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# Nucleophilic Substitution Reactions between Diphosphonate and Orthophosphate Characterized by High-Performance Liquid Chromatography and $^{31}P$ NMR Spectroscopy

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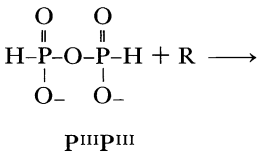
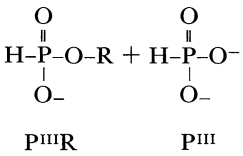
Inorganic diphosphonate with phosphorus of an oxidation number +3 was found to react with orthophosphate (oxidation number +5) at 50 °C to give phosphonylated compounds; dimeric isohypophosphate (oxidation numbers +3 and +5) and a novel trimeric compound (oxidation numbers +3 and +5). Orthophosphate ion is considered to act as a ligand with two or three nucleophilic sites that were phosphonylated stepwise by diphosphonate. Kinetic processes of the nucleophilic reactions were monitored by HPLC and $^{31}P$ NMR. Two products, the dimer having one P–H bond and the trimer with two P–H bonds, were well-resolved by HPLC and their yields, based on orthophosphate applied, were 85% (dimer) and 3% (trimer) when a mixed solution of 0.5 M diphosphonate and 0.1 M orthophosphate was incubated at 50 °C for 21 h. A complicated $^{31}P$ NMR spectrum that indicated a large coupling constant($J_{PH}$=650 Hz) and grew with incubation time is reasonably assigned to the unsymmetrical isohypophosphate composed of both a phosphate group and a phosphonate group. Apparent formation constants of the dimer and trimer were evaluated.

Inorganic diphosphonate,[1,2] $P^{III}P^{III}$, a dimeric form of phosphonate(phosphite), $P^{III}$, has been characterized to be very reactive as an acceptor in a nucleophilic substitution reaction in Eq. 1 and its wide applicability in phosphonylating various nucleophilic biomolecules and inorganic phosphorus compounds(R) has been demonstrated.[3–11]



$P^{III}P^{III}$

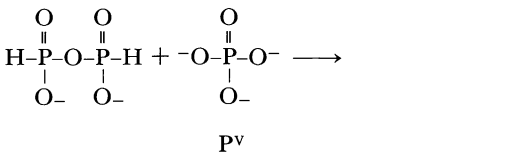


(1)

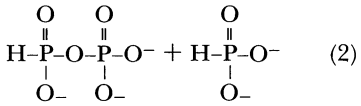
$P^{III}R$ $P^{III}$

Each phosphorus compound was expressed by an abbreviated nonation based on the arrangement of structural constituents; phosphonyl group with a P–H bond, P(III), and/or phosphoryl group, P(V), and was shown for convenience in a dissociated form.

In the nucleophilic reaction in Eq. 1 one of phosphonyl groups in diphosphonate transfers to R to form a phosphonylated compound, $P^{III}R$, as exemplified by the phosphonylation of ADP which resulted in the formation of an ATP analogue having a terminal phosphonyl group, $P^{III}P^{V}P^{V}A$.[9] If orthophosphate ion, $P^{V}$, is used as R, isohypophosphate, $P^{III}P^{V}$, is expected to be formed according to Eq. 2 liberating phosphonate.



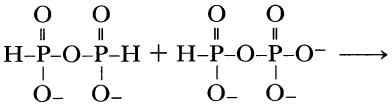
$P^{V}$

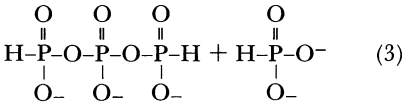


(2)

$P^{III}P^{V}$

Since orthophosphate ion is a ligand with three nucleophilic sites further addition of phosphonyl groups to the $P^{III}P^{V}$ is also expected to produce a trimeric compound in Eq. 3 and, with less probability, a branched compound with three phosphonyl groups, $P^{III}P^{V}(P^{III})P^{III}$.





