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Studies of Nucleosides and Nucleotides. XXXIX.[1] Synthesis of 8-Substituted Purine Nucleotides by the Direct Replacement Reactions

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8-Bromo-AMP and 8-bromo-GMP, which are key intermediate for the synthesis of 8-substituted purine nucleotides, were synthesized directly from AMP and GMP by the bromination with bromine–water in buffer solution. 8-Bromo-MP's were converted to 8-oxy compounds by the reaction with sodium acetate in acetic anhydride or in acetic acid. 8-Dimethylamino-GMP was obtained by replacement of 8-bromo-atom with dimethylamine. These 8-substituted purine nucleoside monophosphates were derived to 5'-diphosphates *via* phosphoromorpholidate.

A convenient synthesis of [α-^{32}P]-8-bromo-ADP was described.

Recently, we have reported a versatile method for the bromination of 8-position of purine nucleosides[3-5] and AMP.[4,6] This method involves treatment of purine nucleosides or nucleotides with bromine–water in buffered solution at pH 3.5—4.0. Analogous methods have been reported from other laboratories.[7,8] The present method seems to be suitable for obtaining a large quantity of material, because of ready accessibility and easy handlings. Since several 8-substituted purine nucleosides were found to be active against mouse sarcoma,[9,10] we are interested in synthesizing polynucleotides containing these compounds as analog of AMP or GMP and studying the nature of the polynucleotides.[11] The use of polynucleotide phosphorylase[12] for the synthesis of ribopolynucleotides requires 8-substituted purine nucleoside 5'-diphosphates as substrate. For this purpose, a procedure for direct bromination at position 8 of AMP and GMP and subsequent replacement of 8-bromo to oxy and dimethylamino groups were developed. Resulting monophosphates were converted to phosphoromorpholidate and led to 5'-diphosphates by the procedure described by Khorana.[13] A synthesis of [α-^{32}P]-8-bromo-AMP, which was required for enzymatic assay, was also described.

The synthesis of 8-substituted purine nucleosides reported previously[8,14,15] would afford starting materials for 8-substituted purine nucleotides. However, as shown in the case of

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[1] Part XXXVIII: M. Ikehara and S. Uesugi, *Chem. Pharm. Bull.* (Tokyo), **17**, 348 (1969).

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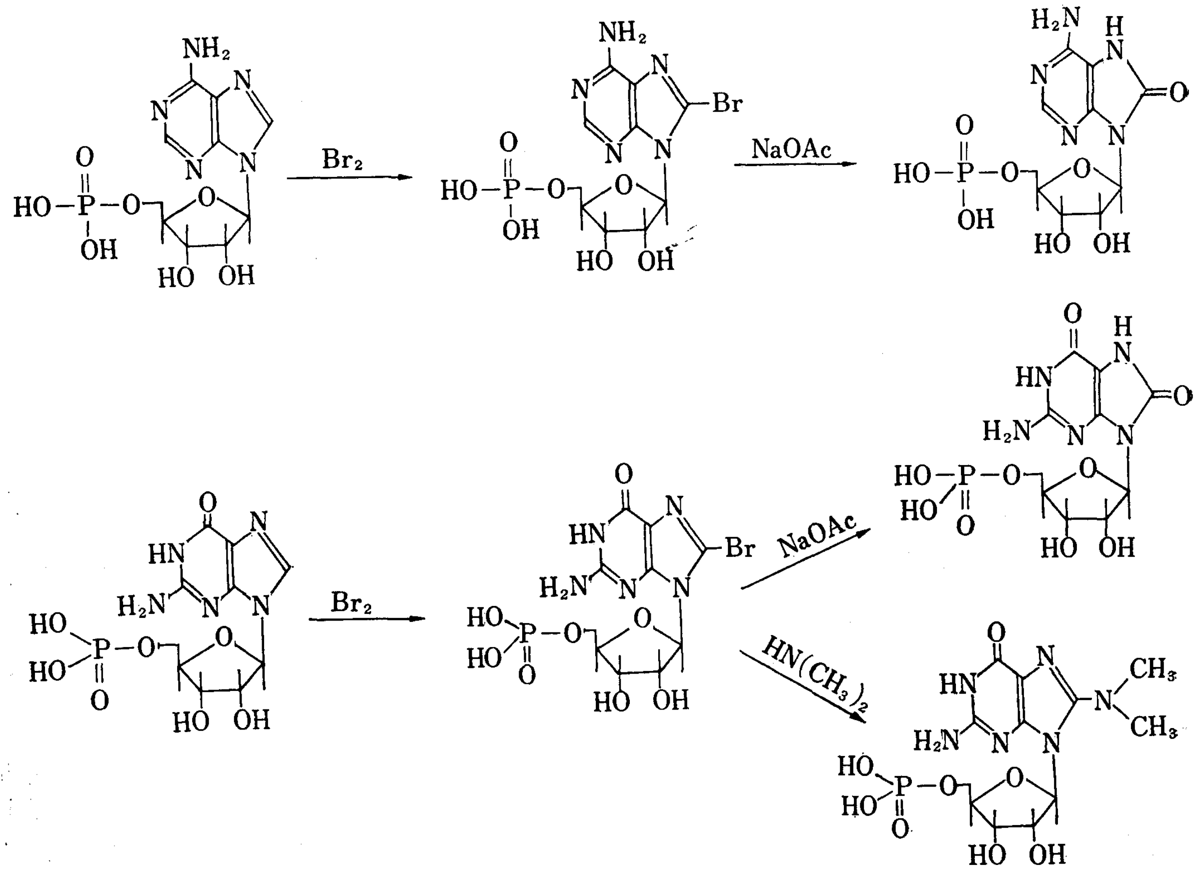
8-bromoadenosine[5)] and 8-oxyguanosine,[16)] nucleosidic linkage of the nucleosides having 8-substituents was frequently labilized against attack of acidic reagent and the base moiety became labile against alkaline treatment. Therefore, deprotection after phosphorylation of these nucleosides will damage the product and lower the yield. Taking a loss during multi-step synthesis and accessibility of AMP and GMP into account, we have chosen direct transformation of monophosphates to the desired nucleotides.



