. The resulting solution was stirred at 0° for 10 min and then treated with an additional 400 μl of methyl vinyl ether. After a total reaction time of 20 min, the solution was treated with 160 μl of concentrated ammonium hydroxide and allowed to warm to room temperature. The solution was treated with water and concentrated *in vacuo* to remove excess ammonia, then purified by chromatography on a DEAE-cellulose column (2.4 × 30 cm), elution with a linear gradient of triethylammonium bicarbonate (2 l. total volume; 0–0.4 M; 15-ml fractions) at a flow rate of 225 ml/hr. The appropriate fractions were combined and desalted by repeated evaporations of portions of water under diminished pressure to afford **4** as a colorless glass, yield 180 $A_{253}$ units (75%; 90% based on consumed starting material).

*2′-O-(1-Methoxyethyl)guanosine 5′-Diphosphate 3′-Diphosphate* (**5**). Phosphoric acid (9 μl; 150 μmol) was converted to anhydrous mono(tri-*n*-butylammonium) phosphate in 1.0 ml of dimethylformamide and treated with 121.5 mg (750 μmol) of 1,1′-carbonyldiimidazole in 1.0 ml of dimethylformamide. The combined solution was maintained under anhydrous conditions for 12 hr and then treated with 25 μl (610 μmol) of methanol for 30 min. The resulting solution was added to 1 ml of a dimethylformamide solution containing 350 $A_{253}$ units of 2′-O-(1-methoxyethyl)guanosine 5′-monophosphate 3′-monophosphate (**4**) as the anhydrous tri-*n*-butylammonium salt. The reaction mixture was maintained at room temperature for 48 hr, then treated with an equal volume of methanol and concentrated to dryness under diminished pressure. The crude product was purified by chromatography on a DEAE-cellulose column (2 × 22 cm), elution with a linear gradient of ammonium bicarbonate (2 l. total volume; 0–0.6 M; 15-ml fractions) at a flow rate of 150 ml/hr. The appropriate fractions (83–92) were combined and desalted by repeated evaporations of portions of water under diminished pressure to afford **5** as a colorless glass, yield 4.0 mg (70 $A_{253}$ units) (20%; 69%, based on consumed starting material).

*Guanosine 5′-Diphosphate 3′-Diphosphate* (**6**; *Guanosine Tetraphosphate; ppGpp*). A solution of 6 $A_{253}$ units (0.5 μmol) of 2′-O-(1-methoxyethyl)guanosine 5′-diphosphate 3′-diphosphate (**5**) in 100 μl of water was adjusted to pH 3.0 with acetic acid. The solution was maintained at 37° for 90 min and then concentrated to dryness under diminished pressure. Traces of acetic acid were removed by repeated evaporations of portions of water and the product was analyzed by thin-layer chromatography on polyethyleneimine cellulose, elution with 1.5 M $KH_2PO_4$, pH 3.4 (Table IV) and by phosphate analysis (Table I).

### *Preparation of a Polyadenylate Primer Terminating in Adenosine 3′-Diphosphate*

Polyadenylic acid, obtained by the polynucleotide phosphorylase-catalyzed polymerization of 5′-ADP, was dissolved in 9M aqueous ammonium hydroxide and maintained at 37° for 12 hr. The solution was concentrated under diminished pressure and analyzed by chromatography on DEAE-cellulose paper, elution with 1.0 M ammonium bicarbonate, which revealed breakdown to afford oligomers with $R_F$ values of 0–0.7 ($R_F$(5′-ADP) 0.7). A portion (250 $A_{258}$ units) of the anhydrous, partially degraded polymer was treated with *p*-toluenesulfonic acid (30 mg; 0.17 mmol) and methyl vinyl ether (0.5 ml) in 150 μl of dimethyl sulfoxide to effect conversion to the 2′(3′)-(1-methoxyethyl) derivative, by analogy with the synthesis of **1**. Purification was effected by chromatography on DEAE-cellulose paper, elution with 1.0 M ammonium bicarbonate. The blocked oligomer was converted to the anhydrous mono(tri-*n*-butylammonium) salt and treated successively with 1,1′-carbonyldiimidazole (40 mg; 0.25 mmol) and mono(tri-*n*-butyl ammonium) phosphate (1.25 mmol) in dry dimethylformamide, by analogy with the conversion of **1** to **2a**. Purification was carried out on DEAE-cellulose paper, elution with 1.0 M ammonium bicarbonate, to afford 250 $A_{258}$ units of oligoribonucleotides which were believed to terminate in adenosine 2′- and 3′-diphosphates on the basis of analogous transformations at the mononucleotide level. The primer was deblocked immediately prior to use by incubation in 0.2 ml of dilute aqueous acetic acid, pH 3.0, at 37° for 90 min. The acetic acid was then removed at 30° under diminished pressure.

