Original Article

Penetration and Metabolism of Bicyclic Phosphorus Esters Which Affect Their Insecticidal Activity against the Housefly

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Pretreatment with the microsomal oxidase inhibitor piperonyl butoxide (pb) greatly improved the topical insecticidal activity of the GABA antagonist, 4-*n*-propyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane 1-oxide (4-*n*-propyl BP),* against the housefly, *Musca domestica* L., when the synergistic ratio was 313. The insecticidal activity was also enhanced by bypassing the integument with injection without pb pretreatment. When tested by four different methods, the order of the 24-hr LD₅₀ values (μg/fly) for 4-*n*-propyl BP was: injection, pretreated with pb (0.169) < topical application with pb (0.212) < injection (0.449) < topical application (66.4). Penetration studies indicated that 4-*n*-propyl BP caused marked mortality at the internal level of 0.2–0.3 μg per fly, although the penetration was slow. The topically applied thiono and phosphite analogs without pb were approximately as insecticidal as the oxon analog injected. The thiono analog had the lowest 24-hr LD₅₀ value (with pb) among its analogs, which was 0.061 μg per fly. The lethal level of the thionate ester in the housefly body was estimated to be one-tenth of that of 4-*n*-propyl BP. The difference in the internal lethal level between 4-*n*-propyl BP and its thiono analog seems to be related to the difference in their insecticidal activity when they are topically applied without pb.

INTRODUCTION

Bicyclic phosphorus esters described here are a group of compounds possessing a structure of 2,6,7-trioxa-1-phosphabicyclo[2.2.2]-octane, including phosphites, phosphates and phosphorothionates. These esters with appropriate substituents are neurotoxic to insects as well as to mammals.^{1,2)} The compounds are different from usual organophosphorus insecticides and quite unique in that they interfere with the inhibitory neurophysiological action of γ -aminobutyric acid (GABA) in synapses.^{3–5)} Also, it is likely that their GABA antagonistic action results from the blockade

of chloride ion channels caused by binding noncovalently to a synaptic site for a plant-origin toxicant picrotoxinin, although there is no conclusive evidence for it such as BP inhibition of chloride uptake.^{6–8)}

We studied the structure-insecticidal activity relationship of bicyclic phosphates with the housefly, *Musca domestica* L., as a part of research on the GABA neurotransmission system of insects.²⁾ Our findings showed that bicyclic phosphates topically applied without any synergists were inactive as insecticides, while they exerted insecticidal activity by injection. The inactivity by topical application was ascribed to their low penetration rate to the housefly body. The present study was initiated to evaluate the influence of penetration and detoxication factors on the insecticidal activity of bicyclic phosphorus esters.

* Bicyclic phosphates are abbreviated as BPs; e.g., 4-*n*-propyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]-octane 1-oxide (**2**) is referred to as 4-*n*-propyl BP.

MATERIALS AND METHODS

1. Chemicals

Bicyclic phosphorus esters were prepared by procedures previously described.⁹⁾ Piperonyl butoxide (pb) was purchased from Tokyo Kasei Kogyo Co., Ltd.

2. Treatment of Houseflies

An acetone solution (1 μ l/fly) of bicyclic phosphorus esters was topically applied on the mesonotum of an adult female housefly (3–5 days old) which had been lightly anesthetized with carbon dioxide. When used, an acetone solution of pb (10 μ g/1 μ l/fly) was similarly applied 1 hr prior to a toxicant application. After treatment, 15 flies were immediately released in a glass jar (5.8 cm in diameter and 10.5 cm in height) containing a cotton pad soaked with sucrose solution, and held at 25°C and 50% R. H. under continuous lighting. Triplicate groups of 15 houseflies were used for each dosage to determine LD₅₀ values. In penetration studies, a group of 15 treated houseflies were removed from the jars at appropriate time intervals and were washed with successive portions of acetone (30 ml of total volume). Then, the rinsed houseflies were transferred into a mortar and ground in acetone with a pestle. Further homogenization was performed with a Teflon-glass homogenizer. The total volume of acetone used for this procedure was 50 ml, including acetone for rinsing of glasswares *etc.*

3. Cleanup of Extracts

The acetone rinse of the housefly surfaces was concentrated to about 1 ml under reduced pressure below 50°C, and then brought to dryness with a gentle jet of air. The residue was transferred into a separatory funnel alternately with three 10-ml portions of hexane and of hexane-saturated acetonitrile, and the funnel was shaken for 10 min. The layer (30 ml) of hexane-saturated acetonitrile was concentrated *in vacuo* below 50°C until most of the solvent was eliminated, and then the remaining solvent was evaporated to dryness with a fine stream of air. The residue was transferred into another separatory funnel with chloroform (30 ml of total volume), to

which 10% (w/v) NaCl solution (20 ml) was added, and then the funnel was shaken for 10 min. Extraction with chloroform was repeated three times and the combined chloroform layer was concentrated in a similar manner after being dried over anhydrous sodium sulfate ("external"). This procedure gave 95.0% and 90.2% recoveries for 4-*n*-propyl BP (**2**) and its thiono analog (**5**) in control experiments, respectively.

