Phosphorylation of Phenols with *cyclo*-Triphosphate

Hideko INOUE,[a] Yoshinobu BABA,[a] Tohru MIYAJIMA,[b] and Mitsutomo TSUHAKO*,[a]

Kobe Women's College of Pharmacy,[a] Kitamachi, Motoyama, Higashinada-ku, Kobe 658, Japan and Department of Chemistry, Faculty of Science, Kyushu University,[b] Hakozaki, Higashi-ku, Fukuoka 812, Japan. *Received May 11, 1992*

Phosphorylation of phenols (phenol, catechol, resorcinol, hydroquinone, cresols, hydroxybenzoic acids, and nitrophenols) with inorganic sodium *cyclo*-triphosphate hexahydrate (P_{3m}), $Na_3P_3O_9 \cdot 6H_2O$, was carried out under various reaction conditions (pH, temperature, and molar ratio). (1) The main products of these reactions were triphosphate derivatives of phenols produced by phosphorylation of a hydroxyl group. Reaction of catechol with P_{3m} gave a five-membered cyclic phosphate formed by an intramolecular cyclization of a triphosphate derivative. (2) The optimum conditions for phosphorylation were found to be pH 12, and a molar ratio of P_{3m}: phenols = 1 : 5. (3) The pK_a values of phenols strongly affected the reaction rate and yield. The reactivity of phenols increased with an increase in their pK_a values. A hydroxyl group on phenols with a pK_a value of more than 8 would be reactive with P_{3m}. (4) The reactivity of *ortho*-substituted phenols was less than those of *meta*- and *para*-substituted phenols, owing to the steric hinderance of *ortho*-substituents. (5) The mechanism of the reaction in the phosphorylation of phenols with P_{3m} is discussed.

Keywords phosphorylation; phenol; *cyclo*-triphosphate; phenyltriphosphate; ^{31}P-NMR

Introdution

Sodium *cyclo*-triphosphate (P_{3m}), which is a simple inorganic condensed phosphate having a six-membered ring, is a very unique phosphorylating agent which produces biologically important triphosphates in aqueous solutions by one-step phosphorylation of several organic and biological compounds[1] having an amino or a hydroxyl group. This is of great advantage in comparison with other phosphorylating agents,[2] which require multi-step reactions to give triphosphates. Additionally, some resultant triphosphates form five-membered cyclic phosphate species *via* an intramolecular nucleophilic attack by the vicinal hydroxyl, carboxyl, or amino group on ribonucleosides,[3,4] ribonucleotides,[5] amino acids,[6-9] glucose,[1] and glucosamine.[1] Much attention has recently been focused on a five-membered cyclic phosphate as an extremely interesting intermediate for elucidating the mechanism in RNA hydrolysis,[10] self-cleavage of RNA,[11,12] and prebiotic formation of oligopeptides.[6,7]

In the present study, phosphorylation of phenols with P_{3m} has been examined in order to develop a selective phosphorylating agent for aromatic compounds. Phenols are model compounds suitable for these purposes because they have sufficient reactivity with P_{3m} in a moderate alkaline medium, and several phenols which have vicinal hydroxyl, carboxyl, methyl, nitro, or amino groups have been well characterized. Although Feldmann[13] has studied the reaction of phenol with P_{3m}, he provided only a semi-quantitative discussion on the product because paper chromatography has been principally used to analyze the reaction product in his study. Both ^{31}P-NMR and HPLC[14,15] techniques used in the present study gave a more improved understanding of the reaction mechanism based on the structural diagnosis and the quantitative measurement of the intermediates and the products.

Results and Discussion

Phosphorylation of Phenol with P_{3m} Figure 1 shows HPLC profiles for the phosphorylated product in the reaction of phenol (2.5 mol dm^{-3}) and P_{3m} (0.5 mol dm^{-3}) at pH 12 and room temperature. As shown in Fig. 1, a peak of the phosphorylated product (designated as **1**) appears at a retention time of about 40 min after one day of incubation. The peak area of **1** increased gradually with the expenditure of P_{3m}. Other chromatographic peaks were assigned to P_{3m} and its hydrolytic products, *i.e.*, mono-(P_1), di-(P_2), and triphosphate (P_3), respectively. The elution order of the phosphorylated product exhibits a strong retention on the ion-exchanger because of an interaction between the phenyl group on the product and the polystyrene skeleton of the ion-exchanger in addition to the ion–ion interaction.