### *Enzyme Assays*

*Polynucleotide Phosphorylase Polymerizations.* METHOD A. To 500 μl (total volume) of 0.2 M Tris-HCl (pH 8.1) containing 0.01 M magnesium acetate, 0.1 mM copper sulfate, and 2 mM [$^3$H]adenosine 3′- or 5′-diphosphate was

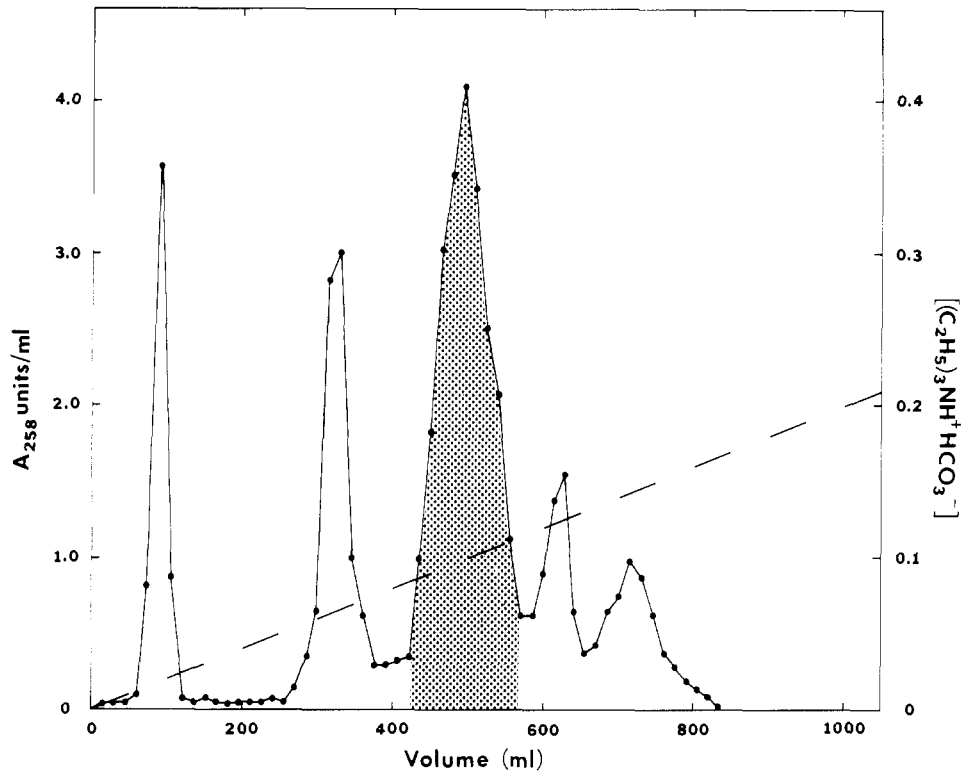



FIGURE 1: Chromatography on a DEAE-cellulose column of the crude reaction mixture containing 2′,5′-di-*O*-(1-methoxyethyl)adenosine 3′-monophosphate (**1**). The first two peaks of uv absorbing material to elute from the column were dimethyl sulfoxide and *p*-toluenesulfonic acid, respectively. The third peak (shaded area) was the desired product and the fractions containing this material were combined, desalted, and concentrated to dryness. Experimental details are given in the text.

added 1 unit[2] of polynucleotide phosphorylase (*Micrococcus luteus*). The solution was incubated at 37°, with or without added primer. Aliquots were removed at seven predetermined time intervals over a period of 60 min and each was quenched by addition to a Whatman GF/A glass fiber disc which had been presoaked with 0.1 ml of 0.05 M cetyltrimethylammonium bromide in 1% aqueous acetic acid. The discs were washed thoroughly with 1% aqueous acetic acid and counted in a toluene-based scintillation fluid. The results of the assay after 60 min are given in Table III.

METHOD B. To 300 μl (total volume) of freshly prepared 0.2 M Tris-HCl (pH 8.1 or 7.0) containing 0.01 M magnesium chloride or manganese chloride and 2 mM [$^3$H]adenosine 3′- or 5′-diphosphate was added 0.75 unit of polynucleotide phosphorylase. The solution was incubated at 37°, with or without added primer, and five aliquots were removed over a period of 60 min and assayed for polynucleotide formation as indicated above. The results are shown in Figure 5 and Table III.