The acetone homogenate of houseflies was filtered to remove debris through Toyo No. 2 filter paper on which sea sand (20–50 mesh) was placed. Other procedures were the same used for the "external" fraction except that fatty materials were removed with a 30-ml portion and additional two 20-ml portions of hexane. This extract from fly bodies is referred to as the "internal" fraction. The recoveries were 87.0% and 81.7% for **2** and **5** in control experiments, respectively.

The inside walls of the jars, in which treated flies were held, were washed with acetone (40 ml of total volume) to collect residues excreted or brushed off by the flies. The samples were cleaned up similarly to the "external" fraction, being designated as the "deposited" fraction.

No loss of **2** was observed when the compound (15 μ g) was kept on a glass plate at 25°C during the period of bioassay. Hence, it was concluded that the loss of **2** by volatilization from the housefly body was negligible.

4. Chemical Analysis

An appropriate volume of acetone was added to the cleaned-up residue. The amounts of the parent compounds, **2** and **5**, were determined with a Shimadzu GC-7A gas chromatograph equipped with an FTD-8 (Rb) detector. The conditions of analysis were: a 3 mm $\times$ 1 m glass column packed with 3% OV-17 on Chromosorb W (AW-DMCS), 80/100 mesh; column temp., 260°C; injection temp., 290°C or 300°C; the flow rate of carrier gas helium, 30 ml/min. An acetone solution of 5 ppm 4-(1-hydroxyethyl)-2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane 1-sulfide (**7**) was used as an internal standard. The retention times of **2**, **5** and **7** were 1.0 min, 0.9 min and 1.5 min under the above conditions, respectively.

RESULTS

1. Insecticidal Activity

Table 1 summarizes the insecticidal activity of various bicyclic phosphorus esters determined by four different methods. As described in our earlier paper,²⁾ 4-*n*-propyl BP was extremely low in insecticidal activity by topical application without any synergists. However, it was found that LD₅₀ values for **2** decreased with the elapse of time after topical application (Fig. 1). Also, the pretreatment of houseflies with the microsomal oxidase inhibitor piperonyl butoxide (pb) enhanced its insecticidal activity up to a level comparable to that determined by injection. The synergistic ratio was 313. When tested by four different meth-

Fig. 1 Topical LD₅₀ values of 4-*n*-propyl BP determined at various intervals after application without synergists.

ods, the toxicity of **2** was in the following order: injection, pretreated with pb > topical application with pb > injection >> topical application.

As for the 4-*n*-propyl thiono (**5**) and phosphite (**6**) analogs, a topical application method was effective, even without pb. Compounds **5** and **6** by topical application were as active as compound **2** by injection. Especially **5** with pb was most potent among bicyclic phosphorus analogs already reported.²⁾ The synergistic ratios of **5** and **6** with pb were 4.59 and 2.18, respectively. Included in Table 1 for comparison are the LD₅₀ values of the most active oxon analog **3** and the GABA antagonist picrotoxinin.

2. Penetration, Accumulation and Mortality

Figure 2 shows the plots of the percentages of internal, external 4-*n*-propyl BP (**2**) and the compound deposited in jars against time (topical application without pb, 1 µg/fly). The percentage of total recovery of **2** depicted by a dotted line gradually declined with the elapse of time. The disappearance of **2** is considered to be due to metabolism. The integument penetration of **2** was found to be slow, judged from the recovery from the housefly surface. It took 2–3 days for 50% of **2** to be absorbed. First-order plots of amount of external **2** indicated that the penetration proceeded in a biphasic

Table 1 Insecticidal activity of bicyclic phosphorus compounds to houseflies.

