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STUDIES ON NUCLEOTIDE ANALOGS

I. SYNTHESIS OF TWO 9-α-D-MANNOFURANOSYLADENINE PHOSPHATES, AND THEIR INHIBITION OF ADENYLATE KINASE*†

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ABSTRACT

9-α-D-Mannofuranosyladenine (**1**) was quantitatively phosphorylated at O-5 by phosphoryl chloride in the presence of triethyl phosphate, giving phosphate **2**. Treatment of 9-(2,3-*O*-isopropylidene-α-D-mannofuranosyl)adenine (**3**) with phosphoryl chloride–trimethyl phosphate, followed by hydrolysis at pH 1.5 to remove the protecting group, yielded mononucleotides **2** and **4** having the phosphate group at C-5′ and C-6′, respectively. These mononucleotides, chromatographically homogeneous in six solvent systems, were further characterized by their patterns of chromatography on Dowex ion-exchange resin, by their mass spectra, and by phosphorus n.m.r. spectroscopy. Both the 5′- and 6′-phosphates are noncompetitive inhibitors of adenylate kinase (for which a sensitive, accurate, and inexpensive assay-system was developed). Of the two, the 6′-mononucleotide was the more potent inhibitor of adenylate kinase.

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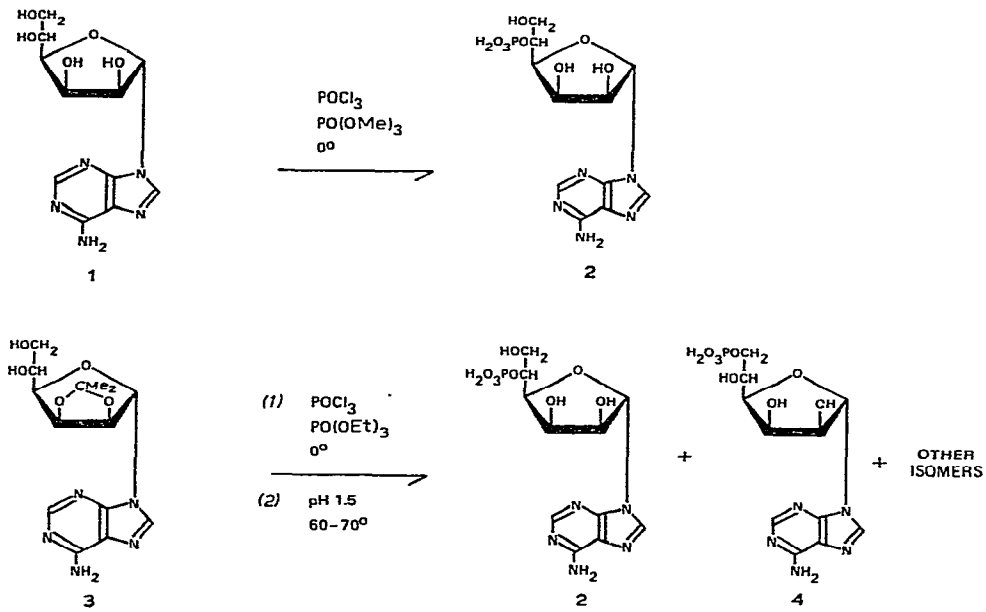
INTRODUCTION

In the past few years, much attention has been given to the synthesis of nucleoside analogs, but a recent, *in vitro* analysis demonstrated that 9-α-D-mannofuranosyladenine (**1**) is of low efficacy as an antineoplastic agent[1].