Bromination of AMP was first investigated according to the procedure described previously.[4)] The best results were obtained in the bromination using 1.2 equivalents of bromine-water in molar acetate buffer solution of pH 4.0. 8-Bromo-AMP was obtained in the yield of 91%. In the case of GMP bromination, also 1.2 equivalents of bromine-water was used in 0.6M acetate buffer of pH 3.0. Purification of the nucleotide by ion-exchange column chromatography gave 8-bromo-GMP in a yield of 64%. When pH of the reaction mixture was changed to 1.0, 2.0, 3.5, 4.0 or 5.0, less amount of BrGMP was obtained. Structure of these bromo derivatives was elucidated by ultraviolet (UV) absorption properties, paper electrophoresis and paper chromatography. The structure of BrGMP was further confirmed by the direct comparison with an authentic sample synthesized from 2,'3'-O-isopropylidene-8-bromoguanosine[3)] phosphorylated with P^1-diphenyl-P^2-morpholinopyrophosphoro chloridate.[17)]

Conversion of 8-bromo- to 8-oxy function in BrAMP and BrGMP was achieved either by treatment with sodium acetate in acetic acid[16)] or in acetic anhydride.[18)] When BrAMP was refluxed for 2 hr in acetic anhydride in the presence of sodium acetate, total conversion

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16) M. Ikehara, H. Tada and K. Muneyama, *Chem. Pharm. Bull.* (Tokyo), **13**, 1140 (1965).
17) M. Ikahara and E. Ohtsuka, *Chem. Pharm. Bull.* (Tokyo), **11**, 961 (1963).
18) M. Ikehara and M. Kaneko, *Chem. Pharm. Bull.* (Tokyo), **15**, 1261 (1967).

of bromo to oxy function was confirmed by ultraviolet absorption spectra. By this reaction, acetylation both on 6-NH_2 and 2'- or/and 3'-OH groups occurred. The acetyl group was removed by 1N sodium hydroxide and the unprotected nucleotide was obtained by a column chromatography on Dowex I resin in a yield of 88%.