LD ₅₀ (μg/fly)		Synergistic ratio
				Alone	With pb	
No.	R ¹	R ²	X			
Injection ^{a)}						
1	<i>t</i> -C ₄ H ₉	H	P=O	0.705	0.124	5.69
2	<i>n</i> -C ₃ H ₇	H	P=O	0.449	0.169	2.66
3	<i>n</i> -C ₃ H ₇	CH ₃	P=O	NT ^{b)}	0.102	—
4	Picrotoxinin			NT	0.106	—
Topical application						
2	<i>n</i> -C ₃ H ₇	H	P=O	66.4	0.212	313
5	<i>n</i> -C ₃ H ₇	H	P=S	0.276	0.061	4.59
6	<i>n</i> -C ₃ H ₇	H	P	0.468	0.215	2.18

a) Taken from Ref. 2) for comparison.

b) Not tested.

Fig. 2 Time course of penetration, accumulation and deposition of 4-*n*-propyl BP.

Totally recovered (.....), external (○—○), internal (●—●) and deposited (□—□) 4-*n*-propyl BP are represented in the case of topical application at the dosage of 1 $\mu\text{g}/\text{fly}$ without synergists.

Fig. 3 First-order plots of 4-*n*-propyl BP (A: 1 $\mu\text{g}/\text{fly}$, alone; B: 1 $\mu\text{g}/\text{fly}$, with pb) and its thiono analog (C: 1 $\mu\text{g}/\text{fly}$, alone) recovered in the external fraction when topically applied.

X_0 : amounts of BP at 0 hr, X_t : amounts of BP at t hr.

manner with steep ($t_{1/2}=26$ hr) and gentle ($t_{1/2}=114$ hr) slopes (Fig. 3A).

Figure 4 shows the time course of the accumulation of **2** in the fly body and that of the housefly mortality when **2** was topically applied at 1 μg per fly (A) and 5 μg per fly (B). The internal amount of **2** reached *ca.* 0.04 μg per fly and *ca.* 0.16 μg per fly at 96 hr when treated with 1 μg per fly and 5 μg per fly, respectively. At these dosages, **2** caused no or little mortality during the 96-hr period of

Fig. 4 Amounts of internal 4-*n*-propyl BP (○) and mortality of houseflies (●) at the dosages of 1 $\mu\text{g}/\text{fly}$ (A) and 5 $\mu\text{g}/\text{fly}$ (B) during the 96-hr period of bioassay.

Fig. 5 Time course of internal accumulation of 4-*n*-propyl BP (○) and mortality of houseflies (●) when topically applied at the dosages of 12 $\mu\text{g}/\text{fly}$ without pb (A) and of 1 $\mu\text{g}/\text{fly}$ with pb (B).

assay. By contrast, treatment with 12 μg per fly markedly increased both the internal accumulation and mortality after 24 hr (Fig. 5A). Mortality attained about 50% when the internal accumulation level reached approximately 0.3 μg per fly. After 96 hr, the level of internal **2** increased to about 0.52 μg per fly to result in 100% mortality. When houseflies were pretreated with 10 μg of pb per fly prior to treatment with 1 μg of **2** per fly, the level of internal **2** reached 0.26 μg per fly, which resulted in 100% mortality as early as 6 hr (Fig. 5B). The amount of **2** that penetrated into the fly body was slightly larger in pb-treated flies than in untreated ones at the dosage of 1 μg per fly. However, the difference does not seem to be significant. The penetration rate was little influenced by pb treatment (Figs. 3A and 3B).

Similar experiments were performed for the thiono analog of **2**, *i.e.*, **5**. The high activity

Fig. 6 Internal amounts of 4-*n*-propyl BP (■) and its thiono analog (□), and mortality of houseflies (○) when the latter compound (1 µg/fly) was topically applied without synergists.

of **5** was not directly associated with its penetration process because little, if any, difference was detected in the penetration rate between **2** and **5** (Figs. 3 A and 3 C). When houseflies were treated with **5** at 1 µg per fly without pb, extracts from acetone-rinsed fly bodies contained **2** which was formed by *in vivo* oxidation of **5**. At 24 hr, the total amount of internal **2** and **5** was about 0.12 µg per fly and the mortality reached 90% (Fig. 6).

