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Chemical Properties of Quinol Phosphate Esters and Inhibition of Alcohol Dehydrogenase by Them

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Some phosphate and phosphorothiolate esters of hydroquinone and related phenols and thiophenols were prepared. Quinol phosphates were stable to alkaline hydrolysis but susceptible to oxidation. They inhibited yeast alcohol dehydrogenase probably owing to the oxidatively produced quinone.

In a previous paper (Eto ***et al.,*** 1975), we found the metabolic formation of a quinol phosphate in houseflies from triphenyl phosphate, which has been known as a malathion synergist (Plapp ***et al.,*** 1963). It has been also reported that the fungicide edifenphos (S, S-diphenyl ethyl phosphorodithiolate ; Hinosan®) was partially metabolized through p-hydroxylation in ***Pyricularia oryzae*** (Uesugi and Tomizawa, 1971). Quinol phosphates are known to be activated oxidatively (Blackburn and Cohen, 1968). By oxidation, a quinol phosphate will give a quinone which may react with SH-compounds. This paper describes that quinol phosphates are susceptible to oxidation and inhibit the SH-enzyme alcohol dehydrogenase.

MATERIALS AND METHODS

Synthesis

Diethyl p-hydroxyphenyl phosphate was prepared by the reaction of diethyl phosphite with p-benzoquinone according to Nishizawa (1961). Yield, 73 %. B. p., 105°C (0.005 mmHg). Anal. Found : C, 47.88 ; H, 6.22 ; P, 12.38. Calcd. for $C_{10}H_{15}O_5P$: C, 48.78 ; H, 6.10 ; P, 12.60 %. Diethyl p-hydroxy-2, 3, 5, 6-tetrachlorophenyl phosphate was similarly prepared from diethyl phosphite and chloranil. Yield, 17 %. M. p., 180-181°C. ***Anal.*** Found: C, 31.43; H, 3.23. Calcd. for $C_{10}H_{11}Cl_4O_5P$: C, 31.28; H, 2.89 %.

Diethyl p-methoxyphenyl phosphate was synthesized by the reaction of diethyl phosphorochloridate with p-methoxyphenol in the presence of pyridine. Yield, 14 %. B. p., 125-130°C (0.1 mmHg). ***Anal.*** Found: P, 11.12. Calcd. for $C_{11}H_{17}O_5P$: P, 11.90 %. Diphenyl p-hydroxyphenyl phosphate was prepared similarly from diphenyl phosphorochloridate and hydroquinone as described in the previous paper (Eto ***et al.,*** 1975). Diethyl S-p-hydroxyphenyl phosphorothiolate was synthesized in the same manner from diethyl phosphorochloridate and monothioquinol.

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The product could not be distilled and was submitted to a column chromatography. The structure was supported by the presence of OH stretching band at 3160 cm^{-1} in infrared spectrum and by the molecular ion of m/e *262* in mass spectrum.

Diethyl S-p-methoxyphenyl phosphorothiolate was prepared according to Hoffmann et al. (1956) by the reaction of p-methoxybenzenesulfonyl chloride and three molar equivalents of triethyl phosphite. Yield, 49 %. B. p., 120-135°C *(0.07* mmHg). *Anal.* Found: C, 47.81; H, 6.21. Calcd. for $C_{11}H_{17}O_4PS$: C, 47.66: H, 6.23 %.

Hydrolysis of phosphorus esters

The rate of hydrolysis of phosphorus esters *(6.25* x 10^{-4}M) was determined at **80°C** in a **50** % **(v/v)** aqueous isopropanol solution of 0.025N sodium hydroxide by colorimetric measurements of liberated phenols or thiophenols. The former was determined by 4-aminoantipyrine and potassium ferricyanide (Eto and Oshima, 1962) and the latter was by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959). A 0.8 ml aliquot withdrawn at appropriate time interval was neutralized by adding 0.2 ml of 0.1N hydrochloric acid. To the neutralized aliquot, 3.8 ml of 0.01 % 4-aminoantipyrine prepared in M/15 phosphate buffer, pH 8, and 0.2 ml of **0.8** % potassium ferricyanide were added. After 15 min. the optical density at **510** mμ was measured. For thiolate esters, 3.9 ml of phosphate buffer (M/15, pH 8.0) and 0.1 ml of a DTNB solution (39.6 mg/10 ml phosphate buffer) were added to the neutralized aliquot of the reaction mixture. The color intensity at 412 mμ was measured after 5 min.