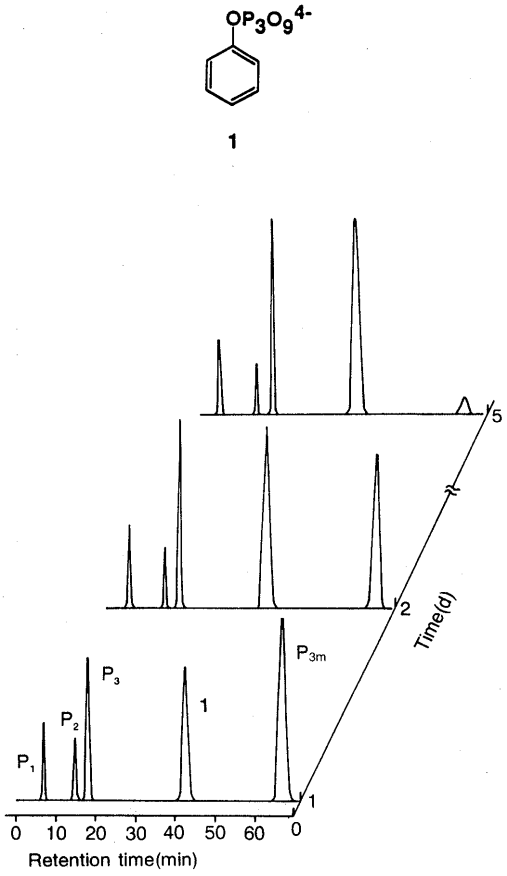


Fig. 1. HPLC Profiles for the Reaction Product of Phenol (2.5 mol dm^{-3}) and P_{3m} (0.5 mol dm^{-3}) at pH 12 and Room Temperature

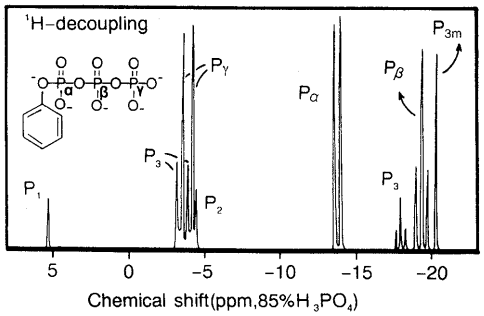


Fig. 2. ^{31}P-NMR Spectrum for the Reaction Product of Phenol (2.5 mol dm^{-3}) and P_{3m} (0.5 mol dm^{-3}) at pH 12 and Room Temperature for 7 d

TABLE I. ^{31}P-NMR Data for Triphosphate Derivatives of Phenols

Product	$\delta(P_\alpha)$	$\delta(P_\beta)$	$\delta(P_\gamma)$	$^2J_{P_\alpha P_\beta}$/Hz	$^2J_{P_\beta P_\gamma}$/Hz
1	−13.8	−19.4	−3.81	17.7	18.6
4a	−13.6	−19.2	−3.64	17.5	18.4
4b	−14.3	−20.2	−4.50	18.0	19.0
4c	−14.5	−20.2	−4.52	18.3	18.4
4d	−14.4	−19.9	−4.02	18.6	19.4
6a	−13.6	−19.6	−3.93	17.9	18.7
6b	−14.6	−20.6	−4.93	18.0	19.0
6c	−15.0	−20.4	−4.56	18.4	18.4
7	−12.6	−19.5	−3.93	18.2	18.7

To determine the molecular structure of the phosphorylated product (**1**), ^{31}P-NMR spectra were measured. Figure 2 shows ^{31}P-NMR spectrum for the reaction mixture of phenol (2.5 mol dm^{-3}) and P_{3m} (0.5 mol dm^{-3}) incubatd at room temperature and pH 12 for 7 d.

The spectrum shows two unknown doublets (−3.8 and −13.8 ppm) and an unknown triplet (−19.4 ppm) in addition to the signals assigned to P_{3m} at −20.1 ppm, P_1 at 5.5 ppm, P_2 at −4.1 ppm, and P_3 at −3.7 and −18.0 ppm on the basis of the chemical shifts of the authentic samples. These unknown peaks are the signals for the phosphorylated product observed by the HPLC measurement.

The phosphorylated product was proven to be phenyl triphosphate (**1**) as shown in Fig. 2 on the basis of the following interpretation of ^{31}P-NMR spectra. The triplet at −19.4 ppm is characteristic of the middle-group phosphorus atom on a triphosphate such as ATP.[16,17] However, such a triplet at around −20 ppm was never observed in the ^{31}P-NMR spectra of mono-, di-, and tetraphosphates. In addition, the coupling constant (18 Hz) of the triplet is approximately equal to that (19.3 Hz) of ATP,[17] the value of which represents the coupling between two phosphorus atoms separated by two chemical bonds through a P–O–P linkage.[18] The triplet, therefore, is assigned to the middle phosphorus atom (P_β) on product **1**. Other doublets at −13.8 and −3.8 ppm are assigned to the phosphorus atoms of P_α and P_γ on **1**, respectively, for the following reason. The δ-value of P_γ (−3.8 ppm) is similar to that of the end-phosphate group on P_3 (−3.7 ppm) because of the similarity in the chemical environment of both phosphorus atoms. The esterification of a phosphorus group (P_α) leads to an upfield shift of δ[19] relative to the free phosphate group (P_γ). The same situation is observed in the chemical shifts of P_α and P_γ on ATP. The coupling constants between P_α and P_β and between P_β and P_γ are 17.7 and 18.6 Hz, respectively. The ^{31}P-NMR parameters of **1** are compiled in Table I.

