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Phosphonylation of Biomolecules with Inorganic Diphosphonate. III.[1] Formation of an H-phosphonate Analogue of Adenosine 5′-Triphosphate by Phosphonylation of Adenosine 5′-Diphosphate

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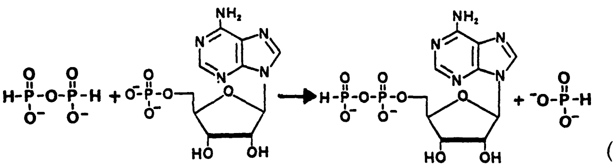
Phosphonylation of adenosine 5′-diphosphate (ADP) with inorganic diphosphonate was investigated by use of ^{31}P NMR spectroscopy and the coupled system of high-performance liquid chromatography and flow injection analysis. ADP was phosphonylated by inorganic diphosphonate to form an H-phosphonate analogue of adenosine 5′-triphosphate (ATP) in aqueous solutions. The yield of the H-phosphonate analogue of ATP varied strongly depending on reaction conditions: pH and initial concentration of inorganic diphosphonate. The maximum yield of the H-phosphonate analogue of ATP was 56% under the following conditions: initial concentration of inorganic diphosphonate and ADP were 1.8 and 0.1 mol dm^{-3}, respectively; 50 °C; pH 5; 90 min. In basic solutions, 2′ or 3′-hydroxyl group on ADP was predominantly phosphonylated.

More recently much interest has been focused on an H-phosphonate approach for the development of new and efficient methods of synthesizing DNA and RNA,[2–4] nucleoside–phospolipid conjugates,[5a] and internucleotide phosphate analogues[5b] which are widely used as metabolic regulators.[6] The increasing significance of H-phosphonate approach has also stimulated further research in organic synthesis. The application of H-phosphonate approach, however, has been limited to the syntheses of monophosphate analogues, such as nucleoside 3′-H-phosphonate.

One of our current projects is the synthesis of H-phosphonates involving a P–O–P linkage as analogues of nucleoside polyphosphates, the analogues which become new important probes for the mechanistic studies on enzymatic reactions. We have recently reported that the phosphonylation of nucleoside 5′-monophosphate such as adenosine 5′-monophosphate (AMP) using a new phosphonylating agent, inorganic diphosphonate (P^{III}–O–P^{III}), gave an H-phosphonate analogue of nucleoside 5′-diphosphate such as adenosine 5′-diphosphate (ADP) in aqueous solutions according to Eq. 1.[1,7,8]



(1)

P^{III}–O–P^{III} AMP P^{III}

This method using P^{III}–O–P^{III} is extended in the present paper to phosphonylation of ADP to obtain an H-phosphonate analogue of adenosine 5′-triphosphate (ATP).

Experimental

Chemicals. Unless otherwise stated, guaranteed reagents from Wako (Osaka, Japan) were used without further purification. Disodium adenosine 5′-diphosphate dihydrate was purchased from Oriental Yeast (Tokyo, Japan).

Disodium inorganic diphosphonate, $Na_2P_2H_2O_5$, was prepared according to the previous paper.[1] The purity of disodium diphosphonate thus obtained was checked by HPLC[9–11] to be 98% (as P).

Phosphonylation of ADP with Diphosphonate. Initial concentration of diphosphonate varied from 0.5 to 1.8 mol dm^{-3}, keeping the initial concentration of ADP at 0.1 mol dm^{-3} constant. The pH of aqueous reaction mixture was ca. 5. If necessary, the reaction mixture was adjusted to the prescribed pH value (10, 8, 7, 6, or 4) with a sodium hydroxide or hydrochloric acid solution. The reaction mixtures with and without pH adjustment were allowed to react at 50, 60, and 70 °C. The pH change during the reactions was not controlled strictly.