Oxidation of phosphorus esters with bromine

To a methanolic solution (10 ml) containing 0.3 mmole of a phosphorus ester were added 37 ml of distilled water and 3 ml of saturated bromine water(0.5 mmole). The reaction mixture was shaken continuously at room temperature in a stoppered flask. After 30 min. a 5 ml aliquot was withdrawn to determine residual bromine as follows: The aliquot was run into 10 ml of 1 % potassium iodide solution and liberated iodine was titrated with 0.002N sodium thiosulfate.

Assay of alcohol dehydrogenase inhibition

The activity of yeast alcohol dehydrogenase (EC 1.1.1.1) (ADH, purchased from Sigma Chemical Co.) was assayed according to Racker (1955). A test compound was placed in a test tube by evaporating the acetone solution under reduced pressure. One ml of a **0.002** % ADH solution in 0.01M potassium phosphate buffer, pH 7.5, was poured into the tube and incubated at 25°C for 1 hr. To the mixture were added successively 0.5 ml of 0.06M sodium pyrophosphate buffer (pH 8.5), 0.1 ml of 3M ethanol, 1.3 ml of distilled water, and 0.1 ml of 0.0015M NAD. The first absorbance reading at 340 mμ was made at 20 sec. after the addition of NAD and further readings were recorded at 15-second intervals.

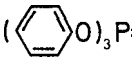
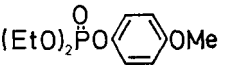
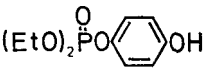
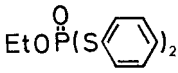
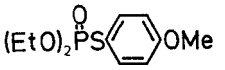
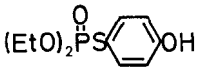
RESULTS

Chemical properties of quinol phosphates and related esters

As expected from the chemical structure of examined phosphate esters, they

were relatively stable to hydrolysis; they were scarcely hydrolyzed in M/15 phosphate buffer (pH 7.7) at 30°C for 3 hr. They suffered hydrolysis in caustic alkaline solutions at higher temperature. The first-order hydrolysis constant (k_{hyd}) and the half-life ($t_{1/2}$) of esters in a ***50 % (v/v)*** aqueous isopropanol solution of 0.025N sodium hydroxide at 80°C were calculated and the results are given in Table 1.

Table 1. Alkaline hydrolysis and bromine oxidation of some phosphorus esters.

Phosphorus ester	Alkaline hydrolysis[a]		Br, consumed[b] (molar equivalent)
	$k_{hyd}(min^{-1})\times10^2$	$t_{1/2}(min)$	
	14.6	4.7	0
	0.88	76.8	1.0
	0.14	505.8	1.3
	>69.3[c]	(1"'	>1.7[e]
	28.6	2.4	>1.7[e]
	<0.57[d]	>120[d]	

a) In 50 % ***(v/v> aq.*** isopropanol solution of 0.025N NaOH at 80°C.
b) 0.3 mmole ester was treated with 0.5 mmole Br_2 in aq. methanol (1 : 5) at room temperature for 30 min.
c) Hydrolysis rate was too fast to be measured.
d) No appreciable hydrolysis occurred within 120 min.
e) Bromine added in the reaction mixture ***was*** consumed completely.

Diethyl p-hydroxyphenyl phosphate resisted greatly to alkaline hydrolysis and was about 100 times more stable than triphenyl phosphate. This may be attributed to the electron-releasing property of p-hydroxyl group (σ = -0.37). The hydroxyl group may ionize under the experiment conditions to release electrons much more efficiently; the σ value of ***p-O-*** is −0.519. The unionizable methyl ether, whose substituent constant σ is -0.263, was hydrolyzed 6 times faster than the hydroxy compound.

Phosphorothiolates were more labile to alkaline hydrolysis than phosphate esters. The effect of ring-substituents on the phosphorothiolate hydrolysis resembles that in phosphate esters. The hydrolysis of S, S-diphenyl ethyl phosphoro-

dithiolate (edifenphos) proceeded too rapidly to be measured accurately by the employed method. Diethyl S-p-methoxyphenyl phosphorothiolate was hydrolyzed 30 times as fast as the corresponding phosphate ester. On the other hand, the hydroxyphenyl phosphorothiolate was not hydrolyzed appreciably under the described conditions.

Table 1 also shows the reactivity of phosphorus esters with bromine in aqueous solutions at room temperature, Triphenyl phosphate labile to alkaline hydrolysis was stable to bromine oxidation. Although resistant to alkaline hydrolysis, diethyl p-hydroxyphenyl phosphate was rapidly decomposed by the action of bromine in an aqueous solution. The oxidative decomposition occurred even with its methyl ether as well, suggesting dehydrogenation might not precede for the reaction. Both the esters consumed approximately equivalent amount of bromine and gave same quinones on the basis of thin layer chromatography. The quinones contained bromine atoms in the molecule. p-Benzoquinone produced by oxidation may add hydrogen bromide to give bromohydroquinones, which may further be oxidized into bromoquinones.