DISCUSSION

Structure-activity relationship studies have established that appropriate hydrophobicity, bulkiness and electronic properties of the bridgehead substituent are very important for high activity.^{2,10)} Such a structural requirement was less strict in the case of insecticidal activity against houseflies than in the case of mammalian toxicity. For instance, 4-*t*-butyl BP (**1**) was outstandingly toxic to mice (i.p. LD₅₀, 0.053 mg/kg),¹⁰⁾ but was not so to houseflies. There was no significant difference in insecticidal activity between 4-*t*-butyl BP and 4-*n*-propyl BP (**2**). When considered with the ratio of mouse-LD₅₀/housefly-LD₅₀ (rs), compound **2** (rs=2.2) appears to have higher specificity in action on houseflies than **1** (rs=0.43).²⁾ This could mean that **2** has high affinity for a target site in houseflies and low affinity for that in mice. In this respect, **2** is likely a better compound for characterization of a housefly-specific site. This compound has also been used as a radiolabeled ligand in our ligand-receptor binding studies.^{11,12)} For these

reasons, 4-*n*-propyl analogs were chosen for this study.

In a previous paper,²⁾ we reported that bicyclic phosphates including 4-*n*-propyl BP (**2**) were not insecticidal as judged from their 24-hr lethal activity when topically applied to houseflies at the dosage of 15 µg per fly without pb. In the present study, however, we found that **2** penetrated and accumulated slowly in the fly body so that the deaths increased gradually until 96 hr after application (Fig. 1). This finding suggests that the rate of detoxication is somewhat lower than that of penetration. Also, **2** was found to exert toxicity by topical application after pretreatment with pb, being comparable to that determined by injection after treatment with pb (Table 1). Thus, the activity of **2** as a contact insecticide was greatly improved by treatment with pb. The integument penetration of **2** is slow, as compared with that of other organophosphorus insecticides such as fenitrothion and methyl parathion.¹³⁾ However, the reason of the improved activity was not the enhancement of the penetration rate of **2** because data failed to show any significant difference in the penetration rate of **2** between pb-treated and untreated fly bodies (Figs. 3 A and 3 B). The absorbed compound appeared to be successively detoxified by microsomal oxidases because the slow penetration of **2**, but its internal concentration was raised to the lethal level by the action of the oxidase inhibitor pb. Pretreatment with pb stabilized a small dose of **2**, which then produced rapid and irreversible intoxication. The high toxicity of **2** was also obtained by injection which bypassed the integument and raised the internal concentration before significant detoxication could occur.

As shown in Fig. 7, the total recovery of **2** applied to houseflies decreased with the elapse of time in houseflies without pb treatment, whereas such a decrease was not noteworthy in pb-treated houseflies. This finding clearly supports the possibility that oxidation by microsomal oxidases plays an important role in detoxication of **2**, and that ester hydrolysis is of minor importance. Oxidized metabolites with the cage structure would be detectable by GC if they were formed, but none of other GC peaks than that of **2** was observed. Hence, it

Fig. 7 First-order plots of totally recovered 4-*n*-propyl BP when topically applied at the dosage of 1 $\mu\text{g}/\text{fly}$ with (○) and without (●) pb pretreatment.

X_{T0} : amounts of total BP at 0 hr, X_{Tt} : amounts of total BP at t hr. The lines were drawn by the least-square method.

is possible to speculate that the cage structure characteristic of bicyclic phosphorus esters may be broken down to a ring-opened structure through oxidation at the endomethylene carbon. The above observation is in marked contrast to the finding that hydrolysis products were main metabolites in mammalian species.¹⁴⁾ With regard to the structure of a possible metabolic intermediate, a bridged bicyclic acyl phosphate synthesized by Casida *et al.*¹⁵⁾ is of great interest in view of its instability. The metabolic pathway proposed here can be confirmed by detailed studies using radio-labeled compounds.

4-*n*-Propyl BP caused mortality after 24 hr at the dosage of 12 μg per fly while **2** at 1 μg or 5 μg per fly exerted little effect (Figs. 4 A, 4 B and 5 A). When the level of **2** in the fly body reached 0.2–0.3 μg per fly, the death was remarkable. At the dosage of 1 μg or 5 μg per fly the internal level of **2** remained below the lethal level, but was easily increased to the lethal level in 6 hr by treatment with 10 μg of pb followed by 1 μg of **2** per fly (Fig. 5 B). It was noted that the same internal level of **2**, e.g., 0.3 μg per fly, resulted in different mortality shown in Figs. 5 A and 5 B. This is probably due to the fact that the internal amount does

not refer to the level at the site of action. In other words, a considerable amount of **2** appears to be taken up by inert tissues other than the target site through gradual accumulation shown in Fig. 5 A.

