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Studies on Pyrophosphates. I. A Selective Degradation of Pyrophosphates by Means of 2,6-Dichloroquinone *N*-Chloroimide

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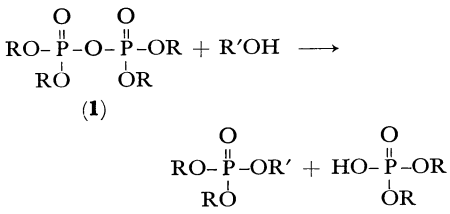
Reaction of P^1,P^2-dialkyl pyrophosphate with alcohols or water in the presence of 2,6-dichloroquinone *N*-chloroimide (CQC) was studied. It was found that alkyl *p*-nitrophenyl phosphates are obtained in high yields by treating P^1,P^2-di-*p*-nitrophenyl pyrophosphate with alcohols in the presence of CQC. Similarly, alkyl phosphates are selectively produced by the reaction of P^1,P^2-dialkyl pyrophosphates with water in the presence of CQC. These reactions afford a new procedure for the selective degradation of P^1,P^2-dialkyl pyrophosphates, especially nucleoside pyrophosphates.

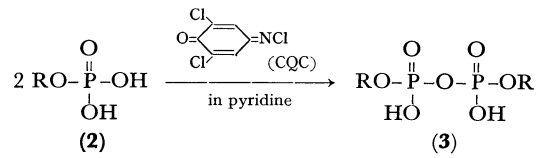
The method of phosphorylation with the use of tetraalkyl pyrophosphates (**1**) is a versatile one[1-3] that has been applied to nucleotide synthesis.[4]



However, only a few studies[5-7] have been reported on P^1,P^2-dialkyl pyrophosphates (**3**) as applied to the phosphorylation of alcohols, since pyrophosphates (**3**) are generally unreactive compared with tetraalkyl pyrophosphates (**1**).

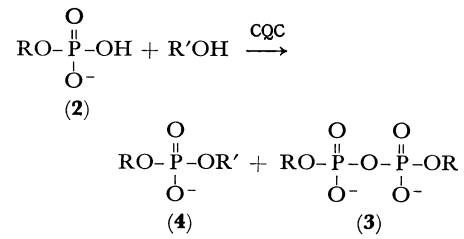
In the present study, phosphorylation of alcohols with P^1,P^2-dialkyl pyrophosphates (**3**) and 2,6-dichloroquinone *N*-chloroimide (CQC) was investigated.

It was found that P^1,P^2-di-*p*-nitrophenyl pyrophosphate (**3a**) was obtained in high yield by treating CQC with *p*-nitrophenyl phosphate (**2a**) in dry pyridine at room temperature for 5 hr.



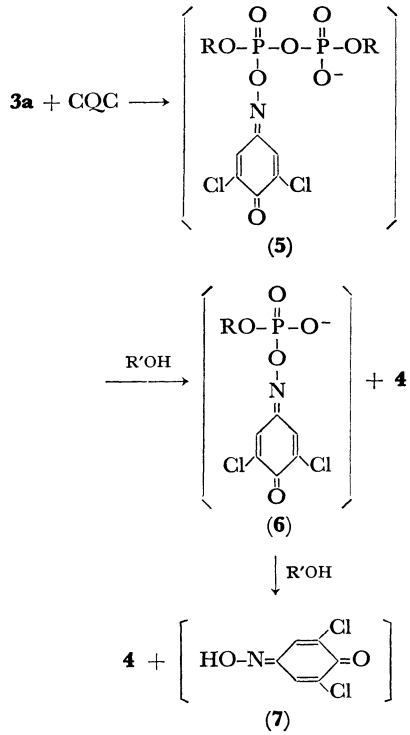
2a and **3a**, R=*p*-nitrophenyl

This result suggests that CQC participates in the activation of *p*-nitrophenyl phosphate (**2a**), which leads us to surmise that it would be used as an active condensating reagent for the preparation of diesters of phosphoric acid (**4**) from **2a** and alcohols. When a mixture of 1 equiv. of **2a** and *n*-octanol in dry pyridine was treated with 3 equiv. of CQC at room temperature for 19 hr, *n*-octyl *p*-nitrophenyl phosphate (**4f**) and **3a** were produced in 15% and 77% yields, respectively.



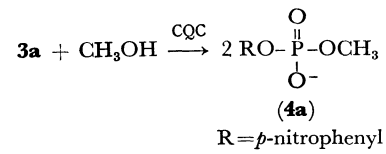
After being kept standing for several days, **3a** decreased significantly and **4f** was obtained as the main product. This indicates that **3a**, initially formed, further reacts with CQC to yield an intermediate (**5**), which in turn reacts with alcohol to give a diester of phosphoric acid (**4**) and *p*-nitrophenyl 2,6-dichloroquinoneoxime phosphate (**6**). **6** thus formed also reacts with another alcohol to yield **4** and 2,6-dichloroquinone oxime (**7**).