HPLC Measurement. Two HPLC systems were used in the present study: 1) an HPLC system with UV-detector for determination of ADP and products; 2) an HPLC system coupled with a flow injection analysis (FIA) designed for the sensitive detection of phosphate and phosphonate groups.[1,9–13]

A TRI ROTAR VI system (Jasco, Tokyo, Japan) was used as an HPLC system with UV-detector. An aliquot (0.1 ml) of reaction mixture was injected onto a separation column (250×4.6 mm I.D.). Two kinds of anion-exchange columns were used: 1) an MCI column (MCI Gel CDR-10, 7 μm, Mitsubishi Chemical Industry, Tokyo, Japan) in Fig. 1; 2) a TSK column (TSKgel SAX, 10 μm, TOSOH, Tokyo, Japan) in Figs. 2 and 3. Flow rate was 1.0 ml min^{-1} and column temperature was kept at 40 °C. Separated ADP and products were detected at 260 nm by use of UV spectrophotometer (UVIDEC-340, Jasco, Tokyo, Japan). Eluent for the MCI column was composed of 0.4 mol dm^{-3} ammonium sulfate, 0.2 mol dm^{-3} potassium dihydrogenphosphate, 0.2 mol dm^{-3} dipotassium hydrogenphosphate, and 4% (V/V) acetonitrile (pH 6.6). Eluent for the TSK column was composed of 0.3 mol dm^{-3} potassium chloride and 0.1% (W/V) tetrasodium ethylenediaminetetraacetate (pH 10).

To detect ADP, diphosphonate, phosphonate, and products simultaneously, an FIU-300 FIA system (Jasco, Tokyo, Japan) was coupled with the TRI-ROTAR VI HPLC system. The separation was performed on the TSK column. Flow rate, column temperature, and eluent were the same as described above. Phosphate and phosphonate groups on separated reactants and products were monitored by the on-line coupled FIA system.[1,9–13] A molybdenum(V)–molybdenum(VI) reagent[12] was used as a chromogenic agent. To oxidize phosphonate to detectable phosphate, sodium hydrogensulfite was introduced into the FIA system.[9–11]

^{31}P NMR Measurement. Pulse FT ^{31}P NMR spectra were recorded at room temperature by use of Varian XL-VXR300 (121 MHz) and Varian XL-200 (81.0 MHz). Orthophosphoric acid (85%) was used as an external standard.

Results and Discussion

Characterization of Phosphonylated Products by HPLC. A chromatographic peak (A) in Fig. 1 for a phosphonylated product appeared after 5 min incubation of ADP (0.1 mol dm^{-3}) with P$^{\mathrm{III}}$–O–P$^{\mathrm{III}}$ (1.8 mol dm^{-3}) at 70 °C and pH 5 in an aqueous solution. Area of the peak (A) increased gradually to a maximum value, 55%, after 15 min incubation and then decreased slowly. The retention time for (A) was 16.4 min measured by the HPLC system with UV-detector using the MCI column. No peaks for other phosphonylated products were observed by the HPLC measurements. A small peak for adenosine 5′-monophosphate (AMP) which would be formed by hydrolysis of ADP was also recorded.

The peak (A) for the phosphonylated product involves both phosphate and phosphonate groups as shown in Fig. 2b, because the solid line represents the sum of the absorbances (830 nm) due to phosphate and phosphonate groups and the dotted line represents the absorbance (830 nm) due to phosphate group measured by the HPLC system coupled with the FIA using the TSK column. The peak (A) (Fig. 2a) measured by use of the HPLC system with UV-detector using the TSK column was also observed at the same retention time as that of the peak (A) in Fig. 2b. The retention time (35.4 min) for (A) measured by use of the TSK column (Fig. 2) was longer than that for (A) measured by use of the MCI column (Fig. 1).