*Degradation of ppGpp.* This procedure is based on the method of Sy and Lipmann (1973). To 10 $A_{253}$ units of synthetic or authentic ppGpp (0.83 μmol) was added 50 μl of 10 mM Tris-HCl buffer (pH 7.0) containing 0.4 mM zinc acetate. The solution was treated with 10 units[3] of inorganic pyrophosphatase and incubated at 37° for 20 min. A 10-μl aliquot of the solution was analyzed by thin-layer chromatography on polyethyleneimine cellulose (1.5 M $KH_2PO_4$, pH 3.4) and most of the guanosine 5′-diphosphate 3′-diphosphate (ppGpp; $R_F$ 0.19) was found to have been converted to material which was identical chromatographically with authentic guanosine 5′-monophosphate 3′-monophosphate (pGp; $R_F$ 0.06)(Table IV). The remaining 40 μl of the reaction mixture was treated with 0.05 unit[4] of rye grass 3′-nucleotidase and incubated at 37° for an additional 30 min. Thin-layer chromatograhy revealed considerable breakdown of pGp to a new product which was identical chromatographically with guanosine 5′-monophosphate ($R_F$ 0.80).

## Results

Adenosine 3′-diphosphate was obtained in 61% overall yield from adenosine 3′-monophosphate *via* the phosphorimidazolidate intermediate (Hoard and Ott, 1965; Kozarich *et al.*, 1973). Prior to activation of the phosphate, the 2′- and 5′-hydroxyl groups were blocked with methyl vinyl ether to prevent the formation of adenosine cyclic 2′,3′-monophosphate. As shown in Figure 1, the diblocked product (**1**) was purified on DEAE-cellulose and shown to reafford 3′-AMP upon treatment with dilute acetic acid (Figure 2). Compound **1** was then activated with 1,1′-carbonyldiimidazole and treated with 5 equiv of mono(tri-*n*-butylammonium) phosphate to afford 75% conversion to diblocked adenosine 3′-diphosphate (**2a**). This intermediate was converted quantitatively to adenosine 3′-diphosphate (**3a**) by incubation in aqueous acetic acid (pH 3.0) at 37° for 90 min. Adenosine 3′-triphosphate (**3b**) was obtained in the same yield by analogous treatment of the phosphorimidazolidate of **1** with mono(tri-*n*-butylammonium) pyrophos-

[2] One unit of polynucleotide phosphorylase is defined as that amount which catalyzes polymerization of 1.0 μmol of 5′-ADP in 15 min at 37° as measured by released $P_i$.

[3] One unit is defined as the amount of inorganic pyrophosphatase which liberates 1.0 μmol of $P_i$/min at pH 7.2 and 25°.

[4] One unit hydrolyzes 1.0 μmol of $P_i$ from 3′-AMP/min at pH 7.5 and 37°.

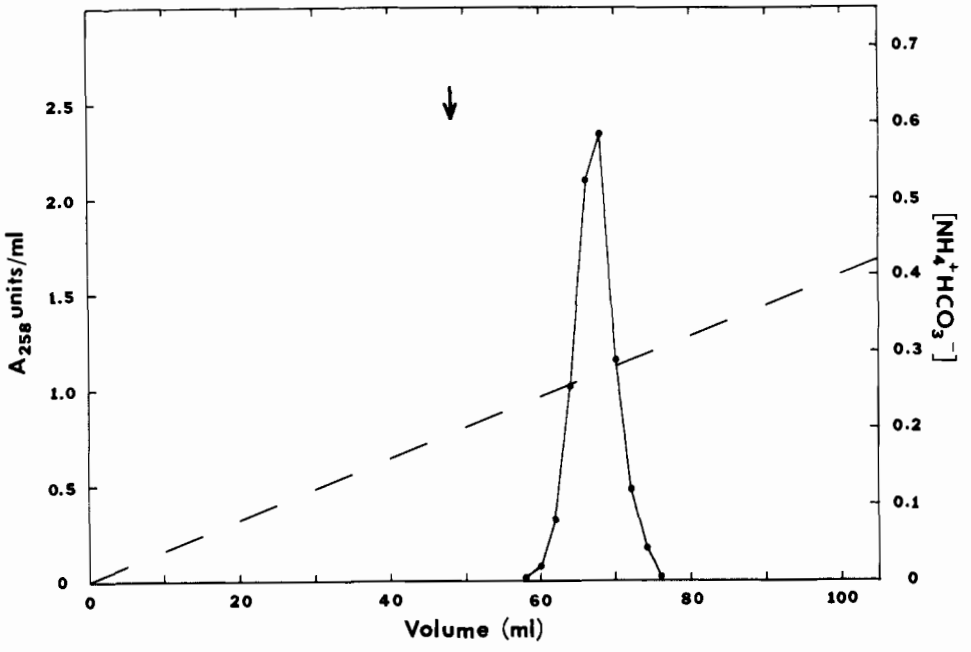



FIGURE 2: Chromatography on DEAE-cellulose of the product (3′-AMP) resulting from treatment of 2′,5′-di-*O*-(1-methoxyethyl)adenosine 3′-monophosphate (**1**) with dilute aqueous acetic acid (pH 3.0) at 37° for 90 min. The arrow indicates the position at which compound **1** elutes under identical conditions. A detailed experimental is given in Materials and Methods.

