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Kinetic Properties of the Inhibition of Juvenile Hormone Esterase by Two Trifluoromethylketones and *O*-Ethyl,*S*-Phenyl Phosphoramidothioate

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Some inhibition kinetic properties and *in vivo* inhibition of the plasma juvenile hormone esterase from the cabbage looper (*Trichoplusia ni* Hübner) by one phosphoramidothioate and two trifluoromethylketones were examined. *O*-ethyl,*S*-phenyl phosphoramidothioate was shown to react irreversibly with the enzyme in a time-dependent manner, and the inhibition reaction can be factored into a reversible step with a dissociation constant, K_d, of 4.55×10^{-5} *M* followed by a phosphorylation step with a rate constant, k_2, of 1.98 min^{-1}. The phosphorylated enzyme did not show spontaneous recovery after 48 hr of dialysis. On the other hand, the two trifluoromethylketones were shown to act as reversible inhibitors, as their inhibited enzyme was regenerated completely after dialysis. However, 1,1,1,-trifluoro-3-thiooctylpropan-2-one, in contrast to 1,1,1-trifluorotetradecan-2-one, showed progressive time-dependent inhibition, and its reaction with the enzyme followed characteristic bimolecular second-order kinetics with a rate constant, k_i, of $3.37 \times 10^7 M^{-1}$ min^{-1}. The *in vivo* inhibition data of topically treated larvae at equimolar amounts of the tested compounds indicated rapid penetration, and the stability of the inhibition was higher for the phosphoramidothioate than for the trifluoromethylketones. The relationship of the mechanism of inhibition and the *in vivo* inhibition of these compounds to the understanding of the interactions between juvenile hormone and juvenile hormone esterase is discussed.

INTRODUCTION

The juvenile hormones (JHs)[3] regulate a myriad of developmental and reproductive events in insects, and metamorphosis in holometabolous insects is certainly among the most striking of these events (1). The reduction in JH titer to initiate metamorphosis in the Lepidoptera examined appears to be caused by degradative metabolism as well as reduction in the rate of biosynthesis (1, 2), and ester cleavage of JHs is apparently the major route of metabolism. In the cabbage looper, *Trichoplusia ni*, much effort has been devoted to the characterization of larval carboxylesterases (3–6), indicating that hydrolysis of JH is due largely to a single enzyme, JH esterase (JHE), mainly present in the hemolymph and fat body. This enzyme was found to be less sensitive to inhibition by the commonly used carboxylesterase inhibitors, i.e., eserine, TOCP, DFP, and DEF (7); however, it is moderately sensitive to some phosphoramidothioates such as EPPAT. Based on the above finding (7), many authors have used EPPAT in their topical application studies to disrupt insect development. Although those studies shed some light on the role of JHE in JH regulation, little is known about EPPAT–JHE inhibition kinetics. As part of a further search for effective inhib-

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[3] Abbreviations used: JH(s), juvenile hormone(s); DFP, *O,O*-Diisopropyl phosphorofluoridate; DEF, *S,S,S*-Tributyl phosphorotrithioate; TOCP, Tri-*o*-tolyl phosphate; EPPAT, *O*-Ethyl, *S*-phenyl phosphoramidothioate; TFT, 1,1,1-trifluorotetradecan-2-one; TFPOS, 1,1,1-trifluoro-3-thiooctylpropan-2-one; L5D2, fifth stadium Day 2 larvae; 4ALO, 4 hours after light on; JHE, juvenile hormone esterase; JH_{III}, juvenile hormone III; α-NA, α-naphthyl acetate; AchE(s), acetylcholinesterase(s); r^2, squared correlation coefficient.

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itors of JHEs, Hammock and co-workers (8, 9) have found trifluoromethylketones to be the most active and selective inhibitors yet developed.

In this report we have chosen 1,1,1-trifluorotetradecan-2-one (TFT) and 1,1,1-trifluoro-3-thiooctylpropan-2-one (TFPOS) as active inhibitors of *T. ni* JHE (8, 9), along with EPPAT, to study the kinetic properties of their interaction with plasma JHE. As these compounds were evaluated for their morphogenetic action in *T. ni* (9), part of the present report describes the *in vivo* JHE inhibition in topically treated *T. ni* larvae in an effort to explain the intrinsic selectivity of these compounds.