When the conversion of 8-BrGMP to 8-HOGMP was carried out by sodium acetate-acetic anhydride procedure, acetylation of 2-NH_2 of guanine moiety occurred. The removal of acetyl group by an alkaline treatment caused the damage of base moiety and resulted in lowering of the yield. Therefore, in this case acetic acid was employed as the solvent. Heating at 70—80° for 2 hr gave 8-HOGMP in 36% yield. The by-product formed was 8-bromoguanine, which indicated the scission of the nucleophilic linkage prior to the hydroxylation. The structure of 8-HOGMP was confirmed by the direct comparison with an authentic sample synthesized from 8-oxyguanosine.[19)]

8-Bromo-GMP was then converted to 8-dimethylamino-GMP. This was achieved by an analogous procedure, which was used in the case of 8-bromoguanosine to 8-dimethylaminoguanosine.[14)] Heating of 8-BrGMP with anhydrous dimethylamine in methanol at 115—125° for 5 hr, followed by ion-exchange column chromatography gave 8-dimethylamino-GMP in a yield of 74%. When heating was carried out at 130—140°, cleavage of phosphomonoester linkage occurred and the yield was dropped to 21%. When 2',3'-O-isopropylidene-8-dimethylaminoguanosine was phosphorylated, deprotection using acidic condition met with little success due to extreme lability of the glycosidic linkage in 8-dimethylamino-GMP.

8-Bromo-, 8-Oxy-AMP and -GMP, as well as 8-dimethylamino-GMP were converted to 5'-phosphoromorpholidate as described by Moffatt and Khorana[13)] and allowed to react with inorganic phosphate to give diphosphates. Reaction mixture was applied to column chromatography on DEAE-cellulose and the diphosphates were obtained in the pure state. Over-all yield of these diphosphates fluctuated between 19% and 69% as to individual 8-substituted monophosphate. The structure of diphosphates was confirmed by ultraviolet absorption properties, phosphate analysis data, paper chromatography and paper electrophoresis.

In order to investigate ^{32}P incorporation into polynucleotide from 8-bromo-ADP by means of polynucleotide phosphorylase, [α-^{32}P]-BrADP was required. 2',3'-O-Isopropylideneadenosine was phosphorylated by Tener's method[20)] using [^{32}P]-cyanoethylphosphate and DCC as the phosphorylating agent. [^{32}P]-AMP, thus obtained, was brominated as described above to give [^{32}P]-BrAMP in a yield of 80%. This compound was further converted to phosphoromorpholidate and allowed to react with inorganic phosphate. By this procedure [α-^{32}P]-BrADP was obtained in over-all yield of 4.2% as calculated from [^{32}P]-cyanoethylphosphate.

Polymerization reaction using these 8-substituted purine ribonucleoside 5'-diphosphates as substrate in polynucleotide phosphorylase systems will be reported elsewhere.[11)]

Experimental[21)]

Paper Chromatography——Solvent A, 1-propanol-conc. ammonia-water, 55:10:35; solvent B, ethanol-1M ammonium acetate (pH 7.0), 1:1; solvent C, 2-propahol-conc. ammonia-water, 7:1:2; solvent D, 1-butanol-acetic acid-water, 5:2:3. All chromatographies were performed on Toyo filter paper No. 51A by the descending technique.

Paper Electrophoresis——Carried out in 0.05M triethyl ammonium bicarbonate (pH 7.5) at 20 V/cm or 60 V/cm for 30—90 min. Toyo filter paper No. 51A was used.

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19) M. Ikehara and K. Murao, *Chem. Pharm. Bull.* (Tokyo), **16**, 1330 (1968).

20) G.M. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961).

21) Ultraviolet absorption was taken with Hitachi automatic recording spectrophotometer ESP 3T or with Shimadzu AOV-50 digital spectrophotometer. Radioactivity was measured with Aloka LSC 501 scintillation counter.

8-Bromoadenosine 5′-Monophosphate——AMP (sodium salt, TOD_{260}[22]=49000, 3.3 moles) was dissolved in 1M sodium acetate buffer (pH 4.0, 200 ml). Into this solution was added bromine–water (containing 0.3 mmole Br_2/ml, 19.8 ml, 2 equiv.). Reaction mixture was kept at room temperature overnight and sodium bisulfate was added until red color discharged. Solvent was evaporated *in vacuo*, the residue was dissolved in water (500 ml), and applied to a column (1.5×50 cm) of Dowex I X8 (formate form) resin. After the water, wash, the column was eluted with 0.1N formic acid. 8-BrAMP was obtained in a yield of TOD_{260} =46600 (91%). Ultraviolet absorption properties, *Rf*'s in paper chromatography, mobility in paper electrophoresis were listed in Table I and III.