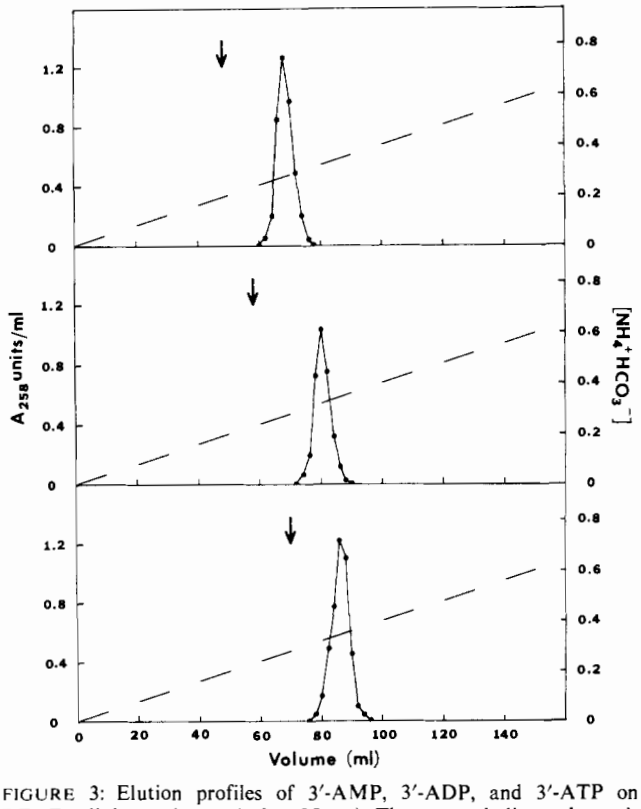



FIGURE 3: Elution profiles of 3′-AMP, 3′-ADP, and 3′-ATP on DEAE-cellulose columns (0.9 × 25 cm). The arrows indicate the positions at which the respective 2′,5′-di-*O*-(1-methoxyethyl) derivatives elute under identical conditions.

phate. The chromatographic properties of 3′-AMP, 3′-ADP (**3a**), and 3′-ATP (**3b**) on DEAE-cellulose are compared in Figure 3. Also shown are the relative mobilities of the 2′,5′-di-*O*-(1-methoxyethyl)adenosine 3′-phosphates. Phosphate analyses of 3′-AMP, 3′-ADP, and 3′-ATP (Table I) indicated the presence of 1, 2, and 3 mol of phosphate per mol of adenosine, respectively.

In agreement with the results of Mitchel *et al.* (1967), we found adenosine 3′-diphosphate to be reasonably stable in neutral, aqueous solution, although heating to 100° caused substantial breakdown to 3′-AMP and adenosine cyclic 2′,3′-monophosphate. The compound was less stable at high or low pH, as shown in Table II. Adenosine 3′-triphosphate was less stable than the diphosphate, affording large amounts of adenosine cyclic 2′,3′-monophosphate when maintained in neutral, aqueous solution or 0.1 M Tris-HCl (pH 7.5). As the data in Table II indicate, the triphosphate was also unstable at elevated temperature at all tested pH values, affording various mixtures of 3′-ADP, 3′-AMP, 2′-AMP, and adenosine cyclic 2′,3′-monophosphate, according to the specific reaction conditions.

Table I: Phosphate Analyses of Ribonucleoside 3′-Di- and -Triphosphates.

| Compd | Amount Analyzed | μmol of P/μmol of Ribonucleoside Phosphate |
|---|---|---|
| | $A_{258}$ Units | |
| 3′-AMP[a] | 5.0 | 1.0 |
| 3′-ADP[a] | 5.0 | 1.9 |
| 3′-ATP[a] | 5.0 | 3.0 |
| | $A_{253}$ Units | |
| 5′-GMP[b] | 5.0 | 1.0 |
| pGp[b] | 5.0 | 1.9 |
| GTP[b] | 5.0 | 2.9 |
| ppGpp[b] | 5.0 | 3.7 |

[a] Determined by the method of Fischer (1961). [b] Determined by the method of Allen (1940).