1) H. S. Mason and A. R. Todd, *J. Chem. Soc.*, **1951**, 2267.
2) G. O. Dudek and F. H. Westheimer, *J. Amer. Chem. Soc.*, **81**, 2641 (1959).
3) J. G. Moffatt and H. G. Khorana, *ibid.*, **79**, 3741 (1957).
4) R. W. Chambers, J. G. Moffatt and H. G. Khorana, *ibid.*, **79**, 3747 (1957).
5) F. Cramer and H. J. Baldauf, *Angew. Chem.*, **72**, 627 (1960).
6) F. Cramer and K. H. Scheit, *Chem. Ber.*, **95**, 1657 (1962).
7) M. Smith, G. I. Drummond and H. G. Khorana, *J. Amer. Chem. Soc.*, **83**, 698 (1961).



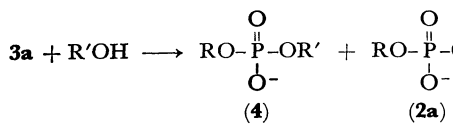
Based on the above mechanism, phosphorylation of alcohols with the use of CQC and P^1,P^2-di-*p*-nitrophenyl pyrophosphate (**3a**), obtained from *p*-nitrophenyl phosphate (**2a**) and dicyclohexylcarbodiimide, was tried.

When a mixture of 3 equiv. of CQC and 1 equiv. of **3a** in methanol was heated at 100°C for 2 hr, methyl *p*-nitrophenyl phosphate (**4a**) was obtained in 91% yield, as expected.



R=*p*-nitrophenyl

This shows that the pyrophosphate can be effectively used as a phosphorylating reagent when it is treated with CQC. By this method, several alkyl *p*-nitrophenyl phosphates (**4**) were obtained in high yields from the corresponding alcohols as shown in Table 1. In the cases of *n*-octanol and *n*-hexadecanol, **2a** was produced as a by-product probably by way of a direct nucleophilic attack of alkoxide anion on phosphorus atom of **3a** to give **2a** and **4**.



R=*p*-nitrophenyl

Table 1. Preparation of alkyl *p*-nitrophenyl phosphate (**4**)

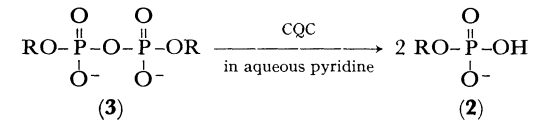
No.	alkyl (R)	yield (%)	R_f value
4a	methyl	91	0.75
4b	ethyl	85	0.76
4c	*n*-propyl	82	0.81
4d	*n*-butyl	93	0.82
4e	*n*-pentyl	72	0.85
4f	*n*-octyl	65	0.91
4g	*n*-hexadecyl	59	0.92

Solvent system: isopropyl alcohol : conc. ammonia : water=8 : 1 : 1 (v/v)

A nucleoside pyrophosphate, P^1,P^2-di-thymidine 5′-pyrophosphate (**8a**) was also degradated in methanol to methyl thymidine 5′-phosphate and thymidine 5′-phosphate by treating with CQC.

A further study to extend the reaction for a selective hydrolysis of the pyrophosphates was carried out with the assumption that 2 mol of alkyl phosphate (**2**) is exclusively produced, when **3** is treated with water in place of alcohols in the above reaction.[8]

When a mixture of 1 equiv. of **3a** and 3 equiv. of CQC in aqueous pyridine was heated at 100°C for 2 hr, **2a** was exclusively obtained in 95% yield as expected. This non-enzymatic method can be applied to the hydrolysis of nucleoside pyrophosphates such as thymidine-, uridine-, adenosine-, guanosine-, and cytidine-pyrophosphate. These results are summarized in Table 2.



In the above reactions, six coloured substances were always detected by paper chromatography. All of the compounds contain no phosphate group and seem to be derivatives of CQC. Failure to isolate and identify the coloured substances makes the interpretation of the reaction mechanism more difficult.

Experimental

General Methods. Paper chromatography was carried out by the descending technique using Toyo Roshi No. 51 and No. 51A papers. The solvents used were isopropyl alcohol-concentrated ammonia - water (7 : 1 : 2 v/v) (Solvent A), or (8 : 1 : 1 v/v) (Solvent B) and isoamyl alcohol-5% disodium hydrogen phosphate (1 : 1 v/v) (Solvent C). The R_f values of different compounds are given in Tables 1 and 2. Except for

8) In general, on hydrolysis in aqueous alkali solution, P^1,P^2-dialkyl pyrophosphate does not afford the single phosphate (**2**), but inorganic phosphate, pyrophosphoric acid and alcohol are always contaminated.