8-Bromoguanosine 5′-Monophosphate——i) Guanosine 5′-monophophate (sodium salt, 720 mg, TOD_{260} =18100) was dissolved in water (30 ml). Into this solution was added 1M sodium acetate buffer (pH 3.0, 60 ml) and saturated bromine-water (8.4 ml, 1.2 equiv.). The reaction mixture was stoppered and kept at room temperature in the dark. After 25 min, reaction was stopped by the addition of 0.1M sodium bisulfate (5 ml) and after 4 min, further 5 ml was added for the complete stop. Adsorption on activated charcoal and extraction with 50% ethanol containing 2% ammonia were performed as described elsewhere.[6] Ethanol solution was evaporated *in vacuo* and the concentrated solution was applied to a column (1.2×32 cm) of Dowex I X8 (formate) resin. After the water wash, the column was eluted with 0.2M ammonium formate (2 liter) to give GMP. Second peak, which was eluted with 0.4M ammonium formate+0.004N formic acid was pooled and evaporated. 8-Bromo-GMP was obtained in a yield of TOD_{260}=16700 (66%).

TABLE I. Ultraviolet Absorption Properties of 8-Substituted Purine Nucleosides

Compound	λ_{max} (mμ) 0.1N HCl	pH 7	0.1N NaOH
BrAMP	263	263	266
[^{32}P]-BrAMP	263	263	265
HOAMP	264.5, 287.5	255 (sh)[a], 270	280.5
BrGMP	263 , 293 (sh)	263, 293 (sh)	273
HOGMP	249.5, 295	249, 295.5	258, 283
Me_2NGMP	265.5, 294 (sh)	264, 294 (sh)	271
BrADP	263.5	265	267
[^{32}P]-BrADP	263	265	267
HOADP	264.5, 286.5	255 (sh), 270	281
BrGDP	263 , 293 (sh)	263, 293 (sh)	273
HOGDP	247 , 296	247, 296	258, 282
Me_2NGDP	266.5, 294 (sh)	264, 294 (sh)	271

a) sh stands for inflection.

TABLE II. Synthesis of 8-Substituted Purine Nucleoside Diphosphates

Starting material	TOD_{260} (mmole)	Morpholine (mmole)	DCC (mmole)	Pi (mmole)	Product	Yield TOD_{260}	%
BrAMP	7800 (0.5)	2	2	2	BrADP	4900	62
BrGMP	15600 (1.0)	6	6	4	BrGDP	11100	69
HOAMP	16300 (1.6)	6.5	6.4	6.3	HOADP	7140	44
HOGMP	3350 (0.35)	2.1	2.1	1.4	HOGDP	2140	52
Me_2NGMP	7030 (0.75)	4.0	4.0	2.0	Me_2NGDP	1340	19

ii) P^1-Diphenyl-P^2-morpholinopyrophosphorochloridate[17] was prepared freshly from diphyenyl phosphate (500 mg, 2 mmoles) and morpholinophosphorodichloridate[23] (408 mg, 2 mmoles) dissolved in dioxane (5 ml), followed by the addition of 2,6-lutidine (428 mg, 4 mmoles). Into the reagent solution was added a DMF (5 ml) solution of 2′,3′-O-isopropylidene-8-bromoguanosine (402 mg, 1 mole). After stirring at room temperature for 2 days, the reaction mixture was evaporated to give a residue. The residue was taken up in a small amount of water and adjusted to pH 1 by the addition of 98% formic acid. After kept at room temperature for 1.5 days, formic acid was evaporated *in vacuo*. The residue was dissolved in water and

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22) TOD_{260} stands for optical density unit measured at 260 mμ in 1 cm path length multiplied by volume (ml) of the solution.

23) M. Ikehara and E. Ohtsuka, *Chem. Pharm. Bull.* (Tokyo), **11**, 435 (1963).

Table III. Properties of 8-Substituted Purine Nucleotides

Compound	Paper chromatography Solvent A	D	Paper electrophoresis (pH 7.5)	Nucleoside/labile P /total P
BrAMP	1.11a)	1.38a)	0.96a)	
[^{32}P]-BrAMP		1.35a)	0.95a)	
HOAMP	0.70a)	0.71a)	1.01a)	
BrGMP	1.02b)		0.91b)	
HOGMP	0.91b)		1.03b)	
$\mathrm{Me_2NGMP}$	1.25b)		0.88b)	
BrADP	1.05c)		0.94c)	1.0 : 1.0 : 2.0
[^{32}P]-BrADP			0.98c)	1.0 : 1.0 : 2.1
HOADP	0.85c)		1.01c)	1.0 : 0.9 : 2.0
BrGDP	1.09d)		0.97d)	1.0 : 1.1 : 2.0
HOGDP	0.94d)		1.04d)	1.0 : 0.9 : 2.0
$\mathrm{Me_2NGDP}$	1.44d)		0.98d)	1.0 : 1.0 : 2.0