Three chromatographic peaks (Fig. 3) for two main

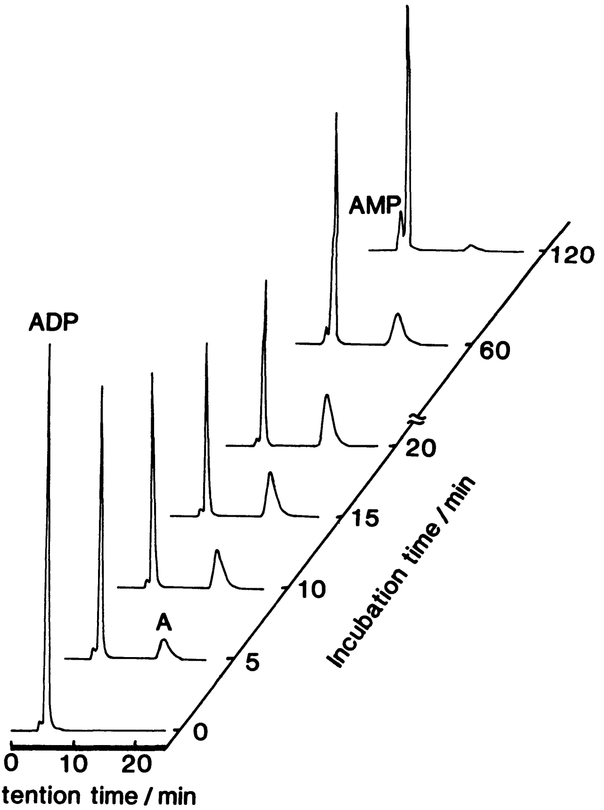


Fig. 1. Kinetic HPLC profile for phosphonylation of ADP (0.1 mol dm^{-3}) with inorganic diphosphonate (1.8 mol dm^{-3}) at 70 °C and pH 5. Column: the MCI anion-exchange column. Eluent: 0.4 mol dm^{-3} $(NH_4)_2SO_4$, 0.2 mol dm^{-3} KH_2PO_4, 0.2 mol dm^{-3} K_2HPO_4, and 4% CH_3CN (pH 6.6). Wavelength: 260 nm.

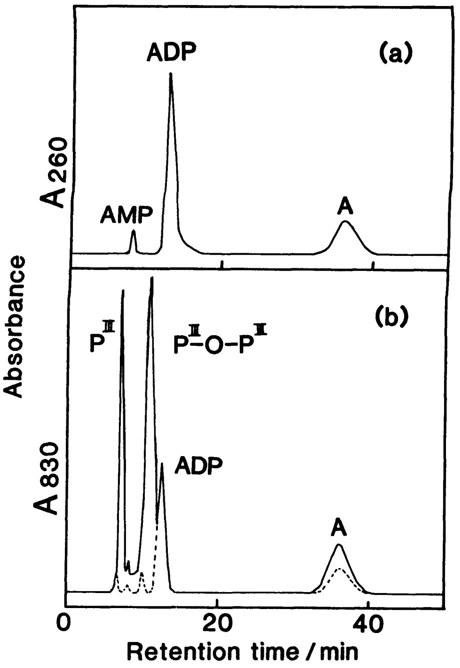


Fig. 2. HPLC profiles for ADP (0.1 mol dm^{-3}) phosphonylated by inorganic diphosphonate (1.0 mol dm^{-3}) at pH 5 and 70 °C for 50 min. Column: the TSK anion-exchange column. Eluent: 0.3 mol dm^{-3} KCl and 0.1% EDTA 4Na (pH 10). Wavelength: 260 nm(a) and 830 nm(b). The solid line in (b) indicates the sum of the absorbances due to both phosphate and phosphonate groups and the dotted line indicates the absorbance due to only a phosphate group.

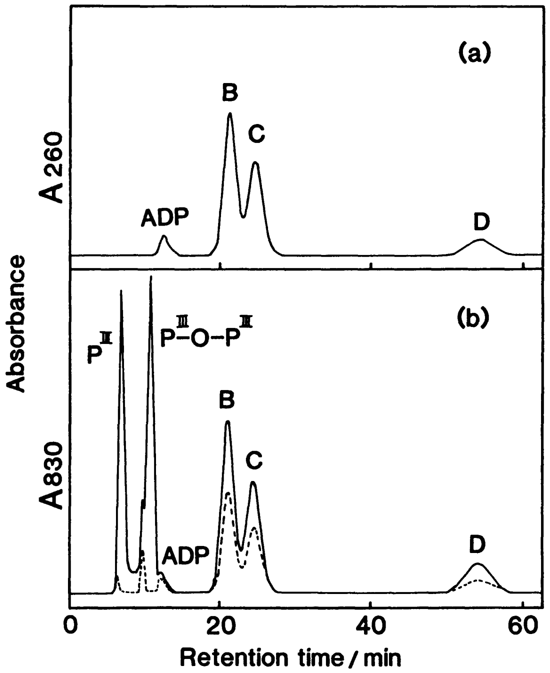


Fig. 3. HPLC profiles for ADP (0.1 mol dm^{-3}) phosphonylated by inorganic diphosphonate (1.0 mol dm^{-3}) at pH 10 and room temperature for 30 min. Other conditions are the same as in Fig. 2.

