TITLE:

Quinol Phosphate as a Metabolite of Triphenyl Phosphate

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in the present experiment, being effective to prevent from disappearance of the red pigment from epidermal cells.

The effects of the other two chemicals, *Cecropia* JH (methyl-12, 14-dihomojuvenate) and 1-(3, 4-methylenedioxyphenyl)-7-epoxy-4, 8-dimethylnona -1, 3-diene (CT 5) on the developmental process were tested in order to compare with those of ZR 512, on the 4th day in the last instar. *Cecropia* JH was active as ZR 512, but CT 5 was inactive up to the dosage of 50 μg.

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Quinol Phosphate as a Metabolite of Triphenyl Phosphate. Morifusa ETO, Hiroto MIYAMOTO* and Yasuaki HASHIMOTO** (Department of Agricultural Chemistry, Kyushu University, Fukuoka, Japan). Received April 28, 1975. *Botyu-Kagaku*, 40, 106, 1975.

20. リン酸トリフェニルの代謝産物としてのキノールリン酸エステル 江藤守総, 宮本公人*, 橋本恭明** (九州大学農学部農芸化学科)

マラソンの共力剤である triphenyl phosphate (TPP) をイエバエに投与し, 代謝産物として diphenyl *p*-hydroxyphenyl phosphate (TPP-OH) を得た. TPP-OH はカルボキシエステラーゼ阻害活性の弱い TPP よりもさらに弱い活性しか示さなかった. TPP のマラソン共力作用はエステラーゼ阻害によらず, 他の機構によるものではないかと推察される.

With a few exceptions, triaryl phosphates which manifest synergistic activity with malathion have at least one alkyl group on the ortho position[1]. They may be biotransformed into active metabolites, i.e. cyclic phosphate esters, which have a high antiesterase activity, as demonstrated with tri-*o*-tolyl phosphate and some related esters[2,3]. The exceptions are tri-*p*-ethylphenyl phosphate and triphenyl phosphate. The metabolic activation of the former has been shown by finding of the formation of α-oxo derivatives[4]. The bioactivation of all these alkylaryl phosphates is initiated by the hydroxylation of the α-carbon of the alkyl group followed by such a subsequent reaction as intramolecular transphosphorylation or dehydrogenation[5]. This paper deals with the metabolic ring-hydroxylation of triphenyl phosphate (TPP) in houseflies to give a quinol phosphate.

Materials and Methods

Syntheses

Triphenyl phosphate was prepared by heating the mixture of phenol and phosphorus oxychloride under reflux. It was distilled under reduced

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pressure, b. p. 180°C (0.08 mmHg), and the solidified distillate was recrystallized from methanol, m. p. 48°C.

Diphenyl p-hydroxyphenyl phosphate (TPP-OH) was synthesized by adding dropwise diphenyl phosphorochloridate (5.4 g) to a stirred mixture of hydroquinone (2.2 g), pyridine (1.6 g), ether (20 ml) and a small amount of acetonitrile in an ice-bath. The reaction mixture was left overnight at room temperature and washed with water, diluted hydrochloric acid, aqueous sodium bicarbonate solution, and water. The solvent of the separated organic layer being removed under reduced pressure after dried over anhydrous sodium sulfate, the residue was solidified. Yield, 4.1 g. It was purified by repeated recrystallization and thin layer chromatography, m. p. 97-98°C. *Anal.* Found: P, 9.22% Calcd. for $C_{18}H_{15}O_5P$: P, 9.07% UV λ_{max}^{EtOH} nm (ε): 280 (2020). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3250 (OH), 1590, 1485, 830 (p-substituted aromatic ring), 1280 (P=O), 1170, 1160, 960 (P-O-C aryl).

Chromatographies

Thin layer chromatography (TLC) was performed by employing silica gel G after Stahl and three kinds of solvent systems: A) chloroform: ether=3 : 1, B) n-hexane : acetone=4 : 1, and C) benzene : n-hexane : ethyl acetate=2 : 2 : 1. Phenolic compounds were visualized by spraying diazotized sulfanilic acid after hydrolysis with potassium hydroxide[3] or by spraying ferric chloride and potassium ferricyanide[6]. The latter was very sensitive to p-hydroxyphenyl phosphates.