Synthesis of related mannonucleotides has now been undertaken on the premise that presence of a phosphate group might enhance the *in vitro* efficacy as compared with that of the analog. Yoshikawa *et al.*[2,3] had reported that phosphoryl chloride plus trimethyl (or triethyl) phosphate causes selective phosphorylation at O-5′ of the unprotected, common ribonucleosides. 9-α-D-Mannofuranosyladenine (**1**) and 9-(2,3-*O*-isopropylidene-α-D-mannofuranosyl)adenine (**3**) were subjected to the phosphorylating conditions of Yoshikawa *et al.*[2,3] to determine whether such selectivity would be obtained with a hexofuranosylnucleoside. The synthesized phosphates were then subjected to the adenylate kinase enzyme system, to ascertain whether they had any biological activity therein.



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EXPERIMENTAL

Synthetic materials and methods. — Percentage yields of individual phosphorylations were determined spectrophotometrically. Total absorbance (*A*) units are defined as the ultraviolet absorption times the volume (in ml) eluted. Molar extinction coefficients were calculated by use of the molecular weight determined by elemental analysis. Optical rotations were measured for 1-ml aliquots in a microtube

(path length, 10 mm) with a Perkin–Elmer, automatic, digital-readout polarimeter equipped with a sodium (589 nm) filter. A Kofler micro-hotstage was used for determining melting points.

Phosphorus nuclear magnetic resonance (n.m.r.) spectra were recorded with a Varian XL 100-15 n.m.r. spectrometer for samples of deuterium oxide (0.5 ml) containing the nucleotide (50 mg) that had been adjusted to pH 9 with sodium deuteroxide. A Chelex ion-exchange resin was used for removing any divalent ions present. The standard used[4] was P_4O_6.

Mass spectra were recorded with an LKB-9000 gas chromatograph–mass spectrometer (1% OV-17). Trimethylsilyl derivatives were synthesized according to Krishna[5]. Typical spectra[6] were obtained for 2′-, 3′-, 5′-, and cyclic AMP.

Paper and thin-layer chromatography. — Descending chromatography on Whatman No. 2 paper was employed with the following solvent systems: solvent *1*, 33:17 (v/v) isobutyric acid–2M ammonium hydroxide; solvent *2*, 6:1:3 (v/v) 2-propanol–conc. ammonium hydroxide–0.1M H_3BO_3; solvent *3*, 6:3:1 (v/v) propyl alcohol–conc. ammonium hydroxide–water; solvent *4*, 125:75:2 (v/v) isobutyric acid–M ammonium hydroxide–0.1M (ethylenedinitrilo)tetraacetatic acid (EDTA). Chromatography was conducted on prewashed paper for 20 h.

Brinkmann thin-layer plates (5 × 20 cm) of silica gel F-254 were used with the following two solvent systems: solvent *5*, 6:3:1 (v/v) propyl alcohol–conc. ammonium hydroxide–water, and solvent *6*, 5% Na_2HPO_4 in water. Spots were located with a Mineralite lamp (254 nm). The $R_{\text{cyclic AMP}}$ value is the distance traveled by AMP or an AMP analog divided by the distance traveled by cyclic AMP.

Preparation of nucleosides. — 9-(2,3-*O*-Isopropylidene-α-D-mannofuranosyl)-adenine (**3**) and 9-α-D-mannofuranosyladenine (**1**) were synthesized according to Lerner and Kohn[7,8], with one minor variation. 2,3:5,6-Di-*O*-isopropylidene-α-D-mannofuranosyl chloride was used immediately after being dried over sodium sulfate (instead of distilling it), thus avoiding loss due to decomposition. The isopropylidene compound **3** had m.p. 252–254° (lit.[7,8] m.p. 249–250°) and $[\alpha]_D^{25}$ +32.2° (*c* 0.72, 0.1M HCl) [lit.[7,8] $[\alpha]_D^{21}$ +32.5° (*c* 1.26, 0.1M HCl)]. Ascending chromatography on Whatman No. 1 paper with 4:1:5 (v/v) butyl alcohol–acetic acid–water gave one spot, R_{adenine} 0.69.

The unprotected nucleoside **1** had m.p. 238–239° (lit.[7,8] 237–237.5°); R_{adenine} 1.33.