a) R_{AMP} b) R_{GMP} c) R_{ADP} d) R_{GDP}

applied to a column (1.2 × 26 cm) of Dowex I X8 (chloride form) resin. A linear gradient elution with 0.2M sodium chloride in 0.003N HCl gave 8-bromo-GMP in a yield of $\mathrm{TOD_{260}}=6900$ (44%). From the accompanying peak, 8-bromo-GMP was obtained ($\mathrm{TOD_{260}}=1800$). The properties of 8-bromo-GMP obtained in i) and ii) were summarized in Table I and III.

8-Oxyadenosine 5′-Monophosphate——8-Bromo-AMP (2.5mmoles) was dissolved in anhydrous pyridine (*ca.* 5 ml) and evaporated to a syrup. This process was repeated to remove traces of water. Into the residue was added freshly fused sodium acetate (410 mg, 5 mmoles) and acetic anhydride (20 ml). The reaction mixture was refluxed for 2 hr, keeping it at room temperature overnight, and methanol (10 ml) was added. Evaporation of methanol and repeated additions and evaporations of methanol gave a residue. The residue was taken up in 1N NaOH (50 ml) and the solution was kept at room temperature for 24 hr. The solution was neutralized with 1N HCl, adjusted to pH 8.0 with conc. ammonia, and diluted with water to 200 ml. The solution was applied to a column (1.7 × 26.5 cm) of Dowex I X8 (chloride form) resin. The column was washed with water, and eluted with 0.003 N HCl (5 l) and 0.003 N HCl + 0.1 M NaCl (5 l) by a linear gradient elution technique. Fractions were collected in 45 ml portions. Fractions No. 49—79 were pooled and evaporated *in vacuo* at below 30°. 8-Oxy-AMP was obtained in a yield of $\mathrm{TOD_{260}}=23200$ (88%). Ultraviolet absorption properties, mobility in paper electrophoresis and paper chromatography were listed in Table I and III.

8-Oxyguanosine 5′-Monophosphate——i) 8-BrGMP ($\mathrm{TOD_{260}}=30000$, 1.9 mmoles) was thoroughly dried by azeotropic distillation with anhydrous pyridine. Into this were added sodium acetate (1.64 g, 20 mmoles) and acetic anhydride (60 ml). The mixture was refluxed for 2 hr. After cooling, methanol was added to decompose acetic anhydride. Solvent was evaporated *in vacuo* and the residue was dissolved in water. Adsorption and extraction from activated charcoal as described above, followed by the evaporation of solvent, gave a residue. The residue was dissolved in conc. ammonia (10 ml) and kept at room temperature 5 days. Ammonia was carefully evaporated and the residual solution was applied to a column (1.25 × 30.5 cm) of Dowex I X8 (chloride form) resin. After the water wash, elution was carried out with 0.003 N HCl + 0.05 M NaCl (3 liter) and 0.003 N HCl + 0.2 M NaCl (3 liter) by a linear gradient elution technique. Fraction was collected in 20 ml portions and No. 50—59 were pooled. Yield was $\mathrm{TOD_{260}}=2760$ (22%).

ii) 8-BrGMP ($\mathrm{TOD_{260}}=40000$, 2.5 mmoles) was azeotropically dried by evaporation with pyridine. There residue was dissolved in glacial acetic acid (300 ml) containing sodium acetate (2.05 g, 25 mmoles). Reaction mixture was stirred at 85—90° for 10 hr. Acetic acid was evaporated by vacuum distillation, the residue was taken up in water, and the insolubles were removed by centrifugation. The solution was passed through a column (2.2 × 17 cm) of Dowex 50 ($\mathrm{H^+}$ form) resin. Effluents were evaporated to give a residue, which was dissolved in water (300 ml) and applied to a column (1.7 × 16.5 cm) of Dowex I X8 (chloride form) resin. After the water wash, the column was eluted with 0.003 N HCl (4 liter) and 0.003 N HCl + 0.2 M lithium chloride (4 liter) by a linear gradient technique. Fractions were collected in 20 ml portions and fractions No. 88—163 were pooled. 8-HOGMP was obtained in a yield of $\mathrm{TOD_{260}}=5250$ (32%).