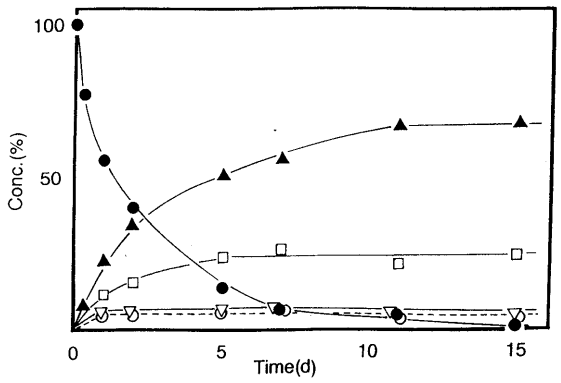


Fig. 3. Changes of the Amounts of Product in the Reaction of P_{3m} (0.5 mol dm^{-3}) with Phenol (2.5 mol dm^{-3}) at pH 12 and Room Temperature

—●—, P_{3m}; —▲—, **1**; —▽—, P_1; —○—, P_2; —□—, P_3.

TABLE II. Yields of Phenyl Triphosphate (**1**) in the Phosphorylation of Phenol with P_{3m}

Reaction conditions				Yield (%)
Mixing ratio	pH	Temp. (°C)	Time	
P_{3m} : phenol				
= 1 : 5 (0.5 M[a] : 2.5 M)	12	Room	11 d	67
	12	50	1 d	43
	12	70	2 h	36
= 1 : 1 (0.5 M : 0.5 M)	12	Room	21 d	28
	12	50	4 d	23
	12	70	5.5 h	18
	10	Room	31 d	20
	7	Room	28 d	0

a) M = mol dm^{-3}.

Figure 3 shows changes in the amounts of the product formed by the reaction of P_{3m} (0.5 mol dm^{-3}) with phenol (2.5 mol dm^{-3}) at pH 12 and room temperature. The amount of **1** is gradually increased with the passage of reaction time, reaching about 67% after 11 d. The maximum amount remained constant after 15 d. Starting material (P_{3m}) decreased with time, and disappeared after 17 d. Reaction of phenol with P_{3m} was further carried out with varying reaction conditions (pH, temperature and molar ratio). The time courses of the reactions under each condition showed the same tendencies as the results shown in Fig. 3. Table II lists the yield of phenyl triphosphate (**1**) under various reaction conditions. The yield and time in Table II represent the maximum amount of **1** and the time required for reaching the maximum amount.

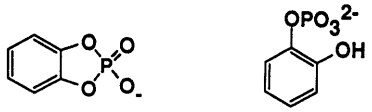
The yield of **1** decreased gradually with a decrease in pH, *e.g.*, 28% at pH 12 and 20% at pH 10, at a molar ratio of 1 : 1 and room temperature. Phenyl triphosphate (**1**) was not obtained at a pH of less than 7. The result clearly shows that P_{3m} does not react with phenol but reacts with a phenoxide ion, because a phenol molecule, of which the pK_a is 10,[20] in an aqueous solution at pH 12, is deprotonated completely into a phenoxide ion, whereas there is

no phenoxide ion in a neutral or acidic solution. The existence of 50% phenol molecules at pH 10 results in the decrease in yield and reaction rate compared with those at pH 12.

The yield of **1** increased with an increase in initial concentration of phenol (molar ratio from 1 : 1 to 1 : 5), where pH and temperature were held constant at pH 12 and room temperature. Phosphorylation in an excess amount of phenol yielded 67% of **1** for 11 d and achieved about a two-fold increase in comparison with that at a molar ratio of 1 : 1. A temperature effect on the yield was also examined at pH 12 when the reaction temperature was changed from room temperature to 70°C. The yield of **1** decreased with an increase in temperature as listed in Table II. A decrease in yield is attributable to the instability of **1** against hydrolysis at higher temperature. To provide further support for the instability of **1**, we carried out an additional experiment involving the hydrolysis of **1**. As a result, **1** decomposed with the progress of the reaction at 70 °C and disappeared after 2-d incubation (data not shown).

In conclusion, the optimum conditions for the formation of **1** were found to be pH 12 and molar ratio of 1 : 5.

Reaction of Catechol, Resorcinol, or Hydroquinone with P_{3m} Figure 4 shows ^{31}P-NMR spectra for the reaction mixture of catechol (0.5 mol dm^{-3}) with P_{3m} (0.5 mol dm^{-3}) incubated at room temperature and pH 12. A proton-decoupling spectrum (Fig. 4A) showed that 30-min incubation gave two products which showed singlets at 2.8 and 15.7 ppm, respectively. The singlet at 15.7 ppm disappeared after 1-d incubation, as shown in Fig. 4B, whereas the intensity of the singlet at 2.8 ppm increased with the expense of the product corresponding to the singlet at 15.7 ppm. The results illustrate that the product ($\delta = 15.7$) is formed at an early stage of the reaction and converted into another product ($\delta = 2.8$).



2

3

These two singlet peaks were confirmed to be assigned to a five-membered cyclic phosphate (**2**: $\delta = 15.7$) and catechol 1-phosphate (**3**: $\delta = 2.8$) on the basis of the experimental and theoretical work[21,22] by Gorenstein on ^{31}P-NMR chemical shifts on O–P–O bond angles in phosphates. His conclusion is that the δ-values of five-membered cyclic phosphates shift downfield from the corresponding open monophosphates, of which the δ-values are around 0—3 ppm, by 15—20 ppm, and such a downfield shift is caused by a decrease in the O–P–O bond angle in five-membered cyclic phosphates. In the reaction of catechol with P_{3m}, **3** is the primary product, whereas **2** is formed at the initial stage of the reaction. Product **2** is unstable and undergoes rapid hydrolysis to **3**. Details of the mechanism will be discussed below.