Gas chromatography was performed by using a Nippon Denshi gas chromatograph JGC-1100-FP. Conditions employed was as follows: column; 10% silicone rubber SE-30 on Diasolid L (60-80 mesh) 3 mm×100cm glass column, 265°C. Detector; thermionic detector with KBr tip. Carrier gas; N$_2$ 0.72 kg/cm^2.

Diphenyl p-hydroxyphenyl phosphate (TPP-OH) and isolated metabolites were submitted to gas chromatograph after trimethylsililation according to Zinkel *et al.* in dry petroleum ether with trimethylchlorosilane and hexamethyldisilazane at 70-80°C[18]. By this treatment the IR band of the hydroxyl group of TPP-OH disappeared completely.

Treatment of houseflies and extraction of metabolites

An acetone solution of TPP (20 mg/ml) was applied on susceptible 2-day-old oriental houseflies (*Musca domestica vicina* Macquart, Sapporo strain), which were anesthetized with carbon dioxide, in a beaker at the dosage of 1mg/g. The houseflies were gently stirred with a spoon in order to spread the chemical over all individuals as homogeneously as possible. The treated flies were reared in a cage at 25°C for 24 hr. They were homogenized in acetone with a Waring blender. The homogenate was filtered and the residue was reextracted with acetone. The combined filtrates were concentrated to a small volume under reduced pressure and extracted with petroleum ether.

Enzyme activity assay

Twenty % rat liver homogenate in 0.25M sucrose obtained after centrifugation at 95,000×g and 10% housefly homogenate in 0.9% sodium chloride were used as esterase sources. Esterase activity was assayed with the Warburg-manometric method employing ethyl malonate (16mM) as the substrate at 37°C and 30°C, respectively, for rat liver esterase and housefly esterase. The final reaction medium contained 14mM sodium bicarbonate and was gassed with a 95% nitrogen-5% carbon dioxide mixture. Antiesterase agent was previously placed on the bottom of the flask by evaporating the desired amount of its acetone solution.

Other methods

Infrared spectra were obtained from chloroform solutions with a Shimazu infrared spectrometer IR-27G. Mass spectra were made with a Nippon Denshi mass spectrometer JMS-01 SG at 75 eV.

Results and Discussion

Isolation of diphenyl p-hydroxyphenyl phosphate as a metabolite of triphenyl phosphate

A total of 530 mg of TPP was applied to about 27,000 houseflies (combined weight, 530g). Metabolites were extracted after 24 hours and purified by TLC. If the biotransformation of TPP into active metabolites occurred, it may not be hydrolytic[5]. Since the most probable metabolic process, besides the ester hydrolysis, is ring

hydroxylation and the authentic sample of TPP-OH has Rf values of 0.55 and 0.11 on TLC developed by the solvent systems A and B, respectively, the corresponding zone was eluted and chromatography was repeated several times on using these two kinds of solvent systems. About 1 mg of the purified metabolite was finally obtained. It gave positive test to ferric chloride-potassium ferricyanide reagent, to which TPP gave negative but TPP-OH positive test. As shown in Table 1, the metabolite was identical with the authentic TPP-OH in the behavior on TLC.

Table 1. Chromatographic behavior of the isolated metabolite of TPP

	TLC[a] Rf			GLC[b] t_R
	A	B	C	
TPP	0.88	0.98	—	0.85
TPP-OH	0.55	0.11	0.22	1.7
Metabolite	0.55	0.11	0.22	1.7

a. Solvent systems
　A. CHCl$_3$: ether=3 : 1
　B. n-Hexane: acetone=4 : 1
　C. Benzene: n-hexane: ethyl acetate=2 : 2 : 1
b. TPP-OH and the metabolite were trimethylsililated.
Column: 10% silicone rubber SE-30 on Diasolid L (60-80 mesh) 3mm×100cm glass column, 265°C
Detector: flame thermionic with KBr tip, 310°C
Carrier gas: N$_2$ 0.72 kg/cm^2

The metabolite was then treated with trimethylchlorosilane and hexamethyldisilazane and submitted to a gas-liquid chromatograph equiped with a thermionic detector. The trimethylsililated metabolite gave a single peak whose retention time (1.7 min) was identical with the authentic diphenyl p-trimethylsililoxyphenyl phosphate as shown in Table 1. The mixture of them gave a single peak. The retention time of TPP under the same condition was 0.85 min. The metabolite gave a molecular ion peak of m/e 340 as was expected of TPP-OH. All these evidence indicates that the metabolite is diphenyl p-hydroxyphenyl phosphate.