MATERIALS AND METHODS

Enzyme assay. JHE analyses were performed using plasma from *T. ni*. This plasma was obtained by centrifuging the hemolymph at 1000*g* for 5 min. Hemolymph was taken by piercing the proleg of fifth instar larvae in their second day of development (L5D2), which were collected 4 hr after the lights were turned on (4ALO) when the JHE titer is very high (4, 8, 10). The plasma was diluted with sodium phosphate buffer (pH 7.4, $I = 0.2$ *M* with 0.01% w/v phenylthiourea) at 4°C.

Candidate inhibitors were added in ethanol solution to 100 μl of 0.2% diluted plasma and preincubated for the indicated times at 30°C. Control experiments received 1 μl ethanol. Substrate (C_{10}–[^{3}H]-JH_{III}, 11 Ci/mmol, New England Nuclear and unlabeled (*2E,6E*)–JH_{III}, Calbiochem) were added so that a final concentration of 5×10^{-6} *M* substrate containing ~25,000 dpm/assay was obtained. The enzyme mixtures were incubated at 30°C for 15 min and halted by the addition of basic methanol and isooctane, and the aqueous phase was analyzed according to the partition assay of Hammock and Sparks (11).

Kinetic analysis. The rate of JHE inhibition by EPPAT, TFPOS, and TFT was evaluated by monitoring the time dependence of the inhibition reaction. Both EPPAT and TFPOS showed progressive inhibition (time dependent); however, TFT showed time-independent inhibition. For the study of the inhibition reaction of the first two compounds with JHE, pseudo-first-order plots of ln percentage residual activity against preincubation time were prepared using five to six inhibitor concentrations ranging from 4.5×10^{-6} to 2.5×10^{-5} *M* for EPPAT and from 2.5×10^{-9} to 1.2×10^{-8} *M* for TFPOS. The plots were sensibly linear, enabling two different kinetic treatments to be evaluated. Those kinetic treatments were the first- (12) and second-order (13) inhibition reactions.

Reactivation of inhibited JHE. Thirty microliters of ethanol or ethanol–inhibitor solution were added to 3 ml of 0.2% diluted plasma to obtain final concentrations of 1.0×10^{-5}, 7.1×10^{-6}, and 1.25×10^{-8} *M*, respectively, for EPPAT, TFT, and TFPOS. These mixtures were preincubated for 10 min at 30°C, resulting in 9, 7, and 2% of the activity of the ethanol-treated enzyme, respectively. Control and inhibited samples were dialyzed identically but separately (in 10-mm-diameter Spectrapor membrane tubing, 12,000–14,000 MW cut off) against 1 liter of phosphate buffer. Aliquots of plasma from each treatment were collected at intervals through 48 hr and immediately assayed for JHE activity as described above. The dialysis experiments were run simultaneously at 25°C and the buffer was changed three times.

In vivo inhibition. L5D1 larvae were treated topically on the dorsum with 1 μl of ethanol or 1 μl of 0.1 *M* of the inhibitor solutions at 4ALO. Then groups of five larvae were bled at four times following treatment as described above, and their hemolymph was frozen at −60°C until assayed. The plasma from hemolymph was prepared as described above and diluted with buffer to 5%. A part of this diluted plasma was used for the measurement of α-naphthyl acetate (α-NA) hydrolysis as described previously (10). Further dilution of the plasma to 0.2% was used for analysis of

JHE activity as described above. The data points were the average of three replicates and at least two determinations.

RESULTS AND DISCUSSION

Kinetic analysis. In previous studies TFPOS and TFT were shown to be the most active congeners in the trifluoropropanone alkyl sulfide and alkyl trifluoromethylketone series, respectively (9). Therefore, these two compounds were chosen, along with a commonly used phosphoramidothioate JHE inhibitor, EPPAT, for detailed inhibition studies. The rates of reaction of these inhibitors with *T. ni* JHE were examined at 30°C, and the reaction was stopped at the desired preincubation time by the addition of JH_{III} at a saturation concentration of final molarity of 5×10^{-6} *M*. JH_{III} at this concentration appears to stop the inhibition of JHE by these compounds for most of the concentrations used. Support for this statement was obtained by adding JHE to a mixture of JH_{III} and inhibitor (data not shown). The results of the rates of reaction are shown in Figs. 1–3 where ln percentage residual activity is plotted against preincubation time. The