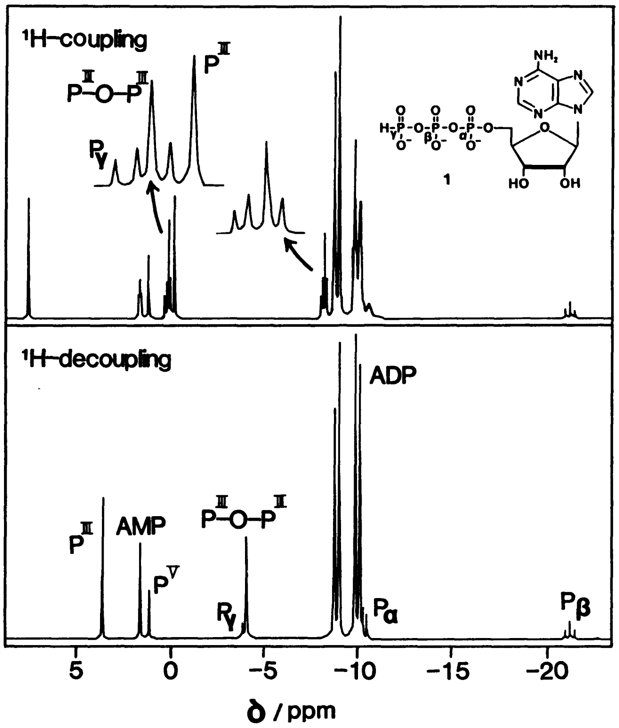


Fig. 4. ^{31}P NMR spectra for ADP (0.5 mol dm^{-3}) phosphonylated by inorganic diphosphonate (0.1 mol dm^{-3}) at pH 5 and 70 °C for 90 min.

products (B and C) and a by-product (D) were observed for the reaction mixture incubated at pH 10 by use of the same HPLC systems in Fig. 2. Although these peaks also involve phosphate and phosphonate groups as shown in Fig. 3b, retention times of these peaks are different from that of peak (A). This shows that in an alkaline solution different products are formed.

Structure of Phosphonylated Products by ^{31}P NMR. To establish the structure of phosphonylated products, ^{31}P NMR spectra were recorded. Figure 4 shows ^{31}P NMR spectra for the reaction mixture of ADP (0.5 mol dm^{-3}) and P^{III}-O-P^{III} (0.1 mol dm^{-3}) incubated at pH 5 and 70 °C for 90 min.

A proton-decoupled spectrum (Fig. 4) represents two unknown doublets (−4.1 and −10.4 ppm) and an unknown triplet (−21.2 ppm) in addition to the signals assigned to phosphonate (P^{III}; 3.5 ppm), AMP (1.5 ppm), orthophosphate (P^{V}; 1.0 ppm), P^{III}-O-P^{III} (−4.2 ppm), and ADP (two doublets at −9.0 and −10.0 ppm) on the basis of the chemical shifts for the authentic samples. These unknown peaks are the signals for the phosphonylated product observed by the HPLC measurement in Figs. 1 and 2. AMP and P^{V} would be formed by hydrolysis of ADP in the course of reaction.

The triplet at −21.2 ppm is the most important signal to characterize the chemical structure of the phosphonylated product, because the triplet at around −20 ppm is caused by the middle-group phosphorus on triphosphate species such as ATP[14,15] and inorganic triphosphate.[16,17] In addition, coupling constant (19.0 Hz) of the triplet is approximately equal to that (19.3 Hz) of ATP.[15] We concluded that, as shown in Fig. 4, product **1** similar in structure to ATP (H-phosphonate analogue of ATP) was fromed by phosphonylation of ADP with P^{III}-O-P^{III} at pH 5. The triplet is assigned to middle phosphorus atom (P_β of **1**). Other unknown doublets at −4.1 and −10.4 ppm would be signals for P_γ and P_α atom of **1**. A signal of the doublet at −4.1 ppm overlapped with that of P^{III}-O-P^{III}.

The doublet at −10.4 ppm is further split into a double-triplet in a proton-coupled spectrum (Fig. 4). The double-triplet is assigned to P_α atom of **1**, because the double-triplet shows a splitting of 4.5 Hz, the value of which is similar to the three-bond coupling constant ($^{3}J_{PH}$) for P_α atom on ATP split by the 5′-hydrogens of the ribose moiety.[16]

Further splitting of the doublet at −4.1 ppm into a double-doublet is shown in a proton-coupled spectrum. The double-doublet is assigned to P_γ atom of **1** for phosphonyl group transferred from P^{III}-O-P^{III} to ADP, because the double-doublet shows the splitting of 669.1 Hz for the hydrogen atom attached directly to a phosphorus atom.[1,18]

The ^{31}P NMR spectra (Fig. 5) for the reaction mixture adjusted to pH 10 differ from those observed at pH 5. A proton-decoupled spectrum shows four singlets and three doublets. The singlets at 6.3 and

−1.8 ppm are assigned to P^{III} and P^{III}-O-P^{III}, respectively. Two unknown singlets (E and F) and three unknown doublets are the signals for the main phosphonylated products observed in Fig. 3.