Adenosine 3′-diphosphate was found to be a good inhibitor of 5′-ADP polymerization, affording 50% inhibition of polymerization after 2 hr of incubation when used in equimolar concentration (5 mM) with the substrate. Under the same conditions 3′-AMP was not inhibitory at all and 5′-AMP afforded only slight inhibition (Figure 4). Polymer-

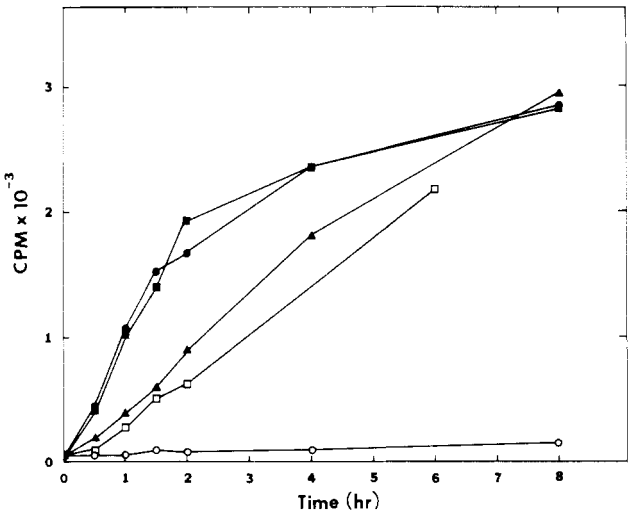



FIGURE 4: Inhibition of polynucleotide phosphorylase-catalyzed polymerization of [$^3$H]-5′-ADP. The reactions were run in 500 $\mu$l (total volume) of 0.2 M Tris-HCl (pH 8.1) containing 10 mM magnesium acetate, 0.1 mM copper sulfate, 5 mM [$^3$H]-5′-ADP and no inhibitor (●), 5 mM 3′-AMP (■), 5 mM ppGpp (□), 5 mM 3′-ADP (▲), or 50 mM 3′-ADP (○). The reactions were initiated by the addition of 0.3 unit of polynucleotide phosphorylase. The combined solution was incubated at 37° and 50-$\mu$l aliquots were removed at predetermined time intervals and applied to glass fiber discs which had been presoaked with 100 $\mu$l of 5% aqueous trichloroacetic acid solution containing 1% sodium pyrophosphate. The dried discs were washed twice with 5% trichloroacetic acid and then with 95% ethanol. Each dried disc was counted in a toluene-based scintillation fluid. Adenosine 5′-monophosphate was also tested as a potential inhibitor and found to be only slightly inhibitory when used at 5 mM concentration.

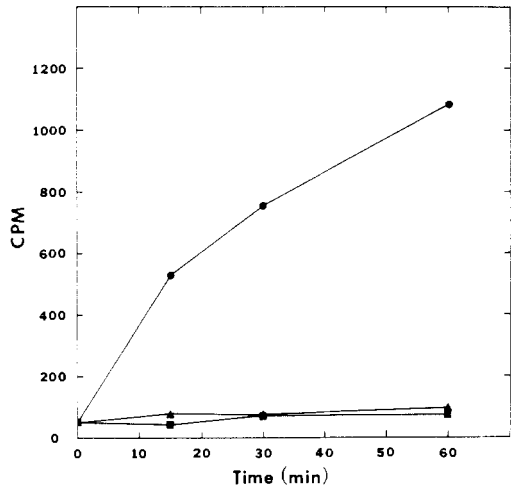



FIGURE 5: Attempted polymerization of 3′-ADP with polynucleotide phosphorylase in the presence of 10 mM $MnCl_2$ at pH 8.1 (■) and 7.0 (▲). A control was run at the same time with 5′-ADP (●) (10 mM $MgCl_2$ (pH 8.1)). Experimental details are given in the text.

ization of adenosine 3′-diphosphate was attempted under a variety of conditions, including the presence of an adenylate primer terminating with adenosine 3′-diphosphate and the substitution of $Mn^{2+}$ for $Mg^{2+}$. As shown in Figure 5 and Table III none of these conditions afforded detectable polymerization.

The synthesis of guanosine tetraphosphate was accomplished by analogy with the synthesis of adenosine 3′-di- and -triphosphates, by initial blocking of the 2′-OH group in guanosine 5′-monophosphate 3′-monophosphate with methyl vinyl ether. Since activation of either phosphate might be expected to afford 2′-*O*-(1-methoxyethyl)guanosine cyclic 3′,5′-diphosphate *via* intramolecular nucleophilic displacement of the activated group by the other phosphate, compound **4** was converted to **5** by treatment with the imidazolidate of phosphoric acid. The product, which formed in 20% yield (69%, based on consumed starting material), was purified by chromatography on DEAE-cellulose. This material was deblocked in aqueous acetic acid to afford ppGpp in an overall yield of 18%. The lower yield of this compound, as compared with those of 3′-ADP and 3′-ATP, may be attributed in part to the more limited solubility of the guanosine phosphates in the reaction medium used for the phosphorylation.