Enzyme assay. — Adenylate kinase catalyzes the reaction: $2ADP \rightleftharpoons AMP + ATP$. As the compounds synthesized are analogs of AMP, the enzymic reaction was conducted in the reverse direction, with AMP, AMP analog, and ATP initially present in the reaction mixture. The standard reaction-mixture consisted of 46mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) (pH 7.8), 7mM $MgCl_2$, 7mM cold ATP, 62 μM [^{14}C]-ATP (Schwartz, 29.2 mCi/mm). The proportions of AMP, AMP analog, and adenylate kinase diluted with EDTA were varied, and used in amounts sufficient to yield linear kinetics. An assay was desired that was sensitive and yet avoided the complexities of a coupled-enzyme system. Separation of radioactive nucleotides on

plastic sheets of poly(ethyleneimine)-impregnated (PEI) cellulose proved to be accurate, sensitive, and convenient.

At designated time-intervals, 2-μl aliquots were withdrawn with Drummond microcapillaries, and spotted, 1.2 cm apart, on PEI-cellulose plates containing nonradioactive ADP carrier. The plates were chromatographed sequentially in 0.5M LiCl (10 min), 1M LiCl (40 min), and 1.5M LiCl (60 min). The R_F values for ATP, ADP, and AMP were 0.11, 0.20, and 0.31, respectively. The t.l.c. assay of radioactive compounds proved to be less laborious than the multiple columns of Noda[9] or Brown[10], in that all of the samples could be chromatographed simultaneously. Nucleotides were located with a Mineralite lamp (254 nm). Zones were excised, and placed in 5 ml of 4.2% liquifluor–toluene. The amount of radioactive ADP formed from radioactive ATP was expressed as the ratio of ADP counts to total counts, thus compensating for minor variations in the 2-μl aliquots. As radioactive AMP was not produced during the linear reaction, it was only necessary to count ADP and ATP spots.

Synthesis of 9-α-D-mannofuranosyladenine 5′-phosphate (**2**). — A mixture of phosphoryl chloride (5 μl; 54 μmoles) and trimethyl phosphate (30 μl) was cooled to 0° in a test tube fitted with a drying tube of silica gel. To this solution was added 9-α-D-mannofuranosyladenine (**1**) (5 mg; 13 μmoles), and the mixture was stirred for 1 h at 0°. The mixture was then poured into ice water (1 ml) to decompose the excess of phosphoryl chloride and to hydrolyze the nucleoside phosphorodichloridate. The resulting products were chromatographed on a column (1.6 × 25 cm) of Dowex 1 X-8 (formate) ion-exchange resin at a flow rate of 0.7 ml.min^{-1} cm^{-2}. One major peak (91% of total *A* units) was eluted with 0.1M formic acid. The eluate containing this compound was evaporated to a thick syrup *in vacuo* at 40°. Acetone (~20 vol) was added, and the resulting, white solid was collected by filtration. The precipitate was redissolved in the minimal volume of water, and reprecipitated four times with acetone (~20 vol). The solid was dried for 4 h at 120° under vacuum.

Progressive decomposition of this product was observed as the temperature was increased, and melting occurred at 214–225°. U.v. data: in H_2O (pH 2) λ_{max} 256.5 nm (ε_{mM} 15.00), and in H_2O (pH 7) λ_{max} 259 nm (ε_{mM} 15.30); $R_{cyclic\ AMP}$ 0.92 (solvent *1*), 0.53 (*2*), 0.47 (*3*), 0.90 (*4*), 0.43 (*5*), and 1.0 (*6*); $[\alpha]_D^{25}$ +1.85° (*c* 3.77, NaOH, pH 8). The retention time in g.l.c. of the per(trimethylsilyl) derivative was 5 min, as compared with 4.5 min for that of AMP. ^{31}P n.m.r. spectroscopy revealed a well defined doublet having a chemical shift of 107.7 p.p.m. and a *J* value of 5.2 Hz, suggesting a 2′-, a 3′-, or a 5′-phosphate. The first two possibilities were eliminated on the basis of arguments presented in the Discussion. The per(trimethylsilyl) derivative gave a parent peak that indicated a molecular weight of 809.