Table III lists the yield of catechol 1-phosphate (**3**) under various conditions. The yield of **2** could not be determined owing to its instability and low yield. The yield of **3** increased with an increase in pH, *e.g.*, 17% at pH 12 and 3.7% at pH 10, and also increased with an increase in initial concentration of catechol as listed in Table III. The yield of **3**, which was 17% (molar ratio of 1 : 5) at room temperature, was reduced to 3.5% (1 : 5) or 1.8% (1 : 1) at 50°C. Due to the hydrolysis of **3**, the yield obtained at a higher temperature is much lower than that obtained at room temperature. The optimum conditions for the phosphorylation of catechol with P_{3m} were pH 12, a molar ratio of 1 : 5, and room temperature.

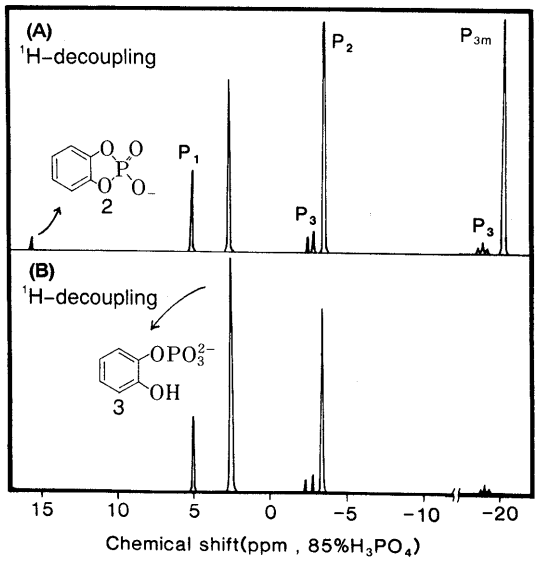


Fig. 4. ^{31}P-NMR Spectra for the Reaction Products of Catechol (0.5 mol dm^{-3}) and P_{3m} (0.5 mol dm^{-3}) at pH 12 and Room Temperature for 30 min (A) and 1 d (B)

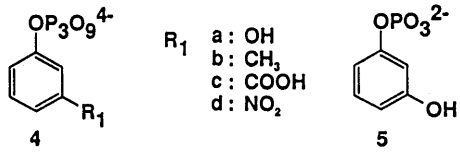
TABLE III. Yields of Phosphorylated Products of Catechol, Resorcinol, or Hydroquinone with P_{3m}

Reaction conditions				Yield (%)
Mixing ratio	pH	Temp. (°C)	Time	
P_{3m} : catechol				
= 1 : 5 (0.5 M : 2.5 M)	12	Room	21 d	17 (**3**)
	12	50	11 d	3.5 (**3**)
	10	Room	17 d	3.7 (**3**)
= 1 : 1 (0.5 M : 0.5 M)	12	Room	27 d	3.7 (**3**)
	12	50	4 d	1.8 (**3**)
P_{3m} : resorcinol				
= 1 : 5 (0.5 M : 2.5 M)	12	Room	7 d	47 (**4a**)
	12	50	3 h	31 (**4a**)
	12	70	6.5 h	39 (**4a**)
	10	Room	28 d	33 (**4a**)
			28 d	4.5 (**5**)
= 1 : 1 (0.5 M : 0.5 M)	12	Room	36 d	5.6 (**4a**)
			44 d	2.2 (**5**)
P_{3m} : hydroquinone				
= 1 : 1 (0.5 M : 0.5 M)	12	Room	5 d	7.2 (**6a**)
			77 d	9.3 (**7**)

Two peaks of a main product and a by-product were observed by HPLC in the reaction of resorcinol (2.5 mol dm^{-3}) with P_{3m} (0.5 mol dm^{-3}) at pH 10 and room temperature. To establish the molecular structure of the phosphorylated products, ^{31}P-NMR spectra were recorded. The main product gave three peaks at −3.6 (doublet), −13.6 (doublet), and −19.2 ppm (triplet), and the by-product gave a singlet peak at 3.2 ppm. The main product was identified as a resorcinol 1-triphosphate (**4a**), similar to

the case of phosphorylation of phenol described in the previous section. The triplet at −19.2 ppm is assigned to the middle phosphorus atom (P_β on **4a**). The doublets at −13.6 and −3.6 ppm are signals for P_α and P_γ atoms on **4a**, respectively. The singlet of 3.2 ppm (by-product) is assigned to resorcinol 1-phosphate (**5**).

Phosphorylation of resorcinol with P_{3m} gave only **4a** in satisfactory yield (47%) at pH 12, a molar ratio of 1 : 5, and at room temperature, and is also compiled in Table III. On the other hand, both **4a** and **5** were formed after a longer incubation time. Resorcinol 1-phosphate (**5**) would be formed by the hydrolysis of resorcinol 1-triphosphate (**4a**). The overall tendencies of the results for resorcinol in Table III are similar to those of phenol in Table II. The optimum conditions for the formation of **4a** were found to be pH 12, a molar ratio of 1 : 5, and room temperature as well as **1** and **3**.

