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3-OCTYLTHIO-1,1,1-TRIFLUORO-2-PROPANONE, A HIGH AFFINITY AND SLOW BINDING INHIBITOR OF JUVENILE HORMONE ESTERASE FROM *TRICHOPLUSIA NI* (HÜBNER)

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Abstract—The nature of the inhibition of juvenile hormone esterase (JHE) from *Trichoplusia ni* (*T. ni*) by 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP) was studied and the kinetic data presented are consistent with the mechanism of a slow and tight binding inhibitor. With the use of the above inhibitor, it was demonstrated that the classical methods, i.e. Lineweaver and Burk, which are based on the assumptions of a steady state are inadequate for determining the mechanism and the inhibition constants for the reaction of OTFP with JHE. The reaction was shown to be reversible based on the non-steady state kinetics with a dissocation constant (K_i) of $\sim 1.2 \times 10^{-10}$ M. Values of $3.37 \times 10^7\,M^{-1}\,min^{-1}$ and $4.1 \times 10^{-3}\,min^{-1}$ for the forward and reverse rate constants were calculated and indicate the nature of slow binding inhibition in comparison to ordinary substrate reactions. An application of Ackermann–Potter plots of tight binding inhibition was presented, and their usefulness in measuring the enzyme molar equivalency and its catalytic number towards JH-**I** and JH-**III** was evaluated. The molar equivalency of JHE in *T. ni* haemolymph which hydrolyzes JH-**III** (at a final concentration of 5.0×10^{-6} M) at a rate of 33.8 nmol/min-ml was found to be 1.6×10^{-6} M. The catalytic numbers for JH-**I** and JH-**III** were 37.1 and 19.4 min^{-1}, respectively. These new kinetic parameters, in addition to the Michaelis constant (K_m) and the maximum velocity (V_{max}) were applied to a study of enzyme–substrate specificity and an evaluation of the role of JHE in the regulation of JH-titre.

Key Word Index: *Trichoplusia ni*, juvenile hormone esterase, inhibition, substrate specificity, kinetics, molarity, slow binding inhibitors, tight binding inhibitors, 3-octylthio-1,1,1-trifluoro-2-propanone

INTRODUCTION

The events which occur during the metamorphosis of lepidopterous insects have long been in question. Certainly one important event is the reduction in juvenile hormone (JH) titre to levels which allow the release of prothoracicotropic hormone (PTTH) (Nijhout and Williams, 1974). It was widely assumed that this decrease in JH titre was due entirely to a cessation of JH production by the corpora allata, thus it was surprising when Williams (1961) reported that the apparent release of JH by corpora allata from *Hyalophora cecropia* continued at a diminished rate during the last larval stadium and did not cease until pupation. Such observations were made subsequently on other species (see deKort and Granger, 1981, for review). It is now becoming more obvious from *in vitro* assays of biosynthetic activity that biosynthesis is reduced but certainly not terminated during the last larval stadium (Kramer and Kalish, unpublished; Granger *et al.*, 1982). Nijhout (1975) further found in *Manduca sexta* that the mere absence of the corpora allata was not sufficient to stimulate an early release of PTTH. The above data then raise the question of how the JH titre is rapidly reduced to levels which allow PTTH release.

In addition a prepupal burst of JH now has been observed in a number of Lepidoptera, and Jones and Hammock (unpublished) have demonstrated that JH has an apparent ecdysiotropic role which is critical for the pupation of *Trichoplusia ni*. The obligate appearance of JH in the prepupa at this time is problematical because a short time later the insect is very susceptible to teratogenic effects caused by physiological levels of the hormone.

It is hypothesized that the rapid reduction in JH titre observed at these two times is due in part to hydrolysis of the methyl ester of JH catalyzed by a large increase in JH esterase (JHE) activity. Evidence for this hypothesis has come from many quarters (see Hammock and Quistad, 1981; Hammock, 1984, for review), but the most direct evidence arises from the JH-like effects observed when JH esterase activity is inhibited (Sparks and Hammock, 1980; Hammock *et al.*, 1984; Prestwich *et al.*, 1984; Sparks *et al.*, 1983; Jones, unpublished). Thus, it becomes more important to understand the biochemical characteristics and physiological actions of JHE.

This problem was aproached by precisely evaluating the kinetic characteristics of the JHE from *T. ni* and then probing its action with a novel inhibitor,

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3-octylthio-1,1,1-trifluoro-2-propanone (OTFP). The interaction of this compound with JHE had been shown earlier to demonstrate unusual kinetics (Abdel-Aal *et al.*, 1984). So in this study we provide an explanation for the kinetic behaviour of OTFP on JHE and then use this probe to demonstrate that JHE is a highly active enzyme which occurs at a molarity sufficient to lead to a rapid reduction in circulating JH titre.

MATERIALS AND METHODS

Haemolymph preparation

Larvae of *Trichoplusia ni* (*T. ni*) were reared at 14 hr L: 10 hr D, 28°C as described by Shorey and Hale (1965) and modified by Roe *et al.* (1982). JHE analyses were performed using plasma from *T. ni* larvae. Haemolymph was from larvae having weights and developmental markers indicative of the peak of prewandering JHE activity during development (Sparks *et al.*, 1979; Jones *et al.*, 1981, 1982). Haemolymph was collected by piercing a proleg and drawing the blood into glass capillary tubes containing a few crystals of phenylthiourea (PTU) to inhibit tyrosinases. Plasma was then obtained by centrifuging the haemolymph at 1000 *g* for 5 min at 23°C. The plasma was diluted with sodium phosphate buffer (pH 7.4, I = 0.2 M with 0.01% w/v PTU) at 4°C. All plasma dilutions are expressed as v/v. As the JHE activity in the haemolymph of larvae drawn from our culture gradually fluctuates over a period of months (Wing *et al.*, 1984) only two enzyme preparations were used in the present work which varied ~2.5 × in their activity towards JH-I and III.