Anal. Calc. for $C_{11}H_{16}N_5O_8P \cdot 1H_2O$: C, 33.42; H, 4.59; N, 17.71; P, 7.83. Found: C, 33.34; H, 4.52; N, 17.74; P, 7.87.

Synthesis of 9-α-D-mannofuranosyladenine 6′-phosphate (**4**). — A mixture of phosphoryl chloride (5 μl; 54 μmoles) and triethyl phosphate (30 μl) was cooled to 0° in a test tube equipped with a drying tube of silica gel. To this solution was added

9-(2,3-*O*-isopropylidene-α-D-mannofuranosyl)adenine (**3**; 5.68 g; 13 μmoles), and the mixture was stirred for 4 h at 0°; the suspension became a solution within the first hour. The excess of phosphoryl chloride was then hydrolyzed by addition of ice-water (1 ml), and the isopropylidene group was removed by heating at pH 1.5 for 40 min at 60–70°. The mixture was then chromatographed under the same conditions as for the 5′-phosphate. Fractions (14 ml each) were collected, and five peaks were eluted, as shown in Fig. 1. Peak 5, in tubes 221–277 (52% of the total *A* units), was adequately separated from the preceding peaks; it chromatographed at the same position as 9-α-D-mannofuranosyladenine 5′-phosphate (**2**), already described.

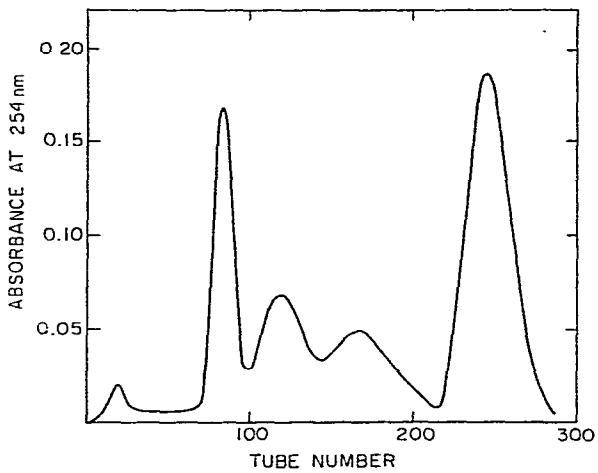

Fig. 1. Chromatography of the 9-α-D-mannofuranosyladenine phosphates on Dowex-1 X-8 (formate). (Peak 1 was eluted with water; for the remaining peaks, 75mM formic acid was used as the eluant).

On rechromatography with the same column, peak 2, tubes 70–95 (19% of the total *A* units), could be adequately separated from peak 3. (Peaks 3 and 4, together totaling 29%, were not studied further.) The eluate containing peak 2 was evaporated at 40° *in vacuo* to a thick syrup, and acetone or ethanol (~20 vol) was added. The resulting, white solid was collected by filtration, and redissolved and reprecipitated as described for compound **2**. The solid was dried overnight at room temperature under vacuum; this compound slowly decomposes on drying at 120° under vacuum. Progressive decomposition of **4** was observed as the temperature was increased, and melting occurred at 173–178°. U.v. data: in H_2O (pH 2), λ_{max} 257 nm (ε_{mM} 14.50) and in H_2O (pH 7), λ_{max} 259 nm (ε_{mM} 15.40); $R_{cyclic\ AMP}$ 0.82 (solvent *1*), 0.46 (*2*), 0.30 (*3*), 0.86 (*4*), 0.38 (*5*), and 1.17 (*6*). No optical rotation could be detected when a concentration of 3.77 g/100 ml of solution was examined. The retention time in g.l.c. of the per(trimethylsilyl) derivative was 7.2 min, as compared with 4.5 min for that of AMP. ^{31}P n.m.r. spectroscopy revealed a well defined triplet having a chemical shift of 108 p.p.m. and a *J* value of 3.7 Hz, indicating a 6′-phosphate. The per(trimethylsilyl) derivative gave a parent peak that indicated a molecular weight of 809.