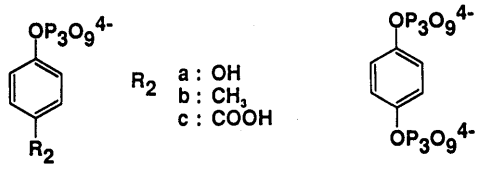


4 5

HPLC analysis (Fig. 5) shows the formation of two products in the reaction of hydroquinone with P_{3m}. The area of the peak at the retention time of about 40 min increased gradually to a maximum value (7.2%) after 5 d of incubation, and then decreased gradually to reach the value of 3.8% after 56 d. Another peak at the retention time of 49 min appeared after 5 d of incubation. The area of the peak at 49 min increased with an elapse of reaction time and reached 9.3% after 77 d of incubation (Table III).

Figure 6 shows ^{31}P-NMR spectrum for the reaction mixture of hydroquinone (0.5 mol dm^{-3}) and P_{3m} (0.5 mol dm^{-3}) incubated at a pH of 12 and room temperature for 9 d. Three unknown doublets and two unknown triplets are the signals for the two phosphorylated products observed in Fig. 5. The spectra illustrate that two kinds of triphosphates are formed in the reaction of hydroquinone with P_{3m} because of the appearance of two triplets at around −19 ppm, which are characteristic of the triphosphate species as discussed above.

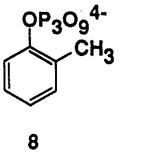
These products must be hydroquinone 1-triphosphate (**6a**) and 1,4-bis(triphosphate) (**7**). The doublet at −13.6 ppm was assigned to the signal of P_α on **6a**, because of the similarity in the δ values of the P_α atoms on **1** and **4a** as listed in Table I. The doublet at −12.6 ppm, therefore, was assigned to P_α on **7**. The triplet at −19.6 ppm was assigned to P_β on **6a** and that at −19.5 to P_β on **7** from the peak intensity of each P_α. The P_γ atoms on **6a** and **7** gave only a doublet at −3.9 ppm, because of the similarity in δ of both P_γ atoms.



6 7

Both hydroxyl groups at the 1- and 4-positions of hydroquinone were phosphorylated by P_{3m}, whereas the reaction of resorcinol with P_{3m} gave no resorcinol 1,3-bis(triphosphate). From the result of HPLC (Fig. 5), the amount of **7** increased after the formation of **6a**, showing that **7** is produced by the phosphorylation of a hydroxyl group at the 4-position on **6a** with P_{3m}. In the reaction of resorcinol with P_{3m}, the 3-OH group on **4a** did not react with P_{3m}, because a negatively charged 1-triphosphate group is strongly repulsed in attacking trianionic P_{3m} in addition to some steric hinderance of 1-triphosphate group. Such repulsion is the most minimum in the reaction of the 4-OH group on **6a** with P_{3m}.

Reaction of Cresols, Hydroxybenzoic Acids (HBAs), or Nitrophenols with P_{3m} A peak of a product was observed by HPLC in the reaction of cresols (*o*-, *m*-, and *p*-methylphenols) (0.5—2.5 mol dm^{-3}) with P_{3m} (0.5 mol dm^{-3}) at pH 12 and room temperature. The ^{31}P-NMR spectra of the products gave two doublets and a triplet as listed in Table I. Each product was identified as a triphosphate derivative (**8**, **4b**, **6b**) similarly to the case of phosphorylation of phenol.



8

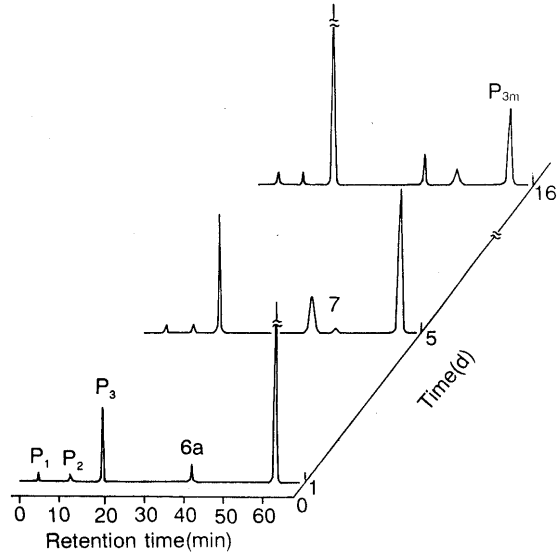


Fig. 5. HPLC Profiles for the Reaction Products of Hydroquinone (0.5 mol dm^{-3}) and P_{3m} (0.5 mol dm^{-3}) at pH 12 and Room Temperature

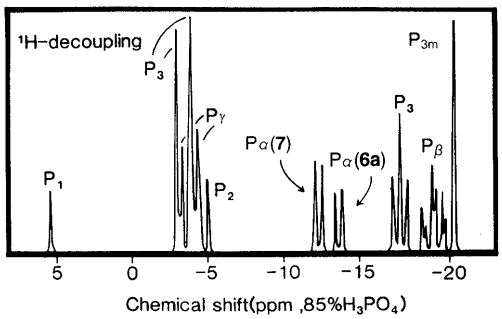


Fig. 6. ^{31}P-NMR Spectrum for the Reaction Products of Hydroquinone (0.5 mol dm^{-3}) and P_{3m} (0.5 mol dm^{-3}) at pH 12 and Room Temperature for 9 d

The yields of **8**, **4b**, and **6b** are summarized in Table IV. In the reaction of cresols with P_{3m}, each phosphorylated product was formed in fairly good yield in an alkaline aqueous solution. Hydroxyl groups on cresols, of which the pK_a values are 10.1—10.3, are deprotonated completely into anionic species at pH 12. The yield of **8** is about a half that of **4b** and **6b**, owing to the steric effect of the *o*-methyl group.