In a proton-coupled spectrum, both singlets (E and F) are split into double-doublets. These double-doublets are assigned to the signals for compounds **2** and **3** (Fig. 5) producing the phosphonylation of 2′- or 3′-hydroxyl group, because each double-doublet shows one-bond coupling constant ($^1J_{PH}$=650 Hz) and three-bond coupling constant ($^3J_{PH}$=10 Hz).[1] Doublet at −7.1 ppm is then assigned to P_α of these compounds and two doublets at −2.4 ppm are assigned to P_β of these compounds. ^{31}P NMR spectrum of a by-product could not be observed, owing to a low yield.

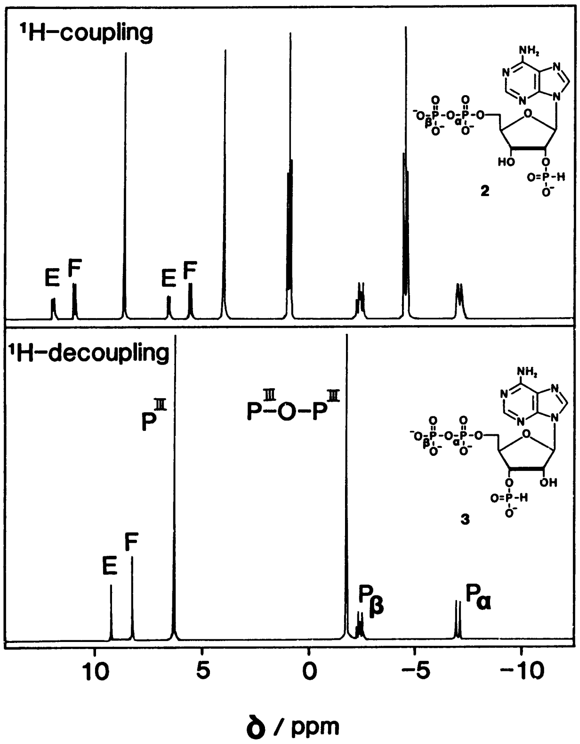


Fig. 5. ^{31}P NMR spectra for ADP (0.1 mol dm^{-3}) phosphonylated by inorganic diphosphonate (0.5 mol dm^{-3}) at pH 10 and room temperature for 20 min.

Effect of pH on the Phosphonylation of ADP. The product **1** was predominantly formed at pH 5 as shown in Figs. 1 and 6. The amount of the product **1** increased gradually with time at pH 5 as shown in Fig. 6. The yield of **1** reached a maximum (about 40%) at 120 min, and then slowly decreased.

In the reaction at pH 10, very little amount of **1** was formed, so that the datum for **1** was omitted in Fig. 7. The amount of both products **2** and **3** showed a gradual increase with time, reaching a maximum (about 74%) after 60 min. The maximum amount remained constant at least after 100 min incubation. The unknown by-product was formed in the amount of ca. 20% after 240 min.

Table 1 which lists the yields of products **1**, **2**, and **3** in the pH 4 to 10 region shows that the yield of product **1** at pH 6 is higher than those at any other pH's. To get product **1** selectively without impurities of **2** and **3**, however, ADP should react in more acidic solu-

Table 1. Yields[a] of Phosphonylated Products in the pH Region from **4** to **10**

pH	Yield/%		Time/min
	1	**2+3**	
4	27	0	80
5	40	0	120
6	45	7	100
7	37	11	100
8	21	30	40
10	0	74	60

a) Reaction conditions: initial concentrations of ADP and P^{III}-O-P^{III} were 0.1 mol dm^{-3} and 1.0 mol dm^{-3}; 60 °C.