On the other hand, phosphorothiolates were oxidized, consuming bromine more than equivalent, regardless of their substituents at para position on the benzene ring. S, S-Diphenyl ethyl phosphorodithiolate yielded phenyl disulfide and benzenesulfonic acid, which were identified by comparison with the authentic samples on thin layer and paper chromatographies. Diethyl S-p-methoxyphenyl phosphorothiolate gave the corresponding disulfide by bromine oxidation.

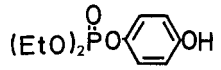
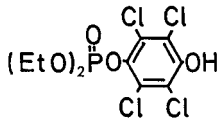
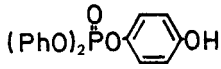
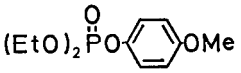
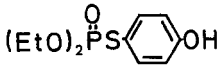
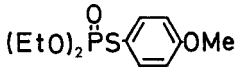
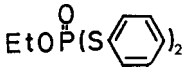
Inhibition of alcohol dehydrogenase

As shown in Table 2, quinol phosphates having a free hydroxyl group on the aromatic ring showed considerable activity to inhibit yeast alcohol dehydrogenase, an SH-enzyme. The corresponding methyl ether had no appreciable activity. The same tendency in the effect of p-substituent on the dehydrogenase inhibition was observed in a series of phosphorothiolates. The phosphorothiolate edifenphos which has no substituent on p-position did not inhibit the enzyme.

As mentioned above, quinol phosphates are oxidatively decomposed to give quinone, which is known to inhibit a variety of enzymes including SH-enzymes (Webb, 1966). Table 3 shows the inhibitory activity of quinones and some related compounds toward alcohol dehydrogenase. Hydroquinone as well as quinones inhibited the enzyme completely at 3.3×10^{-4}M. The corresponding mono-ether and -thiol derivatives had no or very weak activity. Thiophenol and disulfide showed only weak activity.

The effect of some SH-compounds on the inhibition of alcohol dehydrogenase by a quinol phosphate was then investigated. When alcohol dehydrogenase was treated with diethyl p-hydroxyphenyl phosphate in the presence of the equimolar amount of cysteine, no inhibition of the enzyme was observed as shown in Table 4. Similar effect was obtained by another aliphatic mercaptan, 2-mercaptoethanol, but thiophenol had no such ability to protect the enzyme from inhibition by the quinol phosphate. The amino group appeared to have little influence on the inhibitory activity of the quinol phosphate as exemplified by glycine. Plasma albumin has some protective effect for alcohol dehydrogenase.

Table 2. Inhibitory activity of quinol phosphates and related compounds toward alcohol dehvdrogenase.

Inhibitor	I_{50} (M)
	1.3×10^{-5}
	8.5×10^{-5}
	1.5×10^{-4}
	$>3.3 \times 10^{-4}$ (0%)[a]
	5.5×10^{-5}
	$>3.3 \times 10^{-4}$ (10%)[a]
	$>3.3 \times 10^{-4}$ (0%)[a]

a) Inhibition % at 3.3×10^{-4}M.

Table 3. Inhibition of alcohol dehydrogenase by quinones and related compounds at 3.3×10^{-4}M.

Inhibitor	Inhibition (%)
p-Benzoquinone	100
Chloranil	100
Hydroquinone	100
p-Methoxyphenol	0
p-Hydroxythiophenol	25
Thiophenol	8
Phenyl disulfide	36

DISCUSSION

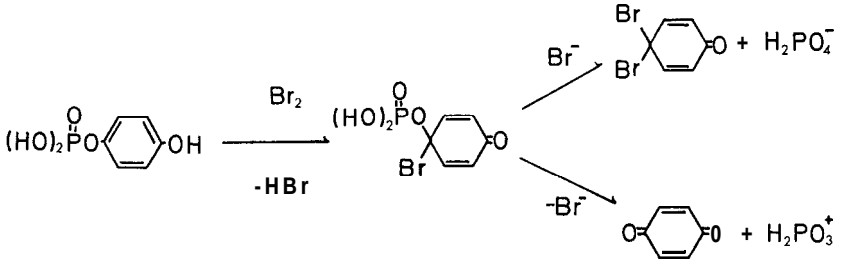
The oxidation of quinol phosphate has been well studied as a model of mitochondrial oxidative phosphorylation reaction (Blackburn and Cohen, 1968). According to the applied conditions, either the P-O or C-O bond is cleaved by

Table 4. Effect of SH-compounds on alcohol dehydrogenase inhibition by diethyl p-hydroxyphenyl phosphate.