(3)

$P^{III}P^{V}P^{III}$

Attempts to characterize the substitution reactions in Eqs. 2 and 3 by gel chromatography had been made in our laboratory.[3,4] Analytical methodologies had been proposed capable of resolving and identifying such reaction products that were complex in structure and included unknown species. A preliminary experiment

with HPLC[5] and detailed experiments to give theoretical discussion about kinetics and mechanisms of the substitution reactions[6] have been reported. Baba's works[6] with advanced techniques, HPLC and $^{31}P$ NMR, presented more improved chromatographic resolution and structural diagnosis of reaction products. The present paper was undertaken to examine the usefulness and reliability of HPLC and $^{31}P$ NMR in practical synthetic experiments with diphosphonate. In accordance with the earlier observations these techniques were confirmed to be valuable for visualizing the reaction processes in Eqs. 2 and 3.

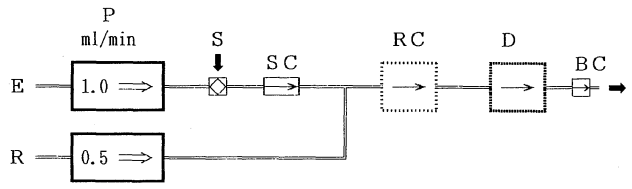



Fig. 1. HPLC system for the selective determination of P(V)-containing compounds. <P> Pump, <S> Sample injector, <SC> Separation column (4 mm I.D.×25 cm, TSK-gel SAX), <RC> Reaction coil (0.5 mm I.D.×20 m, 140 °C), <D> Spectrophotometric detector (820 nm, 650 nm), <BC> Back-pressure coil (0.3 mm I.D.×2 m), <E> Eluent, 0.25 M KCl+0.1% EDTA(4Na), <R> Mo(V)-Mo(VI) reagent.

## Experimental

**Chemicals.** Disodium diphosphonate, $Na_2P_2H_2O_5$, was prepared according to the previous paper.[8] Other chemicals were guaranteed reagents from Wako (Osaka, Japan).

**HPLC Measurement.** Detailed procedures used for the analysis of phosphorus compounds by employing a high-temperature detection system (Fig. 1) have been shown in the previous papers.[7-11]

**NMR Measurement.** $^{31}P$ NMR measurement was made by use of a JEOL JNM-GX-400 (161.8 MHz) spectrometer. Orthophosphoric acid (85%) was used as an external standard.

## Results and Discussion

**Separation by HPLC.** In order to monitor phosphorus compounds in Eqs. 2 and 3 the HPLC system in Fig. 1 was employed. The system consisted of an anion-exchange separation column (TSK-gel SAX) and a post-column detection system using a Mo(V)-Mo(VI) reagent.[7-11] The detection system was able to permit the selective detection of P(V)-units in the presence of P(III)-units. Hence, $P^V$ and $P^{III}P^V$ in Eq. 2 were detectable, while $P^{III}P^{III}$ and $P^{III}$ did not indicate HPLC peaks.

A mixed solution composed of 0.5 M diphosphonate ($Na_2P_2H_2O_5$, M=mol dm$^{-3}$) and 0.1 M orthophosphate ($Na_2HPO_4$) was allowed to stand at 50 °C for 24 h.