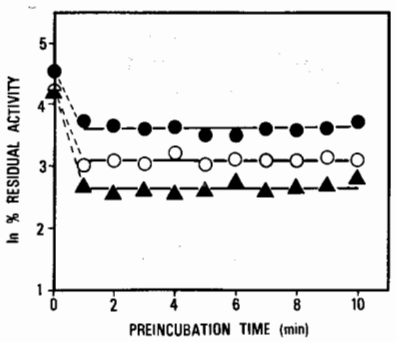

FIG. 1. *Time course of inhibition of JHE from Trichoplusia ni by TFT. To 100 μl of the enzyme in phosphate buffer (pH 7.4, I = 0.2 M), 1 μl of ethanol or ethanol–inhibitor solution was added at a final concentration of* 1.0×10^{-7} (●), 5.0×10^{-7} (○), *and* 1.0×10^{-6} *M* (▲). *At the time indicated 1 μl of* 5.0×10^{-4} *M* JH_{III} *in ethanol was added and the activity was measured as described under Materials and Methods. On all figures each point represents at least three replicates duplicated at least two times.*

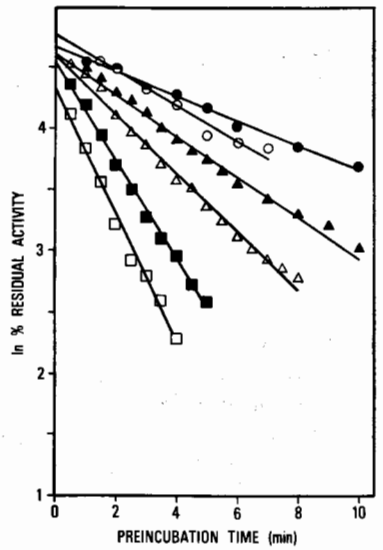

FIG. 2. *Time course of inhibition of JHE by TFPOS. To 100 μl of the enzyme in phosphate buffer 1 μl of ethanol or ethanol–inhibitor solution was added at a final concentration of* 2.5×10^{-9} (●), 3.1×10^{-9} (○), 4.0×10^{-9} (▲), 6.0×10^{-9} (△), 1.0×10^{-8} (■), *and* 1.2×10^{-8} *M* (□), *and the mixture was preincubated at 30°C. At the time indicated, 1 μl of* 5.0×10^{-4} *M* JH_{III} *in ethanol was added and the activity was measured as described under Materials and Methods.*

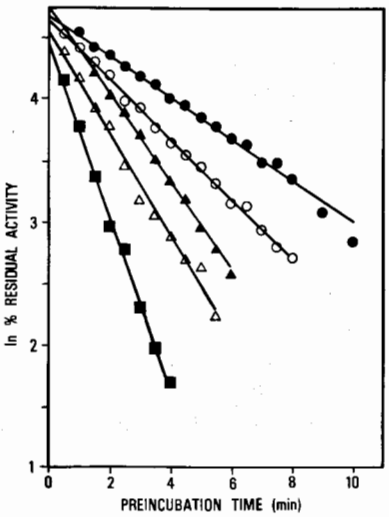

FIG. 3. *Time course of inhibition of JHE by EPPAT. To 100 μl of the enzyme in phosphate buffer, 1 μl of ethanol or ethanol–inhibitor solution was added at a final concentration of* 4.5×10^{-6} (●), 6.2×10^{-6} (○), 1.0×10^{-5} (▲), 1.2×10^{-5} (△), *and* 2.5×10^{-5} *M* (■), *and the mixture was preincubated at 30°C. At the time indicated, 1 μl of* 5.0×10^{-4} *M* JH_{III} *in ethanol was added and the activity was measured as described under Materials and Methods.*

reaction of EPPAT and TFPOS is progressive (time dependent) and shows significant straight lines, with r^2 of 0.99 for most of the inhibitor concentrations used (Figs. 2 and 3). This indicates that the reaction of these two compounds follows the characteristic of pseudo-first-order kinetics ($E \underset{k_{-1}}{\overset{k_1 I}{\rightleftharpoons}} EI$) at each inhibitor concentration. On the other hand, when the rate of reaction of TFT with JHE was examined on the same basis, the lines were unlike those of TFPOS and EPPAT in that they did not pass through the origin (at ln 100), they were almost parallel, and the rate of inhibition is independent of preincubation time (Fig. 1). This behavior is characteristic of classical competitive reversible inhibitors where the two equilibria involving the enzyme and reactants (enzyme–JH and enzyme–TFT) are set up rapidly (14), and the activity was examined under overall steady-state conditions. This observation is in agreement with the finding of Hammock *et al.* (8), who reached the same conclusion by using a different kinetic treatment (double-reciprocal plot). Surprisingly, TFPOS acts differently from TFT although they have the same general structure ($R\overset{O}{\overset{\|}{C}}CF_3$), except that the former has a sulfide bond in the R group, and this will be discussed later.