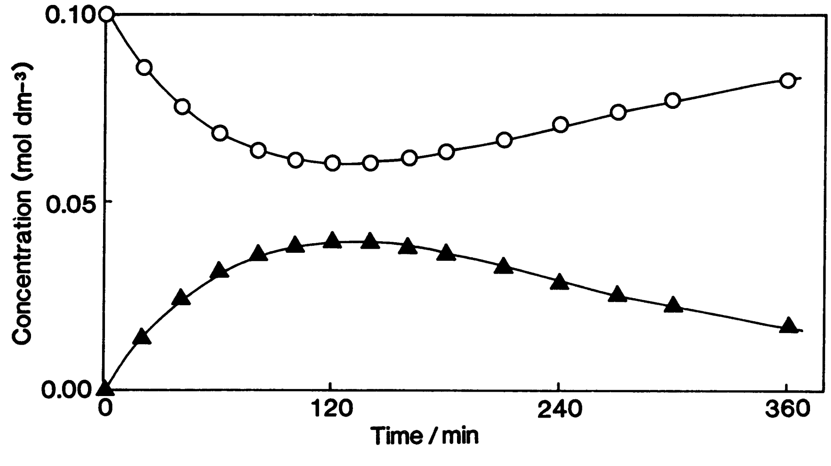


Fig. 6. Time dependence of the reaction components of phosphonylation for ADP (0.1 mol dm^{-3}) with inorganic diphosphonate (1.0 mol dm^{-3}) at pH 5 and 60 °C. Plots of the observed molar concentrations of ADP (○) and product **1** (▲) vs. time.

tions, because small amount of **2** and **3** was formed at pH 6.

Products **2** and **3** were formed in alkaline solutions more favorably than in acidic solutions. For example, the yield of **2** and **3** at pH 6 was only 7%, while the yield reached a maximum value of 74% at pH 10. The results show that the hydroxyl group at 2′- or 3′-position becomes reactive by dissociation of a proton in a basic solution, because such hydroxyl groups have very large protonation constant more than 12 in log scale.[19] Phosphonylation of hydroxyl groups will be described in more detail in a subsequent paper of this series.

It is interesting that the yields of these products strongly depend on pH of reaction solution. The pH dependence can be explained as follows: the degradation of products is in competition with the formation of products. In acidic and neutral solutions, diphosphate group on ADP reacts with P^{III}-O-P^{III} to form product **1**, which is relatively stable as shown in Fig. 6. The hydroxyl groups at 2′- and 3′-positions are protonated in acidic and neutral solutions, so that the hydroxyl groups do not react with P^{III}-O-P^{III}. On the other hand, in basic solution product **1** is very unstable and undergoes rapid hydrolysis to ADP and phosphonate in spite of the formation of **1** from ADP and P^{III}-O-P^{III}. A hydroxyl group is deprotonated in a basic solution, consequently, a deprotonated hydroxyl group reacts with P^{III}-O-P^{III} to form products **2** and **3**, which are very stable even in a basic solution as shown in Fig. 7. To provide further support for the proposed reaction pathway, we carried out an additional ^{31}P NMR experiment by addition of sodium hydroxide solution to the reaction mixture shown in Fig. 4. As a result, the NMR signals for **1** disappeared rapidly and the signals for **2** and **3** appeared.

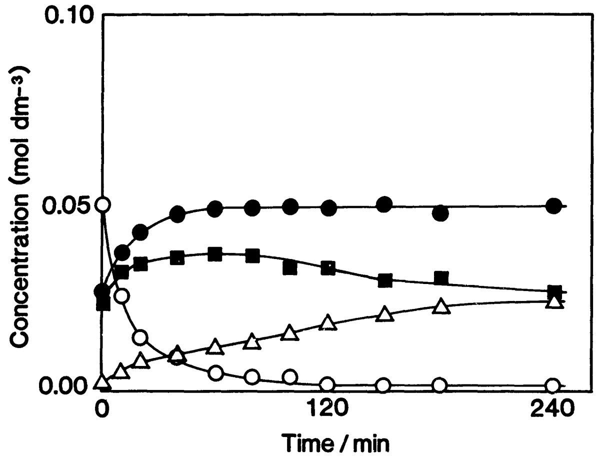


Fig. 7. Time dependence of the reaction components of phosphonylation for ADP (0.1 mol dm^{-3}) with inorganic diphosphonate (1.0 mol dm^{-3}) at pH 10 and 50 °C. Plots of the observed molar concentrations of ADP (○) and product **2** and **3** (●, ■), and unknown product (△) vs. time.