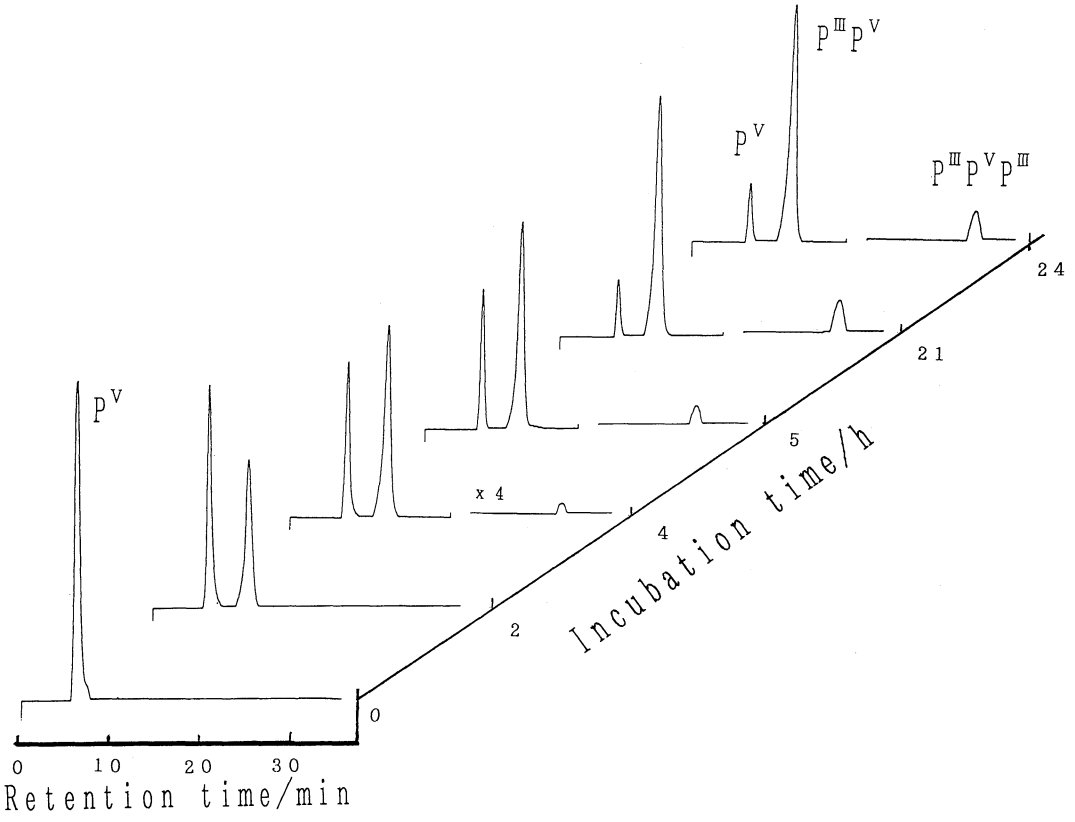



Fig. 2. HPLC profiles indicating progressive phosphonylation of orthophosphate by diphosphonate at 50 °C. <Sample> A mixed solution of 0.1 M $P^V$ and 0.5 M $P^{III}P^{III}$.

The pH of the sample solution decreased with the progress of substitution reactions, from initial pH value ca. 7 to final ca. 5.5, because any buffer agents were not used to avoid their side reactions with diphosphonate. Aliquots of the incubated solution were 100-fold diluted and analyzed by HPLC at desired time-intervals to observe the progressive conversion of phosphorus compounds in Eqs. 2 and 3. As shown in Fig. 2 the peak height for $P^{III}P^{V}$ increased with time, in contrast to the decrease of the peak height for the starting material, $P^{V}$. The peaks for $P^{III}P^{V}P^{III}$ in Eq. 3 were recorded with 4-fold amplification after 4 h incubation. In should be noted that the distributions of $P^{III}$ and $P^{III}P^{III}$ were confirmed by a separate detection method based on the preoxidation of P(III) to P(V) and the chemical compositions of $P^{III}P^{V}$ and $P^{III}P^{V}P^{III}$ were determined by differential analyses of P(III) and P(V) as shown in the previous papers.[3–7]

Since P(III) unit is not to be detected with this spectrophotometric detection method and each species in Fig. 2 includes one detectable P(V) unit per molecule, the relative peak areas of $P^{V}$, $P^{III}P^{V}$, and $P^{III}P^{V}P^{III}$, calculated by a data processor Shimadzu CR1-B, can be used to represent the relative molar concentrations of the three species. About 50% of the initial $P^{V}$ was phosphonylated at 2 h to form $P^{III}P^{V}$. The relative amounts in molar concentration of $P^{V}$(12%), $P^{III}P^{V}$(85%), and $P^{III}P^{V}P^{III}$(3%) tended to become unchanged at 21—24 h, suggesting the attainment of equilibrium or steady states[8,9] of the substitution reactions.