The reaction of *m*-HBA or *p*-HBA with P_{3m} gave respective phosphorylated products by HPLC, whereas the reaction of *o*-HBA with P_{3m} showed no peak corresponding to the product in HPLC. ^{31}P-NMR spectra (proton coupling) of the products in the phosphorylation of *m*-HBA or *p*-HBA, showed two doublets ($\delta = -14$ and -5) and a triplet ($\delta = -20$), respectively. The phosphorylated products, therefore, were proven to be triphosphate derivatives of *m*-HBA(**4c**) and *p*-HBA(**6c**).

Table IV lists the yields of **4c** and **6c**. The yields decreased with an increase in pH, *e.g.*, 28% (**4c**) and 40% (**6c**) at pH 10 and 24% (**4c** and **6c**) at pH 12. This result is different from the pH effect on the yields of other products. The difference is attributable to the different negative charges on phenols, HBA and others, because a more negatively charged HBA is repulsed and slowed down to greater degree in attacking trianionic P_{3m}.

o-HBA did not react with P_{3m} because of its larger pK_a and also the steric effect of its carboxyl group. Since the value of pK_a for a hydroxyl group on *o*-HBA is 13.4, a hydroxyl group is not deprotonated into reactive anionic species at pH 12. On the other hand, hydroxyl groups on *m*-HBA and *p*-HBA, of which the pK_a values are 9.7 and 9.5, are completely deprotonated into anionic species at pH 12. The negatively charged carboxyl group on *o*-HBA also strongly prohibits attacking of the anionic P_{3m} molecule to a hydroxyl group on *o*-HBA in addition to the steric hindrance of the carboxyl group.

TABLE IV. Yields of Phosphorylated Products of Cresols, Hydroxybenzoic Acids, or Nitrophenols with P_{3m}

Reaction conditions				Yield (%)	
Mixing ratio	pH	Temp. (°C)	Time		
P_{3m} : *o*-cresol					
= 1 : 5 (0.5 M : 2.5 M)	12	Room	42 d	32	(**8**)
		50	2 d	17	(**8**)
		70	6.5 h	11	(**8**)
= 1 : 1 (0.5 M : 0.5 M)	12	Room	26 d	3.0	(**8**)
P_{3m} : *m*-cresol					
= 1 : 5 (0.5 M : 2.5 M)	12	Room	32 d	64	(**4b**)
= 1 : 1 (0.5 M : 0.5 M)	12	Room	29 d	16	(**4b**)
P_{3m} : *p*-cresol					
= 1 : 5 (0.5 M : 2.5 M)	12	Room	33 d	66	(**6b**)
= 1 : 1 (0.5 M : 0.5 M)	12	Room	30 d	21	(**6b**)
P_{3m} : *o*-HBA					
= 1 : 1 (0.5 M : 0.5 M)	12	Room	29 d	0	
P_{3m} : *m*-HBA					
= 1 : 1 (0.5 M : 0.5 M)	12	Room	18 d	24	(**4c**)
		50	4 d	18	(**4c**)
	10	Room	39 d	28	(**4c**)
P_{3m} : *p*-HBA					
= 1 : 1 (0.5 M : 0.5 M)	12	Room	27 d	24	(**6c**)
		50	4 d	24	(**6c**)
	10	Room	59 d	40	(**6c**)
	7	Room	35 d	0	
P_{3m} : *o*-nitrophenol					
= 1 : 1 (0.25 M : 0.25 M)	12	50	42 d	0	
P_{3m} : *m*-nitrophenol					
= 1 : 1 (0.25 M : 0.25 M)	12	50	30 d	4.4	(**4d**)
P_{3m} : *p*-nitrophenol					
= 1 : 1 (0.25 M : 0.25 M)	12	Room	23 d	0	

The reaction of nitrophenols (0.25 mol dm^{-3}) with P_{3m} (0.25 mol dm^{-3}) were carried out at pH 12 and room temperature or at 50°C. A peak of the product was observed by HPLC in the reaction of *m*-nitrophenol with P_{3m}, whereas no peak corresponding to the product was observed in the reaction of *o*- and *p*-nitrophenol. The product was identified to be an *m*-nitrophenyl triphosphate (**4d**) by use of ^{31}P-NMR (Table I). The yield of **4d** is much smaller than those of other products, as listed in Table IV. The very low reactivity of nitrophenols is attributable to their low pK_a values. The values of pK_a of *o*-, *m*-, and *p*-nitrophenol are 7.1, 8.1, and 6.9, respectively. In general, the reactivity for the phosphorylation of phenols with P_{3m} increased with an increase in pK_a value of the nucleophilic groups. Therefore, the hydroxyl group of phenols with pK_a values of more than 8 would be reactive with P_{3m}.