Table 2. Hydrolysis of P^1, P^2-di-nucleoside 5′-pyrophosphates (**8**)

No.	Pyrophosphate (NppN)	(μmol)	CQC (mM)	Mixed solvent (m*l*)	Product: Yield of phosphate (pN) (%)	Product: R_f value*	Product: pN/NppN***
8a	Di-thymidine(5′)	4.4	0.1	DMF(2) water (0.05)	80	0.10	0.89
8b	Di-uridine(5′)	5.5	0.1	DMF(2) water (0.05)	72	0.05	0.76
8c	Di-adenosine(5′)	3.5	0.1	pyridine(1.75) DMF (1.75) water (0.05)	77	0.71**	0.72
8d	Di-N^6,*O*,*O*-tribenzoyl guanosine(5′)	19.0	0.25	pyridine(8) water(0.1)	32	0.07	0.74
8e	Di-cytidine(5′)	16.0	0.1	pyridine(8) DMF (2.4) water (1.6)	41	0.08	0.75

* Development with Solvent A.
** Development with Solvent C.
*** Mobility relative to the corresponding pyrophosphate.

the use of Solvent C, phosphorus compounds were made visible on paper chromatograms after spraying the chromatograms with the Isherwood Reagent[9] and exposing them to ultraviolet lamp. The ε_{max} value at 290 mμ used for alkyl *p*-nitrophenyl phosphates (**4**) was 10000 (pH : 7). Paper electrophoresis was carried out in an apparatus in which the paper was immersed in carbon tetrachloride. The buffers used were phosphate 0.05M KH_2PO_4 (pH 6, pH 7 and pH 8).

Materials. 2,6-Dichloroquinone *N*-chloroimide was a commercial sample (Tokyo Kasei Co., Tokyo, Japan). Alcohols and pyridine were purified and dried by ordinary procedures. *N*-Benzoyl guanosine 5′-phosphate,[7] *N*-benzoyl cytidine 5′-phosphate,[7] P^1,P^2-dithymidine 5′-pyrophospate (TppT),[11,12] P^1,P^2-di-adenosine 5′-pyrophosphate (AppA)[7] and P^1,P^2-di-uridine 5′-pyrophosphate (UppU)[10] were prepared by the procedures in literature. P^1,P^2-Di-*p*-nitrophenyl pyrophosphate (**3a**), P^1,P^2-di-*N*-benzoyl guanosine pyrophosphate ($G^{Bz}ppG^{Bz}$) and P^1,P^2-di-*N*-benzoyl cytidine pyrophosphate ($C^{Bz}ppC^{Bz}$) were prepared by a modification of Khorana's method[7,11] as described below.

P^1,P^2-Di-*p*-nitrophenyl Pyrophosphate. To a solution of *p*-nitrophenyl phosphate (**2a**) (0.438 g, 0.2 mM) in dry pyridine (5 m*l*) was added dicyclohexylcarbodiimide (0.247 g, 1.2 mM). To the mixture, after being stirred for 2 hr at room temperature, was added 1 m*l* of water. Stirring was continued for 1 hr at room temperature. A white precipitate, dicyclohexylurea, was filtered and washed with water. The filtrate and the washings were combined and concentrated under reduced pressure at a temperature below 40°C.

Evaporation was then repeated twice or three times with pyridine.

The flask was kept standing in a refrigerator overnight to afford P^1,P^2-di-*p*-nitrophenyl pyrophosphate (0.550 g, 95%) as a white powder: mp 166—167°C (di-pyridinium salt).

Found: C, 45.86; H, 3.59; N, 9.90. Calcd for $C_{22}H_{20}O_{11}N_4P_2$: C, 45.67; H, 3.46; N, 9.70%; mol wt, 578.

P^1,P^2-Di-*N*-benzoyl Guanosine 5′-Pyrophosphate ($G^{Bz}ppG^{Bz}$). *N*-Benzoyl guanosine 5′-phosphate (0.056 mM)[7] was rendered anhydrous by three repeated evaporations of 4 m*l* of dry pyridine. A gummy *N*-benzoyl quanosine 5′-phosphate was dissolved in 10 m*l* of dry pyridine and dicyclohexylcarbodiimide (35 mg) was then added. The reaction mixture was heated at 100° for 20 min and allowed to stand at room temperature overnight. The precipitate was filtered and washed with pyridine. The filtrate and the washings were combined and evaporated to dryness. White powder, thus obtained, was dissolved in 15 m*l* of water. The aqueous solution was washed with four portions of 20 m*l* of petroleum ether. The aqueous layer was treated with 2 m*l* of pyridine and evaporated to dryness. White powder was obtained and dry pyridine was added. The precipitate was filtered and washed with pyridine. The filtrate was evaporated to dryness. P^1,P^2-Di-*N*-benzoyl guanosine 5′-pyrophosphate (0.019 mM, 38%) was obtained as syrupy substance which was chromatographically pure. The yield was estimated spectrophotometrically. $\lambda_{max}^{H_2O}$ 292 mμ (ε : 16200), $\lambda_{max}^{H_2O}$ 259 mμ, $\lambda_{max}^{H_2O}$ 278 mμ.