Anal. Calc. for $C_{11}H_{16}N_5O_8P \cdot 1.5H_2O$: C, 32.67; H, 4.73; N, 17.32; P, 7.66 Found: C, 32.93; H, 4.66; N, 17.16; P, 7.48.

Preliminary studies to determine the mechanism of the phosphorylation reaction. — ^{31}P n.m.r. spectroscopy was utilized to determine whether complex-formation occurred between the phosphorylating agent and the solvent during the phosphorylation of the nucleosides. Phosphoryl chloride and triethyl phosphate were mixed in the ratios of 1:2 and 2:1, with and without the addition of water. In each case, a singlet was observed for phosphoryl chloride and a septet for triethyl phosphate. Each had the same chemical shift as in the pure form, indicating that complex-formation is not involved in the phosphorylation mechanism.

Studies of inhibition of adenylate kinase. — A number of bases other than adenine have been tested in the adenylate kinase system, including cytosine, guanine, inosine, N^6-methoxyadenine, purine, and uracil; only the first base[9] serves as an effective substrate. Two variations in the sugar moiety are substrates: 2′-deoxy-AMP (ref. 11) and 3′-amino-3′-deoxy-AMP (ref. 12). Thus far, nucleotides containing other sugar moieties had not been tested as substrates or inhibitors; hence, the nucleotides synthesized, namely, 9-α-D mannofuranosyladenine 5′-phosphate (**2**) and 9-α-D-mannofuranosyladenine 6′-phosphate (**4**), were tested in the adenylate kinase system. Neither served as a substrate when substituted for AMP. The analogs were then individually added together with the substrates AMP and ATP, to ascertain whether either would alter the kinetics of the enzyme reaction.

A double-reciprocal plot (1/velocity *versus* 1/AMP) of the reaction kinetics[13] demonstrates the effect of 9-α-D-mannofuranosyladenine 5′-phosphate (**2**) on the reaction AMP+ATP⇌2ADP. The results, shown in Fig. 2, were obtained when 3.7 and 18.2mM 9-α-D-mannofuranosyladenine 5′-phosphate (**2**) was used. The slopes

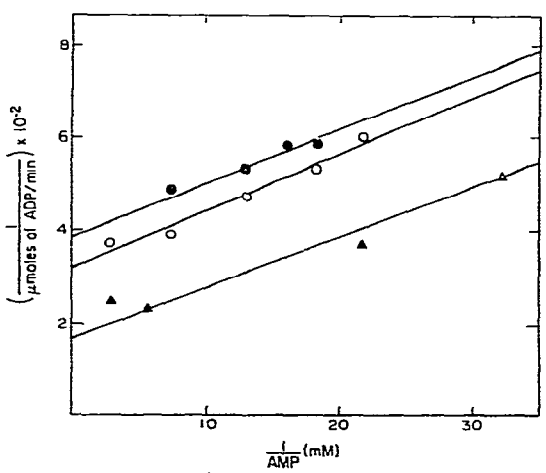

Fig. 2. A Lineweaver–Burk plot of the reverse reaction of adenylate kinase in the presence of 9-α-D-mannofuranosyladenine 5′-phosphate (no inhibitor, ▲; 3.7mM, ○; 18.2mM, ●).

obtained in the presence of the analog are parallel to the slope obtained without the analog, indicating noncompetitive inhibition.