Effects of Initial Concentrations of Diphosphonate and Temperature on Yield of Product 1. As shown in Fig. 8, phosphonylation of ADP gave H-phosphonate analogue of ATP (**1**) at pH 5 selectively, while 2′- or 3′-hydroxyl group was phosphonylated at pH 10. The yields of **1** were measured at pH 5 varying initial concentration of P^{III}-O-P^{III} and temperature to find the optimal conditions for phosphonylation of ADP.

Yield of **1** (Table 2) increased with increasing initial concentration of P^{III}-O-P^{III}: from 28% (0.5 mol dm^{-3}) to 54—56% (1.8 mol dm^{-3}). Yield was found to be maximum at initial concentration of 1.8 mol dm^{-3} in the temperature range studied here. The solution of P^{III}-O-P^{III} is saturated at 1.8 mol dm^{-3}. No improvements of the yields were observed with an increase in temperature, in spite of an decrease in time required to

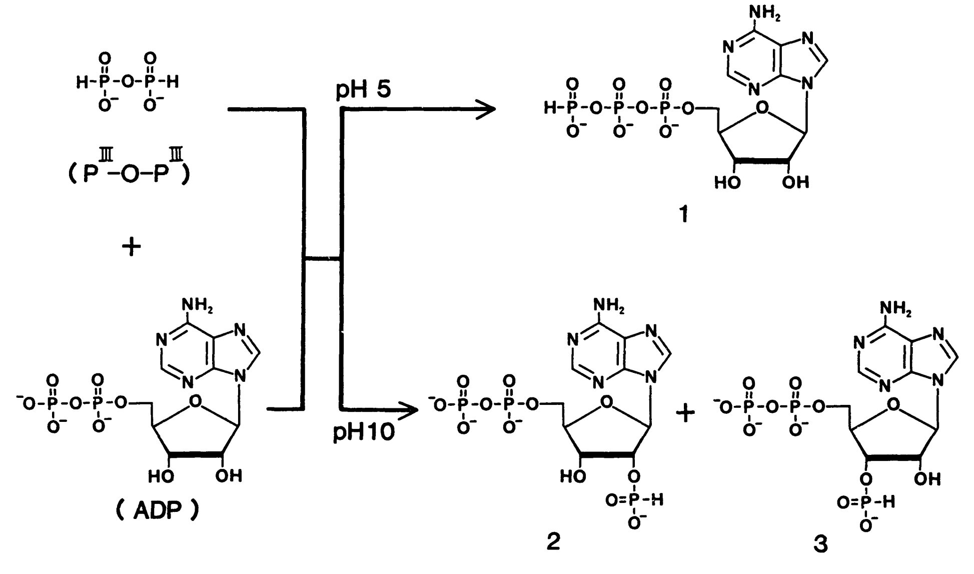


Fig. 8. Reaction scheme for phosphonylation of ADP with P^{III}-O-P^{III} at pH's 5 and 10.

Table 2. Phosphonylation of ADP with Diphosphonate at pH 5

Reaction conditions				Yield of **1**/%
Molarity/mol dm^{-3}		Temp/°C	Time/min	
P$^{\mathrm{III}}$–O–P$^{\mathrm{III}}$	ADP			
0.5	0.1	40	1680	28
		50	420	28
		60	240	28
		70	140	28
1.0	0.1	40	420	42
		50	200	41
		60	120	40
		70	50	41
1.5	0.1	40	330	51
		50	120	51
		60	60	50
		70	30	48
1.8	0.1	40	210	55
		50	90	56
		60	30	54
		70	15	55

reach a maximum amount. The optimal conditions for phosphonylation of ADP with P$^{\mathrm{III}}$–O–P$^{\mathrm{III}}$ were found to be initial concentration of P$^{\mathrm{III}}$–O–P$^{\mathrm{III}}$ 1.8 mol dm^{-3}, pH 5, and 50 °C.

The overall tendencies of the results are similar to those obtained in the phosphonylation of AMP,[1)] except that the yield of **1** is smaller than that of H-phosphonate analogue of ADP (ca. 70%).[1)] The difference in yield between ATP analogue and ADP ananlogue is attributalbe to the different negative charges on nucleophiles, ADP and AMP, because more negatively charged ADP is repulsed and slowed down to higher degree in attacking dianionic P$^{\mathrm{III}}$–O–P$^{\mathrm{III}}$.

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