One of the questions at issue was whether EPPAT and TFPOS follow the first-order kinetics which were developed by Main and Iverson (12) according to their equation [$i\ \Delta t/\Delta \ln v = i/k_2 + 1/k_i$], which was based on the following inhibition scheme:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (EI)_r \xrightarrow{k_2} (EI)_q \quad [1]$$

$$k_i$$

where E, I, $(EI)_r$, and $(EI)_q$ are, respectively, JHE, the inhibitor, the reversible enzyme–inhibitor complex, and the irreversibly inhibited enzyme. The slopes of the pseudo-first-order plots in Fig. 2 and 3 ($\Delta \ln v/\Delta t$) were calculated from linear regression analysis of $\ln v$ vs t and transformed to the form $i\Delta t/\Delta \ln v$. The latter was plotted vs the inhibitor concentration (i) according to the above linear equation (12). In the case of EPPAT the plot fits a straight line rather well (Fig. 4), with $r^2 = 0.986$. The slope of this line gave $1/k_2$, and the intercept on the $i\Delta t/\Delta \ln v$ axis gave $1/k_i$ and the intercept on the i axis gave $-K_d$, where K_d is the dissociation constant of the EPPAT–JHE reversible complex, k_2 is the rate constant of the irreversibly inhibited enzyme formation (phosphorylation rate constant as will be discussed later), and $k_i = (k_2/K_d)$ is the overall bimolecular reaction constant. The kinetic constants calculated for the reaction of EPPAT with JHE are $k_2 = 1.98\ \text{min}^{-1}$, $K_d = 4.55 \times 10^{-5}\ M$, and $k_i = 4.36 \times 10^4\ M^{-1}\,\text{min}^{-1}$. In general terms, this means that the inhibition reaction of JHE by the phosphoramidothioate, EPPAT, involved a reversible step which results in an enzyme–inhibitor complex and precedes the formation of an irreversibly inhibited enzyme. Since the discovery that some phosphoramidates were potent inhibitors of JHE from several insect species, EPPAT has been used as a candidate inhibitor in several studies (7, 15–19). Although it has been suggested by many authors that EPPAT is an irreversible phosphorylating inhibitor of JHE, no kinetic evidence was provided. In this study it is shown clearly that the inhibition of JHE by EPPAT follows the same reaction scheme for the inhibition of acetylcholinesterases (AchEs) by organophosphates (12). Sparks and Hammock (7) found that paraoxon was >50× more active than its phosphorothionate analog, parathion, in inhibiting the JHE of *T. ni* (*in vitro*). The corresponding comparative potency for JHE from *Manduca sexta* was found to be 250× (19). This structural requirement for the inhibition of JHE seems to be identical with that of cholinesterase inhibition. Because of the rela-

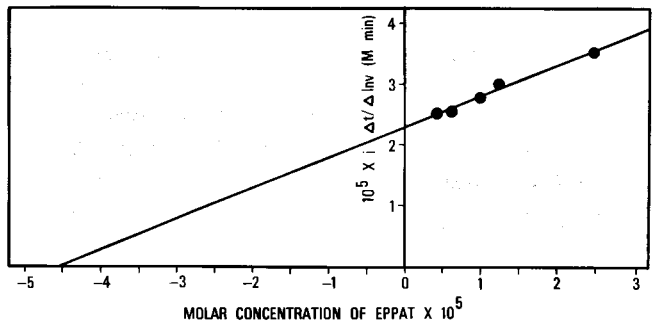

FIG. 4. *Results of plotting $i\Delta t/\Delta 1nv$ against i according to the Main and Iverson (Ref. (12)) inhibition kinetic equation. The results are taken from Fig. 3 for the inhibition of JHE by EPPAT. The line was fitted by a linear regression analysis ($r^2 = 0.986$).*