Fig. 3 shows the effect of the second analog, namely, 9-α-D-mannofuranosyladenine 6′-phosphate (**4**) on the rate of the adenylate kinase reaction. Again, the slope in the double-reciprocal plot is independent of the analog when this is tested at the lower of the two concentrations (350 μM). At the higher concentration, 690 μM, the slope changes slightly. For the 6′-phosphate, the concentrations used were smaller by a log magnitude, and, at 100 μM, a change in slope was seen, whereas no change in slope was noted for the 5′-phosphate at ten times the concentration.

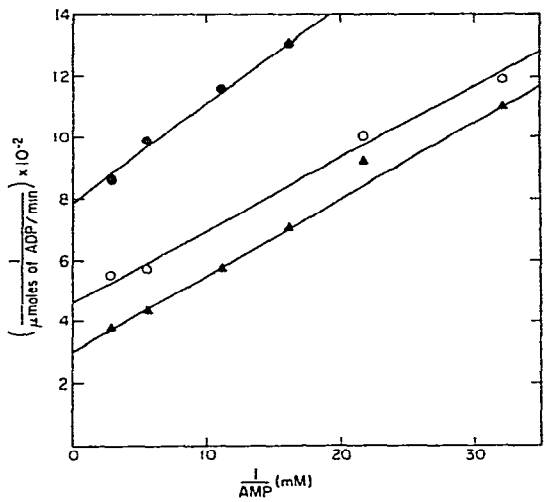

Fig. 3. A Lineweaver–Burk plot of the reverse reaction of adenylate kinase in the presence of 9-α-D-mannofuranosyladenine 6′-phosphate (no inhibitor, ▲; 350 μM, ○; 690 μM, ●).

For the control, AMP–ATP incubations, the K_m values were estimated to be 6.0×10^{-4} and 8.1×10^{-4}, in close agreement with 5.0×10^{-4} found by Callaghan and Weber[14], and 2.6×10^{-4} found by Noda[9].

DISCUSSION

The phosphorylation of 9-α-D-mannofuranosyladenine (**1**) and its 2′,3′-isopropylidene acetal (**3**) was studied by using phosphoryl chloride with trimethyl (or triethyl) phosphate as the solvent, according to the procedure of Yoshikawa *et al.*[2,3]. When the unprotected nucleoside **1** was phosphorylated, one major peak was obtained.

In contrast, two major and three minor peaks were obtained when the isopropylidene-protected nucleoside **3** was subjected to the same conditions of phosphorylation and the product hydrolyzed for 40 min at pH 1.5. The reaction is more complex than one involving mere phosphate migration. When the crystalline material from peak 5 was kept for 40 min at pH 1.5 and 60–70°, no component other than that of

peak 5 was seen on elution from a column of Dowex 1. When the crystalline material from peak 5 was kept at pH 1.5, for the same time at the same temperature, but in the presence of hydrolyzed phosphoryl chloride and trimethyl phosphate, only 3% of the material in peak 2 was formed. It appears, then, to be necessary to subject the protected nucleoside to the action of phosphoryl chloride at 0° in order to obtain a significant amount of the material in peak 2.

In Fig. 1, peak 2 is seen to be cleanly separated after a single rechromatography with the same elution system; it was identified in the following way. The position of elution corresponded to a monophosphate of a primary hydroxyl group, in analogy to the elution of 5′-AMP by Cohn[15]. A discrete zone was given with each of four different paper-chromatographic and each of two different t.l.c. systems. Hence, it may reasonably be assumed that a pure compound was isolated, as suggested by ultraviolet visibilization. The results of elemental analysis corresponded to the values calculated for a monophosphate containing 1.5 molecules of water per molecule. The molecular weight of the per(trimethylsilyl) derivative indicated the presence of six trimethylsilyl groups. A cyclic phosphate was thus eliminated from consideration, as it would acquire four trimethylsilyl groups[6]. ^{31}P n.m.r. spectroscopy showed a well defined triplet having a chemical shift of 108 p.p.m. in the orthophosphate region, indicating that the primary hydroxyl group had been phosphorylated. Hence, the compound eluted in peak 2 is the 6′-phosphate.