The growing rates of the three peaks were dependent on the initial reactant concentrations. With decreased initial sample concentrations, 0.1 M diphosphonate and 0.1 M orthophosphate, the relative amount of $P^{III}P^{V}$ increased more slowly than that in Fig. 2 to attain 42% after 22 h and no peak for $P^{III}P^{V}P^{III}$ was observed. On the other hand, when the initial sample concentrations were increased to 0.25 M orthophosphate and 1.25 M diphosphonate, keeping the same molar ratio of the reactants as in Fig. 2, both peaks of $P^{III}P^{V}$ and $P^{III}P^{V}P^{III}$ appeared as soon as two reactants were mixed. After 3 h the reactions attained to the distribution equilibrium of three species; $P^{III}$(8%), $P^{III}P^{V}$(78%) and $P^{III}P^{V}P^{III}$(14%).

**Characterization by $^{13}$P NMR.** The NMR spectra to indicate the progress of substitution reactions in Eqs. 2 and 3 are shown in Fig. 3. The sample

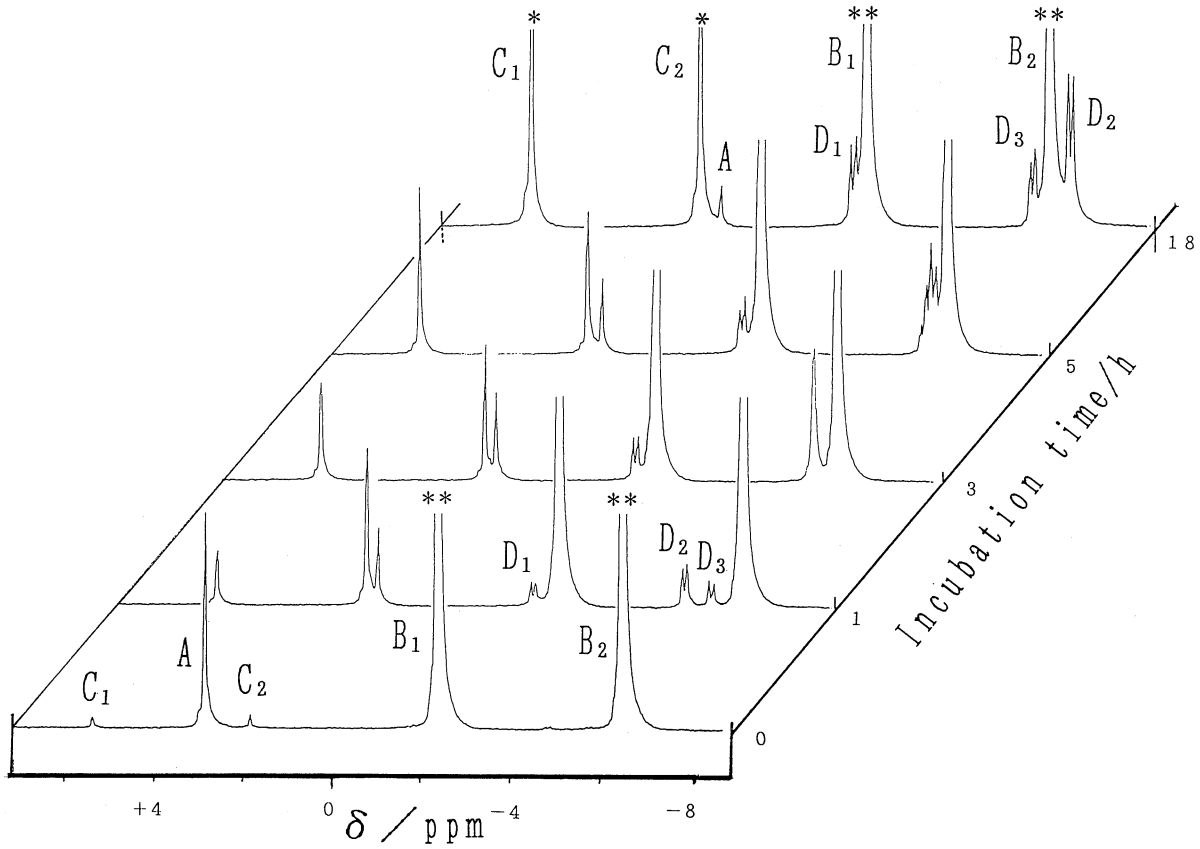



Fig. 3. $^{31}$P NMR spectra showing progressive phosphonylation of orthophosphate by diphosphonate at 50 °C. <Sample> A mixed solution of 0.1 M $P^{V}$ and 0.5 M $P^{III}P^{III}$. <Assignment> Signal A for $P^{V}$; $B_1$ and $B_2$ for $P^{III}P^{III}$; $C_1$ and $C_2$ for $P^{III}$; $D_1$, $D_2$, and $D_3$ for $P^{III}P^{V}$. Single asterisk (*) on the cut peaks of $P^{III}$ refers to singlet and double asterisk (**) of $P^{III}P^{III}$ indicates "doublet" that is apparently observed as a doublet, but has been more finely resolved to be a triplet (double triplet for $P^{III}P^{III}$; Refs. 9 and 12).