Mechanism of the Reaction of Phenols with P_{3m} By comparing the reactivities of several functional groups in the phosphorylation of phenols with P_{3m}, it was found that only the hydroxyl group on phenols was phosphorylated, but the methyl, carboxyl, and nitro groups did not react with P_{3m} at all. Additionally, the reaction of phenols with P_{3m} mainly gave triphosphate derivatives of phenols, except for catechol. The mechanism of phosphorylation of the phenols with P_{3m} was shown in Fig. 7 as exemplified by phenol. The phenol first deprotonates to a phenoxide ion in an alkaline solution such as pH 12. Secondly, the phenoxide ion nucleophilically attacks a phosphorus atom on P_{3m}, since a phenoxide ion is found to indeed be a reactive species; it causes a ring opening of P_{3m}. This single step reaction results in the formation of triphosphate

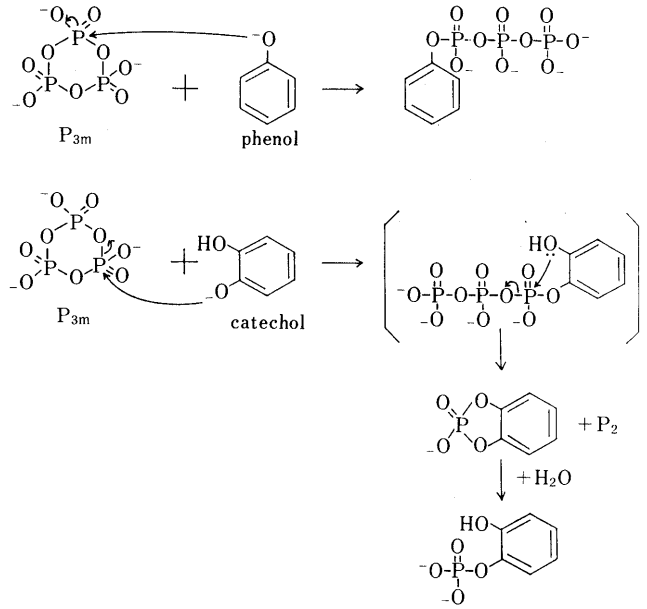


Fig. 7. Mechanism of the Reaction in Phosphorylation of Phenol or Catechol with P_{3m}

derivatives of phenols. The reactivity of the hydroxyl group on phenols depends strongly on the pK_a of their conjugated acids, steric hinderance, and charge repulsion by the neighboring hydroxyl, methyl, carboxyl, and nitro groups.

Catechol is phosphorylated with P_{3m} to produce predominantly two monophosphate derivatives (**2**, **3**), despite the formation of a triphosphate derivative in the reaction of resorcinol or hydroquinone with P_{3m}. The results imply that an *o*-hydroxyl group participates directly in the reaction with P_{3m} as shown in Fig. 7. Catechol, as well as other phenols, deprotonates to form anionic species in an alkaline solution. The anionic oxide group nucleophilically attacks the phosphorus atom on P_{3m} to generate catechol 1-triphosphate as an intermediate, and an intramolecular attack of the *o*-hydroxyl group on a phosphorus atom of the triphosphate group spontaneously occurs to produce a five-membered cyclic phosphate derivative of catechol (**2**). The cyclic phosphate thus formed is quickly hydrolyzed to give catechol 1-phosphate (**3**). In the reaction of resorcinol or hydroquinone with P_{3m}, the participation of an *m*- or *p*-hydroxyl group declines, since a six- or seven-membered cyclic phosphate intermediate is not produced.

Experimental

Chemicals Sodium *cyclo*-triphosphate hexahydrate (P_{3m}), $Na_3P_3O_9 \cdot 6H_2O$, was prepared according to the literature[1,8] and recrystallized three times from an aqueous solution. The purity of P_{3m} thus obtained was checked by HPLC to be 99% (as P). Guaranteed grade phenol, catechol (*o*-benzenediol), resorcinol (*m*-benzenediol), hydroquinone (*p*-benzenediol), *o*-cresol (*o*-methylphenol), *m*-cresol (*m*-methylphenol), *p*-cresol (*p*-methylphenol), *o*-HBA (salicylic acid), *m*-HBA, *p*-HBA, *o*-nitrophenol, *m*-nitrophenol, and *p*-nitrophenol were purchased from Wako Chemical Industries, Ltd. (Osaka, Japan).