The tetraphosphate was shown to be identical with an authentic sample of ppGpp, as judged by thin-layer chromatography on polyethyleneimine cellulose (Table IV). The synthetic and authentic samples were both degraded by inorganic pyrophosphatase to a compound identical with pGp

Table II: Decomposition Profiles of Adenosine 3′-Di- and -Triphosphates.[a]

| Compd | pH | % of Total Reaction Products[b] 3′-ATP | 3′-ADP | 3′-AMP | 2′-AMP | 2′,3′-cAMP |
|---|---|---|---|---|---|---|
| 3′-ADP | 3.5 | | | 12 | | 88 |
| 3′-ADP | 7.0 | | 20 | 42 | | 30 |
| 3′-ADP | 7 M $NH_4OH$ | | 8 | 58 | 35 | |
| 3′-ATP | 3.5 | | | 10 | | 85 |
| 3′-ATP | 7.0 | 10 | 5 | 45 | | 40 |
| 3′-ATP | 7 M $NH_4OH$ | 5 | | 48 | 40 | |

[a] Each reaction mixture contained 20 $A_{258}$ units of ribonucleotide in 1 ml of the indicated aqueous solution. The reaction mixtures were maintained at 100° for 1 hr, then adjusted to pH 7.0 where necessary and analyzed by chromatography on a column of DEAE-cellulose (0.9 × 25 cm), elution with a linear gradient of ammonium bicarbonate (200 ml total volume; 0–0.8 M; 2-ml fractions). [b] The total recovery of products was quantitative in each case.

Table III: Attempted Polymerization of [$^3$H]Adenosine 3′-Diphosphate with Polynucleotide Phosphorylase.[a]

| Ribonucleotide | Primer ($A_{258}$ Units) | Conditions | Maximum % Incorp. into Polynucleotide |
|---|---|---|---|
| Method A | | | |
| 5′-ADP | | 10 mM $Mg^{2+}$, pH 8.1 | 24 |
| 3′-ADP | | 10 mM $Mg^{2+}$, pH 8.1 | <0.10 |
| 3′-ADP | 8 | 10 mM $Mg^{2+}$, pH 8.1 | <0.10 |
| 3′-ADP | 16 | 10 mM $Mg^{2+}$, pH 8.1 | <0.15 |
| 3′-ADP | 32 | 10 mM $Mg^{2+}$, pH 8.1 | <0.10 |
| Method B | | | |
| 5′-ADP | | 10 mM $Mg^{2+}$, pH 8.1 | 31 |
| 5′-ADP | | 10 mM $Mn^{2+}$, pH 8.1 | 7.5 |
| 5′-ADP | | 10 mM $Mn^{2+}$, pH 7.0 | 5.7 |
| 3′-ADP | | 10 mM $Mn^{2+}$, pH 8.1 | <0.15 |
| 3′-ADP | | 10 mM $Mn^{2+}$, pH 7.0 | <0.25 |
| 3′-ADP | 16 | 10 mM $Mn^{2+}$, pH 8.1 | <0.20 |
| 3′-ADP | 16 | 10 mM $Mn^{2+}$, pH 7.0 | <0.40 |

[a] Experimental details are given in the text.

Table IV: Thin-Layer Chromatographic Values of ppGpp and Its Enzymatic Products.[a]

| Sample | $R_f$ Value(s) | |
|---|---|---|
| | Synthetic | Authentic |
| ppGpp (6) | 0.19 (0.25)[b] | 0.19 (0.25)[b] |
| ppGpp + inorganic pyrophosphatase | 0.19, 0.60[c] | 0.19, 0.60[c] |
| pGp | | 0.60 (0.64)[b] |
| ppGpp + inorganic pyrophosphatase + rye grass 3′-nucleotidase | 0.19, 0.60,[c] 0.80[c] | 0.19, 0.60,[c] 0.80[c] |
| 5′-GMP | | 0.80 |

[a] Polyethyleneimine, elution with 1.5 M $KH_2PO_4$ (pH 3.4). [b] $R_F$ value obtained when the tlc plate was pretreated with 0.01% Triton X-100 in 50% methanol (Cashel, 1974). [c] Major spot.

and to 5′-GMP by further incubation with rye grass 3′-nucleotidase. Base hydrolysis in 1M $NH_4OH$ at 37° for 90 min also afforded large amounts of pGp from both samples as determined by tlc on polyethyleneimine cellulose. Finally, phosphate analysis of synthetic ppGpp indicated the presence of 4 mol of phosphate per mol of guanosine (Table I).