In analogy to the sugar phosphates[16], it was predicted that the 6′-nucleotide would be the compound eluted first, as the other monophosphates (2′, 3′, and 5′) are all capable of forming a seven-membered ring involving the hydroxyl group of the phosphate group with the ring-oxygen atom. Such hydrogen-bonded structures would have a decreased ability to dissociate and, hence, would be eluted from the column after any compounds unable to form such stable, cyclic structures.

The sole peak arising from the phosphorylation of the unprotected nucleoside **1** chromatographed in the same position as the major peak (peak 5) arising from the phosphorylation of the protected nucleoside **3**. The position of elution corresponded to a monophosphate formed from a secondary hydroxyl group, in analogy to the elution[15] of 2′- and 3′-AMP. Only one product was seen in paper chromatography with four different solvent-systems, and in t.l.c. with two different solvent-systems. As with the phosphate in peak 2, the results of elemental analysis and the formation of a hexakis(trimethylsilyl) derivative indicated that it was a monophosphate. ^{31}P n.m.r. spectroscopy showed a doublet, in the orthophosphate region, having a chemical shift of 107.7 p.p.m.; this corresponds to a split by one proton, and, hence, could be the spectrum of the hexakis(trimethylsilyl) derivative of a 2′-, 3′-, or 5′-phosphate. When the 2′- and 3′-hydroxyl groups were protected by isopropylidenation, the compound in peak 5 was still the major product of the reaction. It is probable that peaks 3 and 4 contain products phosphorylated at O-2 and O-3, formed after hydrolysis of the isopropylidene group. As the compound in peak 2 was identified as the 6′-phosphate (by n.m.r. spectroscopy), the assignment of peak 5 to the 5′-phosphate is plausible.

It is interesting that the exocyclic, secondary hydroxyl group was mainly phosphorylated. Although the reactions are not strictly analogous, our results contrast with the selective systems of Yoshikawa *et al.*[2,3] and Honjo *et al.*[17], wherein the primary hydroxyl group was almost quantitatively phosphorylated. Barker and Foll[18], however, reported formation of adenosine 2′- and 3′-phosphate as the major products on phosphorylation with phosphoryl chloride–aqueous barium hydroxide. Hence, both the mechanism of selectivity of the triethyl and trimethyl phosphates and the physical properties of the hexofuranosyl nucleotides need further investigation.

When 9-α-D-mannofuranosyladenine 5′-phosphate (**2**) was tested in the adenylate kinase system, a double-reciprocal plot revealed no change in slope in its presence. As both the K_m and V_{max} values are proportionally changed, this type of mixed inhibition may be designated uncompetitive. In the more general case, the inhibitor affects the breakdown of the enzyme–substrate complex, with K_m equal to the velocity constant of the formation of the enzyme–inhibitor complex divided by the velocity constant of the formation of the enzyme–substrate complex[19]. The less general case implies the inability of the enzyme–substrate complex to break down[20].

The second analog, 9-α-D-mannofuranosyladenine 6′-phosphate (**4**), also shows a simultaneous change in the values of both K_m and V_{max} when tested at the lower concentration of 350 μM. Hence, at this concentration, uncompetitive inhibition was again observed. When this concentration is doubled, however, such proportional changes are not seen, as the slope is no longer parallel to that without analog. Hence, the inhibitory effect on K_m is lessened, and the slope begins to approach that given by a noncompetitive inhibitor. As the 6′-phosphate (**4**) shows a tendency for the influence of K_m on its inhibition to be eliminated at a concentration lower than that for the 5′-phosphate (**2**), the former may be regarded as the more potent inhibitor. These results suggest that the 6′-phosphate **4** might be of value in biological systems as an inhibitor of metabolic pathways of nucleotides.

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