Aromatic hydroxylation is a rather common

metabolic pattern of aromatic foreign compounds. However, it is rarely observed with organophosphorus pesticides. As a minor metabolite of the fungicide edifenphos (S, S-diphenyl ethyl phosphorodithiolate, Hinosan), its p-hydroxy metabolite was produced in vivo in rats, cockroaches, and the fungus *Pyricularia oryzae*[1].

Udenfriend's model system[8] for biological oxidation consisting of ascorbic acid (14mM), ferric sulfate (1.3 mM), EDTA (6.5 mM) in phosphate buffer (250 ml, 1M, pH 6.7) was applied for the oxidation of TPP (6 mM) and the formation of TPP-OH was confirmed by TLC after 2hr shaking in an atomosphere of oxygen at 37°C.

Biological activities of diphenyl p-hydroxyphenyl phosphate

It has been believed that the degradation of malathion in mammals and resistant insects owes mainly to carboxyesterase activity[5]. The esterase is inhibited by some malathion synergists such as the oxon analog of EPN, n-propyl paraoxon and saligenin cyclic aryl phosphates[9,10].

The antiesterase activity of TPP and TPP-OH was assayed on using rat liver homogenate and housefly homogenate as enzyme sources and diethyl malonate as the substrate[11]. The liver esterase was so sensitive to both the phosphate esters as to be inhibited 50% at concentrations of about 10^{-7}M. On the other hand, housefly carboxyesterase resisted to the phosphate esters; I$_{50}$ values were about 10^{-4}M. The quinol phosphate TPP-OH was always twice less active than the parent compound TPP (Table 2). This appears

Table 2. Antiesterase activity of triphenyl phosphate (TPP) and diphenyl p-hydroxyphenyl phosphate (TPP-OH)

Inhibitor	I$_{50}$ Carboxyesterase (M)	
	Rat liver	Housefly
TPP	2×10^{-7}	1×10^{-4}
TPP-OH	4.3×10^{-7}	2×10^{-4}

reasonable from the electron-releasing property of p-hydroxyl group; the substituent constant σ is −0.357. The less activity of TPP-OH coincides with the higher stability of quinol phosphates against alkaline hydrolysis as will be demonstrated in the following paper[12].

The poor activity in insect esterase inhibition of TPP[13] and its hydroxy metabolite suggests that the malathion synergism of TPP may be due to other metabolites with antiesterase activity or to an unknown mechanism other than carboxyesterase inhibition proposed by Plapp[14]. The latter appears to be more likely to occur than the former. Dyte and Rowlands reported that TPP retarded the in vivo production of dimethyl hydrogen phosphorothioate from malathion in the resistant strain of *Tribolium castaneum*, suggesting the inhibition of P–S bond cleavage by TPP or its metabolite(s)[15]. Whether the P–S bond is splitted by hydrolases or other enzymes such as oxygenases is not yet esterblished.

TPP-OH did not synergize malathion against susceptible houseflies, and both susceptible and resistant strains of leafhoppers (*Nephotettix cincticeps*) and planthoppers (*Laodelphax striatella*) by contact application[16]. Thus, the ring-hydroxylation does not appear to cause the activation of TPP. However, the high polarity of the hydroxylated product should be taken into account for the poor biological activity by the contact application. Although quinol phosphates are generally stable to nucleophilic attack, they are very active under oxidative conditions[17]. As will be demonstrated in our following papers, quinol phosphates have ability to inhibit alcohol dehydrogenase, an SH-enzyme, and some related analogs have considerable biological activities[12,16].

Summary

In vivo transformation of triphenyl phosphate (TPP) into diphenyl *p*-hydroxyphenyl phosphate (TPP-OH) was confirmed by isolating the metabolite from houseflies treated with TPP. TPP-OH was less active as antiesterase agent than TPP, which itself was only a poor inhibitor of esterases. The synergism of malathion by TPP appears to be due to other mechanism than carboxyesterase inhibition.

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