tively lessened electronegativity differences between P(2.1) and S(2.5), the phosphorus atom in P=S compounds is of low electrophilicity as compared to analogous P=O compounds in which the electronegativity difference (3.5 for oxygen) is much greater (20). More pertinent to our discussion is that the electronic requirement for nucleophilic attack by the serine hydroxyl in the AchE active site on the phosphoryl group (21) can be accepted for inhibition of JHE by organophosphates. According to this indirect line of evidence, along with the kinetic evidence reached from this study, it is possible that EPPAT phosphorylates a serine residue on the JHE active site. However, since substituents on EPPAT are not strong electron delocalizers, a low phosphorylation rate constant is expected and explains the low k_2 value (1.98 min^{-1}) obtained. Assuming that EPPAT reacts with serine as a neutral molecule, thermochemistry favors P-S cleavage over P-N and P-O by a substantial margin under conditions of neutral pH (22). Therefore, the kinetic evidence obtained from our study, along with the above lines of evidence, support the proposed scheme for the inhibition of JHE by EPPAT (23), as seen in Fig. 5 with some modifications.

In constrast to the reaction of EPPAT with JHE, TFPOS showed almost constant values of $i\Delta t/\Delta 1nv$ ($\sim 2.45 \times 10^{-8}$ M min) for the range of concentrations used (Fig. 6), which means that, although the reaction of TFPOS with JHE is time dependent and follows the characteristics of a pseudo-first-order reaction at any particular concentration, it does not fit first-order kinetics (12) when the inhibitor concentration changes from 2.5×10^{-9} to 1.2×10^{-8} M. Therefore, TFPOS either reacts directly with the enzyme to form a stable enzyme–inhibitor configuration with a very slow dissociation rate (off rate), or inhibition of the enzyme involves more than one step where the formation of the stable inhibited enzyme is of second order. The bimolecularity of such

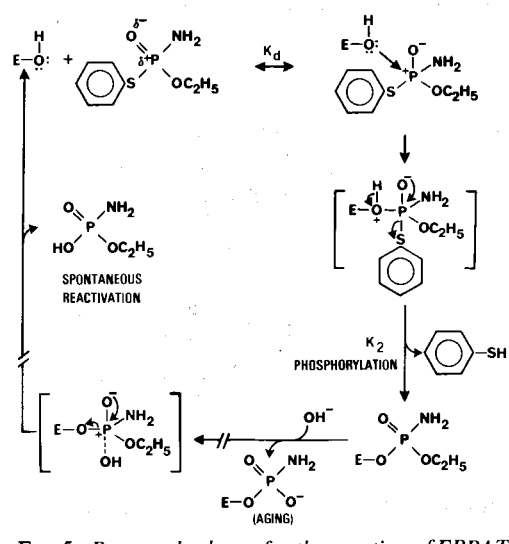

FIG. 5. *Proposed scheme for the reaction of EPPAT with JHE based on the kinetic analysis (see text).*

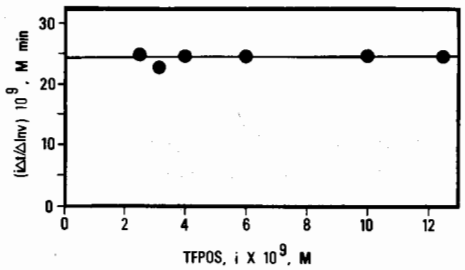

FIG. 6. *Results of plotting $i\Delta t/\Delta \ln v$ against i according to the Main and Iverson (Ref. (12)) inhibition kinetic equation. The results are taken from Fig. 2 for the inhibition of JHE by TFPOS.*

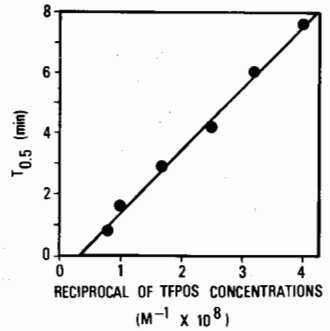

FIG. 7. *Plot of time for 50% inhibition against the reciprocal of TFPOS molar concentrations according to Eq. [3]. Data are taken from Fig. 2 for the inhibition of JHE. The line was fitted by linear regression analysis ($r^2 = 0.995$). From the slope of this line the bimolecular reaction rate constant, k_i, was calculated ($k_i = 3.37 \times 10^7\ M^{-1}\ min^{-1}$).*