composition, 0.1 M $P^V$ and 0.5 M $P^{III}P^{III}$, was the same as that in Fig. 2. If the reactants and products are composed of P(III) units and/or P(V) units, it is useful in assigning the NMR spectra to keep in mind two general characteristics[2,6-8,11-14] arising from the P–H coupling and the pH dependence of chemical shift: (1) P(V) group gives a singlet signal or a multiplet with small peak splitting, ca. 18 Hz, due to P–P coupling and its chemical shift ($\delta$ value) shifts to the high field with decreasing pH, from the initial pH 7 to the final pH 5.5 in Fig. 3 and (2) P(III) group having a P–H bond gives a doublet signal with a coupling constant ($J_{PH}$ value) as large as ca. 600 Hz and its chemical shift is less dependent on pH. Additional small peak splittings due to P–P couplings are also expected to be observed for P(III) groups in polymers.

As shown in Fig. 3 a signal A for $P^V$($\delta$=2.84 ppm) and a pair of two signals, $B_1$ and $B_2$, for $P^{III}P^{III}$ ($\delta$=−4.46 ppm, $J_{PH}$=670 Hz) were observed as the principal signals when NMR spectra were recorded as soon as $P^V$ and $P^{III}P^{III}$ were mixed (ca. pH 7). A pair of small signals $C_1$ and $C_2$ are for $P^{III}$($\delta$=3.47 ppm, $J_{PH}$=580 Hz). With increasing incubation time the signal A as well as $B_1$ and $B_2$ decreased and the signals $C_1$ and $C_2$ increased as could be expected from Eq. 2. Probably due to the decrease in pH to ca. pH 5.5 (final, 18 h), $P^{III}$ indicated small change in $\delta$ value (final, 3.2 ppm) and considerable change in $J_{PH}$ value (final, 620 Hz), while both $\delta$ value and $J_{PH}$ value for $P^{III}P^{III}$ remained unchanged. The peak A for $P^V$ shifted across the signal $C_2$ to the high field after 3 h and finally appeared at +0.86 ppm.