The same reactions were carried out at 70—80° for 12 hr and the product was obtained in the yield of 36%. The properties of 8-HOGMP were listed in Table I and III.

8-Dimethylaminoguanosine 5′-Monophosphate——8-Bromoguanosine 5′-monophosphate (ammonium salt, 0.238 g, 0.5 mmole) was dissolved in anhydrous methanol (15 ml) containing dimethylamine (15 g). The reaction mixture was sealed in a steel tube and heated at 115—125° in an oil bath for 5 hr. After cooling, the solvent was evaporated and the residue was dissolved in water (*ca.* 20 ml). The solution was applied to a column (1.25×26.5 cm) of Dowex I X8 (chloride form) resin. After the water wash, elution was carried out with 0.003 N HCl (2 liter) and 0.003 N HCl+0.1 M NaCl (2 liter) by a linear gradient elution technique. 8-Dimethylamino-GMP was obtained in a yield of $TOD_{260}=6740$ (74%).

Heating at 130—140° of the same reaction mixture gave a yield of 21%. Properties of dimethylamino-GMP were listed in Table I and III.

General Procedure for the Synthesis of 8-Substituted ADP and GDP——Essentially the method described by Moffatt and Khorana[13] was used. 8-Substituted AMP or GMP, synthesized as described above, was dissolved in water-*t*-butanol mixture (30 ml for 1 mmole, 1:1, v/v) containing 4 equivalents of morpholine. A solution of DCC (4 equivalents) in *t*-butanol (5—20 ml) was added dropwise during 6 hr to the above solution at reflux temperature. After 5—10 hr, reaction mixture was examined for confirming the complete conversion to morpholidate. When necessary, appropriate amount of DCC was further added and reflux was continued until all of the monophosphate converted to the morpholidate. Reaction mixture was kept at room temperature overnight, dicyclohexylurea was removed by filtration, and the filtrate and washings (*t*-butanol) were combined. Evaporation of the solvent gave phosphoromorpholidate as crystalline powder. Morpholidate, thus obtained, was thoroughly dried by azeotropic distillation with anhydrous pyridine and combined with a pyridine solution of anhydrous (made by evaporation with pyridine) inorganic phosphoric acid (4 equivalents to morpholidate, as tri-*n*-butylammonium salt). Mixture was dried again with pyridine and final residue was taken up in dry pyridine (5—10 ml). Reaction mixture was tightly stoppered and set aside at room temperature in the dark for 3 days. Reaction was stopped by addition of water and the solvent was evaporated *in vacuo*. The residue was dissolved in water and applied to a column of DEAE-cellulose (bicarbonate form). Linear gradient elution with triethyl ammonium bicarbonate (pH 7.5) gave 8-substituted diphosphates in the yield as summarized in Table II. The effluents were evaporated to dryness and residue was dissolved in a small amount of water and methanol. Precipitation by the addition of acetone gave white powder, which was collected by centrifugation. The properties of diphosphates, thus obtained, were listed in Table II.

[^{32}P]-8-Bromoadenosine 5′-Monophosphate——2′,3′-O-Isopropylideneadenosine was phosphorylated with [^{32}P]-cyanoethyl phosphate (5 mC) and DCC as described by Tener.[20] [^{32}P]-Adenosine monophosphate (1.17 mmoles), thus obtained, was dissolved in a buffer (14 ml) of 1.5 M KH_2PO_4+1.5 N NaOH (pH 8.0). To this solution dioxane (7 ml) containing bromine (0.12 ml, 2 equivalents) was added. The mixture was kept at room temperature for 17 hr in the dark. Work-up procedure as described above, followed by column chromatography on Dowex I X8 (formate form) resin gave [^{32}P]-8-BrAMP in a yield of 80%. Properties were listed in Table I and III.

[^{32}P]-BrAMP, thus obtained, was converted to morpholidate as described above in the general procedure and the morpholidate was allowed to react with inorganic phosphate. Column chromatography on Dowex I X8 (chloride form) ion-exchange resin gave BrADP in a yield of $TOD_{260}=2660$ (0.17 mmoles). Yield as calculated from [^{32}P]-cyanoethylphosphate was 4.2%. Radioactivity of [^{32}P]-BrADP was 7.9×10^4 cpm/μmole. Properties were listed in Table I and III.

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