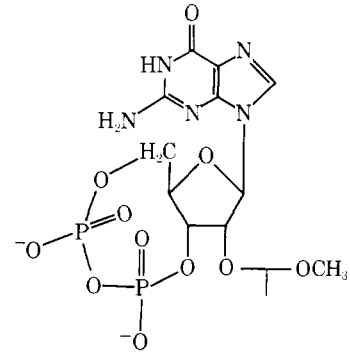
## Discussion

Although the synthesis of deoxyribonucleoside 3′-polyphosphates is relatively straightforward, the tendency of activated ribonucleoside 3′-phosphates to undergo nucleophilic attack on phosphorus by the vicinal hydroxyl group limits the synthetic approaches which can be utilized for the construction of ribonucleoside 3′-polyphosphates. Of particular importance is the choice of a blocking group which permits activation of the ribonucleotide and subsequent coupling with other phosphates, but which can be removed under conditions which do not cause decomposition of the newly formed product. As shown in this report, the acetal derived from methyl vinyl ether and the sugar hydroxyl moieties of ribonucleotides constitutes a suitable protecting group for the synthesis of ribonucleoside 3′-diphosphates and triphosphates. For adenosine 3′-monophosphate, the corresponding 2′,5′-di-*O*-(1-methoxyethyl) ribonucleotide (**1**) can be prepared in 81% yield, after purification by chromatography on DEAE-cellulose (Figure 1). As indicated in Figures 1 and 2, the chromatographic mobilities of 3′-AMP and **1** are sufficiently different to permit recovery of unreacted starting material. Conversion of **1** to the corresponding di- and triphosphates was accomplished by activation with 1,1′-carbonyldiimidazole and treatment of the activated ribonucleoside phosphate with inorganic phosphate or pyrophosphate, respectively. The overall yield for the synthesis of adenosine 3′-di- and -triphosphates was 61%, including the final deblocking step in aqueous acetic acid.

Adenosine 3′-mono-, -di- and -triphosphates exhibited the expected chromatographic properties on DEAE-cellulose, eluting in order of increasing charge in a linear salt gradient (Figure 3), although somewhat less quickly than the corresponding adenosine 5′-phosphates. They were further characterized by phosphate analysis according to the method of Fischer (1961). Adenosine 3′-diphosphate was reasonably stable in neutral, aqueous solution at 40°, reflecting little decomposition after 6 hr. The diphosphate was much less stable at elevated temperature and decomposed to three major products, the relative proportions of which were pH dependent. The diphosphate was found to be resistant to hydrolysis by rye grass 3′-nucleotidase, affording less than 1% of the amount of adenosine obtained from adenosine 3′-monophosphate under comparable conditions (Hecht and Hawrelak, 1975). Adenosine 3′-triphosphate was less stable than 3′-ADP in aqueous solution, affording substantial amounts of adenosine cyclic 2′,3′-monophosphate even in distilled water at 25°. In fact, hydrolysis of the triphosphate by rye grass 3′-nucleotidase could not be estimated due to extensive chemical decomposition of the nucleotide during the course of the incubation.

Oligonucleotides and polynucleotides which have 3′-*O*-monophosphate groups on the 3′-terminal nucleotide moiety have been noted to inhibit both the polymerization of ribonucleoside 5′-diphosphates and the phosphorolysis of certain oligonucleotides by polynucleotide phosphorylase (Hendley and Beers, 1959; Beers, 1959; Godefroy, 1970; Chou and Singer, 1970). The observed inhibition by these polymers prompted us to assay certain ribonucleoside 3′-phosphates as potential inhibitors of polynucleotide phosphorylase. Polymerization of [$^3$H]adenosine 5′-diphosphate by polynucleotide phosphorylase was inhibited by 3′-ADP and ppGpp[5] (Figure 4), but not by 5′-AMP or 3′-AMP. This finding suggested that it might be reasonable to utilize 3′-ADP itself as a possible substrate for polymerization by the enzyme. The polymerization of [$^3$H]-3′-ADP was therefore attempted with and without the additional presence of a polyadenylate primer terminating in an adenosine (2′)3′-diphosphate moiety. Additional experiments utilized $Mn^{2+}$ in place of $Mg^{2+}$. As shown in Figure 5 and Table III, none of these experiments resulted in detectable levels of polymer.