Conc. of quinol phosphate ($M \times 10^5$)	Compound added (conc. $M \times 10^5$)	Inhibition (%)
33	none	100
3.3	none	74
3.3	cysteine (3.3)	negligible
3.3	mercaptoethanol (3.3)	22
3.3	thiouhenol (3.3)	89
3.3	glycine (3.3)	69
3.3	$NaHSO_3$ (3.3)	54
33	albumin[a)]	negligible
330	albumin")	79

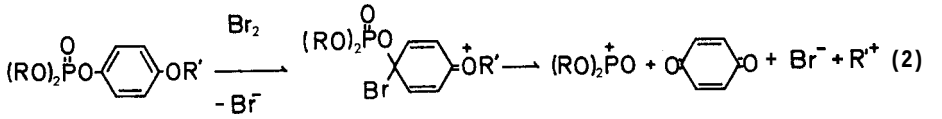
a) 0.033 %

oxidation (Clark *et al.*, 1961; Dürckheimer and Cohen, 1964). Lapidot and Samuel (1964) suggested that there were two parallel reaction pathways *via* a quinonoid intermediate for the breakdown of a quinol phosphate in the presence of bromine in a dimethylformamide solution (Scheme 1).



(1)

We found that the methyl ether derivative as well as a quinol phosphate was oxidized to give same quinones. This suggests that dehydrogenation does not necessarily precede for the bromine oxidation of quinol phosphates. Thus, the following scheme is proposed for the reaction.

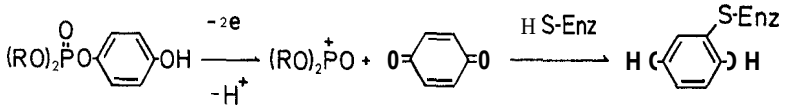


(2)

Thiol esters were very different in behavior toward the oxidant. The P-S bond was oxidatively cleaved by the action of bromine to give disulfide and sulfonic acid. This indicates that phosphorothiolates are activated by oxidation regardless of the absence of any electron-releasing substituent on the benzene ring. The oxidative activation of phosphorothiolates was applied for the synthetic purpose of phosphate esters by Eto et *al.* **(1974).**

For the inhibition of alcohol dehydrogenase, however, the free hydroxyl group is necessary on the benzene ring of phosphate esters. From the facts

that benzoquinone is a strong inhibitor of alcohol dehydrogenase as shown in Table 3 and reacts readily with SH-compounds to give addition products (Snell and Weissberger, 1939), it is reasonable to presume that quinol phosphates may be converted at first into a quinone to inhibit alcohol dehydrogenase through reaction with the SH-group of the enzyme (Scheme 3).



(3)

This presumption was supported by the protective effect of some SH-compounds such as cysteine for alcohol dehydrogenase inhibition by a quinol phosphate. The quinol phosphate itself did not react with cysteine, but gave an additive product with cysteine immediately after treatment with bromine water. The product was identical with that from p-benzoquinone and cysteine. The inhibition of alcohol dchydrugenasc by the quinol phosphate was not due to quinone which might be contained in the ester preparation as a possible impurity, but probably due to that produced in the course of reaction from the parent phosphate ester; although a mixture of diethyl p-hydroxyphenyl phosphate and cysteint did not show any inhibitory activity as shown in Table 4, an ether extract of the mixture inhibited the enzyme as well as the non-treated quinol phosphate. It is not known how the quinone was produced from the ester. It was interesting to examine if quinol phosphates could be dehydrogenated by alcohol dehydrogenase. By the ordinary assay method for alcohol dehydrogenase activity, it was not observed even in the presence of cysteine that the quinol phosphate served as the substrate of the dehydrogenase. Autoxidation may be one of the other possible mechanisms for the production of quinone.

Many neutral organophosphorus esters show strong inhibitory activity against serine enzymes including esterases and proteinases. Quinol phosphate esters are the poorer inhibitors of esterases than the corresponding phenyl ester, owing to the electron-releasing property of the hydroxyl group, as demonstrated in the previous paper (Eto *et al., 1975).* Some phosphorus esters are known to inhibit glutamate dehydrogenasc (Freedland and McFarland, 1965). In previous papers, we indicated that some saligcnin cyclic phosphorus esters inhibited alcohol dehydrogenase *via* the partial hydrolysis product o-hydroxybenzyl phosphates (Ohkawa and Eto, 1969 ; Ohkawa *et al.,* 1972) .Quinol phosphates are an additional example of phosphorus esters which inhibit dehydrogenases.

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