reactions can be proven by the Aldridge kinetic treatment (13) according to

$$k_i = \frac{1}{[i]t} \ln \frac{100}{b}, \qquad [2]$$

where b = percentage residual activity, [i] = molar inhibitor concentration, t = preincubation time, and k_i is the bimolecular rate constant. Furthermore, when $b = 50$ then $t = t_{0.5}$, and Eq. [2] becomes

$$t_{0.5} = \frac{1}{k_i[i]} \ln 2. \qquad [3]$$

On plotting $t_{0.5}$ calculated from Fig. 2 against the reciprocal of the TFPOS concentration according to Eq. [3], a straight line with $r^2 = 0.995$ was obtained (Fig. 7), indicating that the reaction or one of the slowest of a series of reactions is bimolecular and second order. The bimolecular rate constant for the inhibition reaction of JHE by TFPOS was calculated from the slope of the line (Fig. 7) to be $3.37 \times 10^7\ M^{-1}\ min^{-1}$. Interestingly, the average $i\Delta t/\Delta 1nv$ value (Fig. 6) is about $2.45 \times 10^{-8}\ M$ min, which represents a $1/k_i$ value of $4.08 \times 10^7\ M^{-1}\ min^{-1}$, in good agreement with the value obtained from Fig. 7. To test the hypothesis that the reaction of TFPOS with JHE follows bimolecular second-order kinetics, a 10-min preincubation inhibition curve (Fig. 8) at 30°C was theoretically calculated from Eq. [2] using the k_i value calculated from Fig. 7. Included on the graph are experimentally determined values of inhibition (10-min preincubation, 30°C) from separate studies. Good agreement between the theoretical curve and the experimental points is seen (Fig. 8), and this gives further evidence for bimolecular second-order kinetics.

Different modes of AchE inhibition (time-dependent and time-independent inhibition) have been observed before for fluorinated and unfluorinated aldehydes and ketones, respectively, and could be explained by differences in the rate of hydration of both groups (24). However, the sit-

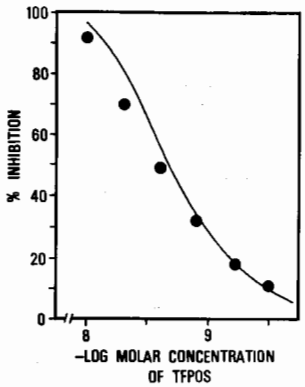

FIG. 8. *Theoretical curve for the inhibition of JHE calculated from Eq. [2], and $k_i = 3.37 \times 10^7\ M^{-1}\ min^{-1}$ for a preincubation time of 10 min at 30°C. Experimentally determined points have been added to show agreement with bimolecular second-order kinetics.*

uation for TFPOS and TFT seems more complicated. The difference in the time dependency of inhibition between TFT and TFPOS might be due, in part, to differences in their detailed inhibition mechanism, particularly if we assume that the presence of a sulfide bond in TFPOS would not greatly affect its extent or rate of hydration. On the other hand, one would expect differences in the intermolecular forces attracting TFPOS to the enzyme active site due to the presence of the sulfide bond. This situation is quite clear from the higher potency of the sulfur-containing compounds as compared to their alkyl analogs of the same molecular volume (9).

The above kinetic treatments cannot answer whether TFT, TFPOS, and EPPAT have some common points of attachment to the enzyme active site. Therefore, substrate protection of JHE from inhibition by these compounds was examined by monitoring the inhibition caused by two concentrations of each inhibitor when the inhibitor was either added 10 sec before or after the substrate at a final concentration of 5×10^{-6} *M*. The results from this experiment are shown in Table 1. Although high inhibitor concentrations were used, and keeping in mind that the substrate is being continuously depleted, less inhibition was obtained when the substrate was added first. This protection from inhibition by JH_{III} indicates that this substrate and the inhibitors used are likely having some common points of attachment to the enzyme active center, possibly the serine hydroxyl.