In contrast to 4-*n*-propyl BP, its thiono (**5**) and phosphite (**6**) analogs applied topically were as insecticidal as **2** injected (Table 1). The structural difference between **2** and **5** is just that the phosphoryl oxygen atom of **2** is replaced by the thiophosphoryl sulfur atom of **5**. The following explanation seems to be possible for the difference in the insecticidal activity between the two compounds by single topical application. First, the markedly high activity of **5** cannot be accounted for by its integument penetrability, which was as low as that of **2** (Fig. 3 C). Second, the inhibitory activity of specific [^3H]4-*n*-propyl BP binding to housefly head membranes has been measured as an indication of the intrinsic activity of **2** and **5** at the target site.¹²⁾ The inhibition constants K_i were $4.3 \pm 0.9 \text{ nM}$ and $27.2 \pm 6.2 \text{ nM}$ for **2** and **5**, respectively. 4-*n*-Propyl BP was more potent than **5** in displacing specifically bound [^3H]**2**, but the potency of **2** may be overestimated because this is the case that the displacer is the same compound as the radio-labeled ligand. With this fact taken into account, there seems to be no essential difference in the intrinsic activity between them. Third, Fig. 6 shows that the mortality at 24 hr was near 100% while the internal level of **5** and its oxidized metabolite **2** was lower than the lethal level described above (0.2–0.3 $\mu\text{g}/\text{fly}$) in the case of topical application with 1 μg of **5** per fly. At this level, **2** is not very effective but **5** appears to be critical. The internal lethal level of **5** is considered to be approximately 0.03 μg per fly. This is close to the low LD_{50} value (0.061 $\mu\text{g}/\text{fly}$) for **5** with pb (Table 1). As mentioned above, the accumulation of such an amount of **2** was also observed in topical application of **2** at 1 μg per fly (Fig. 4 A), although it was much lower than the lethal level. One-tenth of the internal lethal dose of **2** is enough for **5** to cause death in houseflies. The thionate ester may reach the target in nerve tissue more efficiently than **2** because of its higher hydrophobicity ($\log P = 1.4$) than that of **2** ($\log P = 0.0$). In order to

increase the internal amount ten times in 24 hr without pb treatment, more than ten times dose is needed for topical application due to its slow penetration (Figs. 4 and 5). This is a possible cause of the big difference in insecticidal activity between **2** and **5**.

Figure 3 A shows the biphasic proceeding of penetration of **2**. The data in Figs. 3 B and 3 C may be also biphasic although the lines were drawn by the least-square method. Biphasic phenomena have been also reported in the penetration of various insecticides.^{16,17)} Acetone in which **2** was dissolved may work as a carrier to produce the first steep phase. Alternatively, there might be a saturable pool which absorbs a definite amount of chemicals rapidly in the integument, and then releases them inside the body. The former explanation seems to be more conceivable but no detailed studies have been done in this regard.

In conclusion, 4-*n*-propyl BP was so slow in penetration to the housefly body and was so susceptible to microsomal mixed-function oxidases that it was inactive as a contact insecticide. However, the activity was greatly improved by pretreatment with the microsomal oxidase inhibitor pb or by injection, though the ester was still less active to houseflies than to mice. The topical insecticidal activity of the thiono and phosphite analogs was in a range similar to that of the oxon analog by injection. The high activity of the thiono analog is associated with its lower internal lethal level than that of 4-*n*-propyl BP.

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要 約

イエバエに対する二環リン酸エステル類の殺虫活性に影響を及ぼす皮膚透過と代謝

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オキシソソ型 4 位 *n*-プロピル基置換二環リン酸エステル (4-*n*-プロピル BP) のイエバエへの局所施用法による殺虫活性はピペロニルブトキシド (pb) の作用で著しく高められた (共力効果比 313)。4 種の異なった施用法による 4-*n*-プロピル BP の殺虫活性 (24 時間後の LD₅₀ 値, $\mu\text{g}/\text{頭}$) は, pb 処理注射 (0.169) > pb 処理塗布 (0.212) > 注射 (0.449) > 塗布 (66.4) の順となった。イエバエにおける 4-*n*-プロピル BP の代謝には酸化酵素が非常に大きな役割を果たしている。皮膚透過の速度は遅いが, 酸化酵素の阻害あるいは注射法によって 4-*n*-プロピル BP の体内レベルを 0.2~0.3 $\mu\text{g}/\text{頭}$ まで高めると著しい致死が見られた。一方, チオノ体およびフォスファイト体は単独局所施用でも高い殺虫活性を示した。チオノ体の高い殺虫活性は皮膚透過性, あるいは作用部位での活性が高いためではなく, 致死を引き起こす体内レベルが低いことと関連している。