Guanosine tetraphosphate was synthesized from guanosine 5′-monophosphate 3′-monophosphate, the initial step involving blocking of the 2′-hydroxyl group with methyl vinyl ether. Although this conversion generally proceeded in satisfactory yield, the timing of the reaction was critical. Those runs which were permitted to proceed too long produced at least two by-products, which could not be converted back to pGp by incubation in aqueous acetic acid. These were presumably derivatized on $N_2$. The blocked diphos-



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[5] Guanosine tetraphosphate was also tested as an inhibitor of the polymerization of 5′-ADP by polynucleotide phosphorylase from *E. coli* and found to be slightly inhibitory. The physiological significance of these findings is uncertain.

phate (**4**) was treated with the imidazolidate of phosphoric acid to form 2′-*O*-(1-methoxyethyl)guanosine 5′-diphosphate 3′-diphosphate (**5**) by analogy with earlier work. This procedure was employed in lieu of the activation of **4** with carbonyldiimidazole in the belief that the latter procedure might have resulted in the formation of 2′-*O*-(1-methoxyethyl)guanosine cyclic 3′,5′-diphosphate (**7**). The overall yield from pGp to ppGpp was 18%, including deblocking in aqueous acetic acid. The structure was verified by phosphate analysis, by comparison of chromatographic properties with those of an authentic sample of ppGpp and by parallel degradation of the two samples with inorganic pyrophosphatase or aqueous base to afford pGp and additionally with rye grass 3′-nucleotidase to give 5′-GMP.

The procedure reported here for the synthesis of guanosine tetraphosphate is thus similar in approach to that utilized by Simoncsits and Tomasz (1974), the latter involving blocking of the 2′-hydroxyl group of guanosine 5′-monophosphate 3′-monophosphate with ethyl vinyl ether and subsequent conversion to the blocked tetraphosphate by sequential treatment with dibenzyl phosphochloridate and inorganic phosphate. The enzymatic synthesis of guanosine tetraphosphate has also been reported (Sy *et al.*, 1973; Cashel, 1974; Murao *et al.*, 1974).

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References

Allen, R. J. L. (1940), *Biochem. J. 34,* 858.
Beers, R. F., Jr. (1959), *Nature (London) 183,* 1335.
Canellakis, E. S., Kammen, H. O., and Morales, D. R. (1965), *Proc. Nat. Acad. Sci. U.S. 53,* 184.
Cashel, M. (1969), *J. Biol. Chem. 244,* 3133.
Cashel, M. (1974), *Anal. Biochem. 57,* 100.
Cashel, M., and Gallant, J. (1969), *Nature (London) 221,* 838.
Cashel, M., and Kalbacher, B. (1970), *J. Biol. Chem. 245,* 2309.
Chou, J. Y., and Singer, M. F. (1970), *J. Biol. Chem. 245,* 995.
Coutsogeorgopoulos, C., Hacker, B., and Mantsavinos, R. (1965), *Biochem. Biophys. Res. Commun. 20,* 129.
Coutsogeorgopoulos, C., Hacker, B., and Mantsavinos, R. (1966), *Biochim. Biophys. Acta 119,* 439.
Fischer, R. B. (1961), Quantitative Chemical Analysis, Philadelphia, Pa., W. B. Saunders, pp 402–404.
Godefroy, T. (1970), *Eur. J. Biochem. 14,* 222.
Haseltine, W. R., and Block, R. (1973), *Proc. Nat. Acad. Sci. U.S. 70,* 1564.
Hecht, S. M., and Hawrelak, S. D. (1975), *Biochemistry 14,* 974.
Hendley, D. D., and Beers, R. F., Jr., (1959), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 18,* 245.
Hoard, D. E., and Ott, D. G. (1965), *J. Amer. Chem. Soc. 87,* 1785.
Josse, J., and Moffatt, J. G. (1965), *Biochemistry 4,* 2825.
Kozarich, J. W., Chinault, A. C., and Hecht, S. M. (1973), *Biochemistry 12,* 4458.
Mackey, J. K., and Gilham, P. T. (1971), *Nature (London) 233,* 551.
Mitchel, R. E. J., Ward, D. C., and Tener G. M. (1967), *Can. J. Biochem. 45,* 89.
Murao, S., Nishino, T., and Hamagishi, Y. (1974), *Agr. Biol. Chem. 38,* 887.
Que, L. Jr., Willie, G. R., Cashel, M., Bodley, J. W., and Gray, G. R. (1973), *Proc. Nat. Acad. Sci. U.S. 70,* 2563.
Shelton, K. R., and Clark, J. M., Jr. (1967), *Biochemistry 6,* 2735.
Simoncsits, A., and Tomasz, J. (1974), *Biochim. Biophys. Acta 340,* 509.
Sy, J., and Lipmann, F. (1973), *Proc Nat. Acad. Sci. U.S. 70,* 306.
Sy, J., Ogawa, Y., and Lipmann, F. (1973), *Proc. Nat. Acad. Sci. U.S. 70,* 2145.