Reactivation of inhibited JHE. The differentiation between reversible and irreversible inhibitors can be tested by removal of the inhibitors from the enzyme inhibitor species by physical or chemical means (25). Since the exact structure of the JHE active site is lacking at the present time, the removal of the inhibitor by physical means, i.e., gel filtration (8) or dialysis (in the present study), was used. The results of the dialysis experiments are shown in Fig. 9. As seen in this figure, the control enzyme shows some loss in activity, and this loss was fast in the first 8 hr and then slowed down. However, the activity of the TFT-inhibited and TFPOS-inhibited enzyme was restored gradually to the same activity as in the uninhibited enzyme 48 hr after starting the dialysis. EPPAT, in contrast, was characterized by almost complete loss of enzyme activity even 48 hr after starting

TABLE 1
Substrate Protection of JHE from Inhibition by EPPAT, TFT and TFPOS

Inhibitor (*M*)	Percentage inhibition	
	Inhibitor added 10 sec before substrate[a]	Inhibitor added 10 sec after substrate[a]
EPPAT		
1.0×10^{-5}	39.9	5.0
2.5×10^{-5}	63.4	8.3
TFT		
1.0×10^{-7}	40.5	28.7
5.0×10^{-7}	77.2	61.4
TFPOS		
1.0×10^{-8}	19.8	6.8
1.0×10^{-6}	98.1	79.2

[a] JH_{III} was used as substrate at a final molar concentration of 5×10^{-6}.

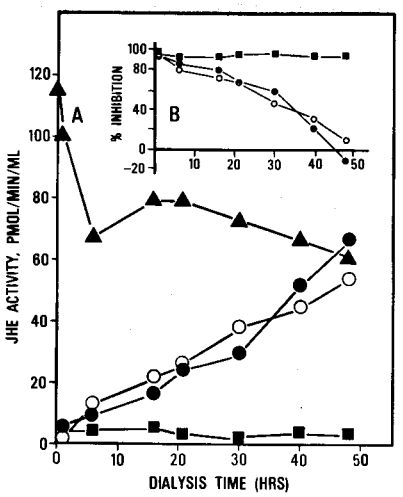

FIG. 9. *Spontaneous reactivation of inhibited JHE following dialysis against phosphate buffer (pH 7.4, I = 0.2 M). (A) JHE activity against dialysis time in control (▲), TFT-inhibited (●), TFPOS-inhibited (○), and EPPAT-inhibited (■) enzyme samples. (B) inset shows percentage inhibition vs dialysis time in hours.*

dialysis. Quistad *et al.* (26) reported that methamidophos-inhibited fly head AchE did not regenerate spontaneously and that pralidoxime gave only partial reactivation. This result was attributed to a rapid aging (demethylation) of the phosphorylated enzyme, which might be the case for phosphorylated JHE as the phosphoryl group on its active site is ethoxy instead of methoxy in methamidophos-phosphorylated AchE. The stability of EPPAT-inhibited JHE to reactivation indicates that this compound is an irreversible inhibitor of JHE and supports the conclusion for its mechanism of inhibition which was reached from the kinetic analysis. The same conclusion has been formulated before (18) based on a different kinetic treatment (27). On the other hand, TFT and TFPOS seem to be more reversible inhibitors of JHE as their inhibited enzyme was totally regenerated by dialysis. However, the rate of reactivation seems to be very slow, as the $t_{0.5}$ is more than 20 hr under these conditions. This is not surprising if we assume that these compounds act as transition-state analogs (8) which do not have all the essential structure features of the transition-state configuration of JH_{III}. Therefore, their dissociation reaction rate would be slow because that rate is not enhanced through product formation as in the case of carbamate insecticides. Also, it is widely known that highly lipophilic compounds are difficult to remove by dialysis. It is rather interesting to note that, although TFT and TFPOS have some common behavior in their reaction with JHE, i.e. reactivation of inhibited enzyme and substrate protection from inhibition, which means that they react with the enzyme in a competitive reversible manner, they differed in at least two aspects. First, in contrast to TFT (8), TFPOS acts in a way resembling noncompetitive inhibition by using Lineweaver and Burk treatment (Abdel-Aal, unpublished data, this laboratory). Such misinterpretation was avoided since the slopes of the lines of a double-reciprocal plot did not vary as a linear function of the TFPOS concentration, a characteristic of tight-binding inhibition (14, 28–30). This tight-binding inhibition may result from the presence of sulfur in TFPOS which allows it to assume a conformation which mimics the α-enoic ester group in the JH structure, which was found to be of high importance for the interaction with the JHE active site (31). The second difference between TFT and TFPOS is the time course of their inhibition, as the latter is time dependent, which is also a characteristic of tight-binding inhibition (30, 32).