Reaction of Phenols with P_{3m} in an Aqueous Solution The initial concentration of P_{3m} was kept at a constant value of 0.5 mol dm^{-3} and those of phenol, catechol, resorcinol, and cresols were varied from 0.5 to 2.5 mol dm^{-3} to change the mixing ratio. Hydroquinone and HBAs reacted with P_{3m} at the fixed mixing ratio of 1:1 (0.5 mol dm^{-3} : 0.5 mol dm^{-3}) and nitrophenols with P_{3m} at the mixing ratio of 1:1 (0.25 mol dm^{-3} : 0.25 mol dm^{-3}). The reactions of hydroquinone, HBAs, and nitrophenols were carried out without increasing their concentrations, because they were not easily soluble in an aqueous solution at higher concentrations. The pH of the mixed solutions was adjusted to the desired values (12, 10, and 7) with 6 mol dm^{-3} sodium hydroxide solution or hydrochloric acid. The mixed solutions were allowed to react at room temperature (20—25 °C) or at specified temperatures (50 and 70 °C) controlled with a thermostated bath within ±2 °C. Since the pH of the mixed solution was gradually decreasing with the progress of the reaction, the pH of each mixed solution was constantly adjusted to the prescribed pH by adding a sodium hydroxide solution.

HPLC Measurement HPLC analysis was carried out with a JASCO HPLC-Twincle system (Tokyo, Japan) coupled with a JASCO FIU-300 flow injection system as a post-column reaction detector[14,15] to detect phosphates. An aliquot (0.1 ml) of the reaction mixture was injected. A column (250 × 4.6 mm i.d.) was packed with a polystyrene-based anion-exchanger (TSK gel, SAX, 10 μm, Tosoh, Tokyo, Japan) and the column temperature was maintained at 40 °C. Flow rate was 1.0 ml min^{-1}. The convex gradient elution technique using 0.2 and 0.45 mol dm^{-3} potassium chloride solutions was used. The absorbance of an effluent was monitored continuously at 830 nm.

^{31}P-NMR Measurement Pulse FT ^{31}P-NMR spectra were recorded at room temperature by use of a Varian XL-VX 200 (81 MHz) spectrometer. Phosphoric acid (85%) was used as an external standard.

References

1) Y. Baba, M. Tsuhako, and N. Yoza, "Trends in Organic Chemistry," ed. by J. Menon, Research Trends, Trivandrum, 1990, pp. 53—75.
2) Y. Mizuno, "The Organic Chemistry of Nucleic Acids," Kodansha, Tokyo, 1986, p. 161.
3) R. Saffhill, *J. Org. Chem.*, **35**, 2881 (1970).
4) M. Tsuhako, M. Fujimoto, S. Ohashi, H. Nariai, and I. Motooka, *Bull. Chem. Soc. Jpn.*, **57**, 3274 (1984).
5) M. Tsuhako, R. Kunitomi, Y. Baba, and T. Miyajima, *Bull. Chem. Soc. Jpn.*, **64**, 490 (1991).
6) J. Rabinowitz, *Helv. Chem. Acta*, **53**, 1350 (1970).
7) N. M. Chung, R. Lohrmann, L. E. Orgel, and J. Rabinowitz, *Tetrahedron*, **27**, 1205 (1971).
8) M. Tsuhako, N. Fujita, A. Nakahama, T. Matsuo, M. Kobayashi, and S. Ohashi, *Bull. Chem. Soc. Jpn.*, **53**, 1968 (1980).
9) M. Tsuhako, A. Nakajima, T. Miyajima, S. Ohashi, H. Nariai, and I. Motooka, *Bull. Chem. Soc. Jpn.*, **58**, 3092 (1985).
10) *a*) L. Stryer, "Biochemistry," 3rd ed., W. H. Freeman and Company, New York, 1988, p. 211; *b*) H. Dugas and C. Penney, "Bioorganic Chemistry A Chemical Approach to Enzyme Action," Springer-Verlag, New York, 1981, p. 111.
11) J. Haseloff and W. L. Gerlach, *Nature* (London), **334**, 585 (1988).
12) K. Taira, M. Uebayashi, H. Maeda, and K. Furukawa, *Protein Eng.*, **3**, 691 (1990).
13) W. Feldmann, *Chem. Ber.*, **99**, 3251 (1966).
14) Y. Hirai, N. Yoza, and S. Ohashi, *Anal. Chim. Acta*, **115**, 269 (1980).
15) Y. Baba, M. Tsuhako, and N. Yoza, *J. Chromatogr.*, **507**, 103 (1990).
16) H. J. Vogel, "Phosphorus-31 NMR Principles and Applications," ed. by D. G. Gorenstein, Academic Press, New York, 1984, p. 114.
17) M. Cohn and T. R. Hughes, Jr., *J. Biol. Chem.*, **235**, 3250 (1960).
18) D. G. Gorenstein, "Phosphorus-31 NMR Principles and Applications," ed. by D. G. Gorenstein, Academic Press, New York, 1984, p. 43.
19) J. R. Van Wazer, C. F. Callis, J. N. Shoolery, and R. C. Jones, *J. Am. Chem. Soc.*, **78**, 5715 (1956).
20) A. Streitwieser, Jr. and C. H. Heathcock, "Introduction to Organic Chemistry," 3rd ed., Macmillan, New York, 1985, p. 813.
21) *a*) D. G. Gorenstein, *J. Am. Chem. Soc.*, **97**, 898 (1975); *b*) *Idem*, "Phosphorus-31 NMR Principles and Applications," ed. by D. G. Gorenstein, Academic Press, New York, 1984, p. 10.
22) D. G. Gorenstein and D. Kar, *Biochem. Biophys. Res. Commun.*, **65**, 1073 (1975).