P^1,P^2-Di-*N*-benzoyl Cytidine 5′-Pyrophosphate (CppC). *N*-Benzoyl cytidine 5′-phosphate[7] (0.046 mM) and 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine(13.5 mg) were dissolved in 10 m*l* of pyridine under anhydrous conditions at 100°C. After addition of dicyclohexylcarbodiimide (25 mg, 0.12 mM), the mixture was kept at 100°C for 10 min and was allowed to stand at room temperature for 1 day. After removal of pyridine, 10 m*l* of water was added. The gel was filtered and washed with three portions of petroleum ether. The aqueous layer was concentrated to dryness. The gummy residue was dissolved in 0.5 m*l* of ethanol and treated

9) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).
10) M. Smith, J. G. Moffatt and H. G. Khorana, *J. Amer. Chem. Soc.*, **80**, 6204 (1958).
11) H. G. Kohrana and J. P. Vizsolyi, *ibid.*, **81**, 4660 (1959).
12) M. W. Moon and H. G. Khorana, *ibid.*, **88**, 1798 (1966).

with 1 m*l* of concentrated ammonium hydroxide. The mixture was allowed to stand at room temperature for 3 days. A white precipitate appeared and the suspension was treated with 2 m*l* of pyridine. After removal of solvent, the residue was dissolved in 3 m*l* of water and neutralized with Dowex 50W-X8 (pyridinium form). The resin was filtered and washed thoroughly with water. The filtrate and the washings were concentrated to a small volume. Electrophoresis showed that the product contained almost pure P^1,P^2-di-cytidine 5′-pyrophosphate. The solution was passed through a column (45 cm×2 cm dia.) of commercially available diethylaminoethyl cellulose (DEAE) (bicarbonate form), elution being carried out with a linear salt gradient, 1*l* of water and 1*l* of 0.05M triethylammonium bicarbonate in a reservoir. About 15 m*l* fractions were collected every 10 min. Fractions 47—56 contained P^1,P^2-di-cytidine 5′-pyrophosphate. The yield of this product, as estimated spectrophotometrically, was 69%. The product obtained as the triethylammonium salt was converted in to the pyridinium salt by treatment with Dowex 50W-X8 (pyridinium form) and the solution was lyophilized.

Alcoholysis of P^1,P^2-Di-*p*-nitrophenyl Pyrophosphate (3a) in the Presence of CQC. To a solution of **3a** (0.134 g, 0.2 mM) and an alcohol (2 mM) in 20 m*l* of dry pyridine was added CQC (0.132 g, 0.63 mM), and the mixture was heated at 100°C for 2 hr. The solution was concentrated to a syrup, which was washed thoroughly three times with chloroform. The chloroform was combined and evaporated to dryness. The residue was washed three times with benzene. The benzene was evaporated and the residual oil was dissolved in 30 m*l* of water and washed with two portions of 30 m*l* of ether. After removal of water under reduced pressure, chromatographically pure alkyl *p*-nitrophenyl phosphate (**4**) was obtained as shown in Table 1. Estimation of the yield of this product was carried out spectrophotometrically after elution of spot from paper chromatogram run in Solvent A.

Hydrolysis of P^1,P^2-Di-*p*-Nitrophenyl Pyrophosphate (3a) in the Presence of CQC. To a solution of **3a** (0.314 g, 0.2 mM) in aqueous pyridine was added CQC (0.132 g, 0.63 mM), and the mixture was heated at 100°C for 2 hr. *p*-Nitrophenyl phosphate (**2a**) was obtained in 93% yield which was estimated spectrophotometrically after elution of the spot from paper chromatogram run in Solvent A.

Hydrolysis of P^1,P^2-Di-nucleoside 5′-Pyrophosphate in the Presence of CQC. In Table 2 are summarized the reaction under various conditions. A typical procedure is described below.

Hydrolysis of P^1,P^2-Di-adenosine 5′-Pyrophosphate. To a solution of P^1,P^2-di-adenosine 5′-pyrophosphate in 2.5 m*l* of pyridine-dimethylformamide (1 : 1) was added water and then CQC. The mixture was heated on a boiling water bath for 2 hr. Adenosine 5′-phosphate was obtained (see Table 2) which was estimated spectrophotometrically after elution of the spot from Toyo Roshi No. 51A paper run in phosphate buffer (pH 6) by electrophoresis.

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