In conclusion, EPPAT inhibits the JHE of *T. ni* as an irreversible inhibitor, and the kinetics can be factored into a reversible step followed by an irreversible phosphorylation step. In contrast, TFT and TFPOS act as reversible competitive inhibitors, with the latter being a tight-binding inhibitor.

In vivo JHE inhibition. In their studies, Hammock *et al.* (9) found that EPPAT and TFPOS delayed the pupation of *T. ni* while TFT showed no effect when the compounds were applied topically. This was explained as due to maintenance of a high JH titer. In the present report this hypothesis was tested indirectly by monitoring the *in vivo* inhibition of JHE following a single dose (0.1 μmol/larva) of the aforementioned compounds (Fig. 10). Interestingly, high inhibition of JHE (Fig. 10A) was obtained within 1 hr of topical application, which indicates a high rate of penetration. For the trifluoromethylketones, the carbon–fluorine bonds substantially increase lipid solubility (33) and thereby are likely to enhance the rate of bioabsorption. Although phosphoramidates of simple structure are hydrophilic (22), they do not have any problem penetrating the insect cuticle, and rapid penetration of methamidophos in house flies reaching maximum internal levels within 2 hr (34) was reported. The picture for general esterases hydrolyzing α-NA (Fig. 10B) is quite different using the trifluoromethylketones, as there was no clear and consistent inhibition of these en-

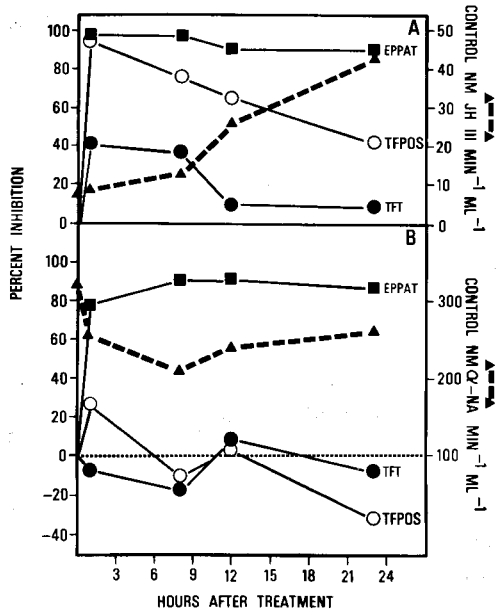

FIG. 10. *Comparison of in vivo inhibition of JHE (A) and α-naphthyl acetate esterase(s) (B) following topical application to L5D1 larvae of 0.1 μmol/larva of EPPAT* (■), TFPOS (○), *and TFT* (●). *Also shown in this figure are the activities of these enzymes in ethanol-treated larvae* (▲) *throughout the course of the experiment.*

zymes. However, EPPAT clearly inhibited general esterases at a higher level. The fact that the *in vivo* response (delayed pupation) as found by Hammock *et al.* (9) is well correlated with JHE, rather than general esterase, inhibition is evidence that JHE plays a specific integral role in the regulation of JH in this insect species. Furthermore, such compounds promise to be useful probes in studying the role of JHE in JH regulation of other insect species. As the JHE activity increased in untreated animals as they matured to L5D2 (Fig. 10), it is not clear whether the decrease in *in vivo* inhibition associated mainly with TFT and TFPOS is due to spontaneous *in vivo* recovery of the inhibited enzyme, biosynthesis of new enzyme, and/or short half-life of these compounds in the insect hemolymph. The elimination of these compounds from the insect hemolymph might be due to complex factors of transport to lipophilic compartments and metabolism which requires further study. However, the higher level of *in vivo* inhibition of JHE (90%) by EPPAT 23 hr after treatment indicates higher stability of the compound in *T. ni* hemolymph. This stability, compounded with its irreversibility in inhibiting JHE, gives the compound a superior opportunity to build up a sufficient concentration in the hemolymph to overcome its kinetically weak inhibition. This relationship can be seen by assuming full systemic distribution of the applied dose (0.1 μmol/larva) in a 200-mg larva of unit tissue density. These conditions translate to a body concentration of 5×10^{-4} *M*, a value 50,000× the TFPOS *in vitro* I_{90} and only 100× that of EPPAT. Such data demonstrate that reversible inhibitors must be very potent to elicit effects *in vivo*.

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