Three signals, $D_1$ (doublet: $\delta$=−1.84), $D_2$ (doublet: $\delta$=−5.25), and $D_3$ (doublet: $\delta$=−5.85), appeared at 1 h incubation with intensity ratio 1:2:1 ($D_1$:$D_2$:$D_3$), were assigned to $P^{III}P^V$. The signal $D_2$ that shifted remarkably with time, as well as A, to appear finally at the right hand side of $B_2$ ($\delta$=−6.99) was assigned to the P(V) unit of $P^{III}P^V$. The pair of $D_1$ and $D_3$ attributable to the P(III) unit of $P^{III}P^V$ indicated a large coupling constant ($J_{PH}$=650 Hz) that was slightly variable with incubation time. The change in $\delta$ value from −3.8 ppm to −4.1 ppm (final) was small. The splitting of each $D_1$, $D_2$, and $D_3$, 17.8—18.5 Hz, was ascribed to P–P coupling of unsymmetrical $P^{III}P^V$.[2] In addition to the proton-coupled experiments in Fig. 3 proton-decoupled experiments[8,9] were also made. As expected the paired signals of $D_1$ and $D_3$ for $P^{III}P^V$ in Fig. 3 disappeared to give a signal (doublet) at −3.8 ppm (initial). Similar effects of proton-decoupling were also observed for $P^{III}$ and $P^{III}P^{III}$. Unfortunately, $^{31}P$ NMR signals for the trimeric $P^{III}P^VP^{III}$ could not be recorded, probably due to its low concentration that can be estimated from the chromatographic data in Fig. 2 to be less than 0.003 M.

**Equilibrium Constants.** In designing synthetic experiments to obtain high yields of products it is convenient to have information about apparent or conditional equilibrium constants of the substitution reactions of interest, even if the constants are based not strictly on thermodynamic consideration. This section deals with the evaluation of such apparent equilibrium constants for Eqs. 2 and 3 on the assumption that no side reactions such as hydrolysis of diphosphonate take place.[6,7]

Equilibrium constants, $K_{III,V}$ and $K_{III,V,III}$, for the formation of $P^{III}P^V$ and $P^{III}P^VP^{III}$ in Eqs. 2 and 3 are given as follows in terms of molar concentrations of each species at equilibrium conditions.

$$K_{III,V} = \frac{[P^{III}P^V][P^{III}]}{[P^V][P^{III}P^{III}]}, \tag{4}$$

$$K_{III,V,III} = \frac{[P^{III}P^VP^{III}][P^{III}]}{[P^{III}P^{III}][P^{III}P^V]}. \tag{5}$$

$[P^V]$, $[P^{III}P^V]$, and $[P^{III}P^VP^{III}]$ can be directly calculated from the relative peak areas in Fig. 2 since the total concentration of three species having P(V) units or the initial concentration of orthophosphate,$[P^V]_0$, is known. The initial concentration $[P^{III}P^{III}]_0$ is also known and hence two unknown concentrations are calculated as follows.

$$[P^{III}] = [P^{III}P^V] + 2[P^{III}P^VP^{III}], \tag{6}$$

$$[P^{III}P^{III}] = [P^{III}P^{III}]_0 - [P^{III}]. \tag{7}$$

The equilibrium constants thus obtained with the sample solution of 0.1 M orthophosphate and 0.5 M diphosphonate in Fig. 2 were; $K_{III,V}$=1.7 and $K_{III,V,III}$=0.008. Higher values, $K_{III,V}$=2.7 and $K_{III,V,III}$=0.05, were obtained for the sample of higher initial concentrations, 0.25 M orthophosphate and 1.25 M diphosphonate, while the sample of lower initial concentrations, 0.1 M orthophosphate and 0.1 M diphosphonate, gave a lower constant, $K_{III,V}$=0.50. The remarkable variation in the equilibrium constant with sample concentrations is not surprising because the experiments were made at high sample concentrations and the variable effects of pH, sodium ion and hydrolysis of diphosphonate[6-10] were not taken into consideration. The equilibrium constants provided by the simple chromatographic procedures are valuable from the practical viewpoint of predicting yields of the reaction products. Extended experiments based on the above prediction are in progress to prepare so much amount of the novel $P^{III}P^VP^{III}$ that is enough to do NMR expepriment for its structural characterization.

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