Enzyme assay

JHE activity was assayed by the partition method of Hammock and Sparks (1977). For routine analysis a final concentration of 5.0×10^{-6} M JH-III was added to 100 μl diluted plasma and the reaction mixture was incubated for 10–15 min at 30°C unless otherwise noted. Esterase assays with JH-I as substrate were run using the same procedure except that carbowaxed (polyethylene glycol, mol. wt 8000, Aldrich Chemical Company, Inc.) tubes were used to prevent adhesion of JH-I to glass (Hammock *et al.*, 1975; Kramer *et al.*, 1976). Preliminary experiments showed that using carbowaxed tubes is critical when JH-I was used as substrate, however, no effect was observed with JH-III even when used at very low concentration (~10^{-8} M). For example rate of JH-I hydrolysis using an 8×10^{-9} M substrate concentration was 40% lower when the tubes were not carbowaxed. The racemic substrate [10 – ^{3}H]JH-III, 11Ci/mmol or JH-I, 15.3 Ci/mmol, New England Nuclear and unlabelled [2*E*, 6*E*]JH-III and JH-I, respectively (Calbiochem) was added in 1 μl ethanol so that each assay contained ~25,000 dpm/100 μl reaction mixture. After incubation at 30°C, the enzyme reaction was halted by the addition of 50 μl basic methanol (methanol–water–ammonium hydroxide, 10:9:1 respectively), pH 11.2 and 250 μl isoctane or dodecane, and the radioactivity in the aqueous phase was measured.

Kinetic analysis

Steady state kinetics. K_m (apparent Michaelis–Menten constant) and V_{max} (maximal enzymic velocity) for JHE activity in the haemolymph of *T. ni* were determined for JH-I and JH-III by using a double reciprocal treatment (Lineweaver and Burk, 1934). JH concentrations varied from 1.7×10^{-8} to 5.2×10^{-7} M. The plasma used for steady state kinetics was diluted to 0.033% with PTU buffer since the enzyme activity at that time was high (the enzyme hydrolyzed 5.0×10^{-6} M of JH-I and JH-III at rates of 124 and 79 nmol/min-ml respectively). In order to estimate the initial velocity accurately, the incubation time used was only 1 min at 30°C. Least squares linear regression analysis was used for maximal fit of the experimental values from which K_m and V_{max} values were obtained. The same kinetic treatment was also performed in the presence of 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP) as an inhibitor of JHE (Hammock *et al.*, 1984; Abdel-Aal *et al.*, 1984) to test the behaviour of this inhibitor under classical steady state conditions. The inhibitor was added in 1 μl ethanol solution to 100 μl diluted plasma and the reaction mixture was pre-incubated for 10 min at 30°C. The reaction was then started by adding JH-III as substrate and the activity was measured after incubation for 1 min at 30°C.

Ackermann–Potter plot. The plasma used in this kinetic treatment had a JHE activity of about 45 and 34 nmol/min-ml respectively for JH-I and JH-III at a final concentration of 5.0×10^{-6} M. In this treatment, various amounts of plasma in a final reaction mixture of 100 μl were pre-incubated with OTFP for a period of time sufficiently long to approach equilibrium (30 min). Then the reaction was started by adding 1 μl of JH-I or JH-III in ethanol solution at a final concentration of 5.0×10^{-6} M. The activity was then measured at several times from 7.5 to 60 min depending on the substrate used and the enzyme dilution. The velocity against the amount of plasma/assay was plotted for a series of inhibitor concentrations (2.5×10^{-10}–8.0×10^{-9} M). Control experiments received only 1 μl of ethanol and the assay was performed under identical experimental conditions. The straight portion for each curve (for inhibited enzyme) was fitted by regression analysis to determine the molar equivalency of JHE and its catalytic number to JH-I and JH-III according to the kinetic treatments of Ackermann and Potter (1949) and Cha (1975).

Non steady-state kinetics. In this kinetic treatment the same enzyme preparation that was used in the Ackermann–Potter plot was diluted in PTU buffer so that 0.2% plasma was obtained. The diluted plasma was added to a mixture of OTFP and JH-III. The inhibitor concentrations used were from 6.0×10^{-9} to 1.0×10^{-7} M, however, the substrate was kept constant at a final concentration of 5.0×10^{-6} M. Ethanol was used as a solvent for both substrate and inhibitor and never exceeded 2% of the reaction mixture. Equivalent volumes of ethanol were added instead of the inhibitor solution for control experiments. The reaction mixture was incubated at 30°C and at different time intervals from 6 to 72 min, 100 μl were withdrawn and added directly to the basic methanol and isooctane mixture and agitated to stop the reaction and the product was measured as described above. The relation between the amount of substrate hydrolyzed/ml plasma was plotted against the incubation time to give downward curves for the inhibited enzyme with an asymptote (linear portion) at long incubation times. The linear portions of the curves were subjected to a linear regression analysis to calculate the activity of inhibited and uninhibited enzyme for further kinetic measurements (Cha, 1975; Morrison, 1982) as will be mentioned later. Each data point in the present work represents the mean of at least four replicates with an average standard deviation of less than 5.0% of the mean.

RESULTS

JHE activity

The activity of JHE from plasma of *T. ni* towards JH-I and JH-III was examined using a single concentration (5.0×10^{-6} M) of each substrate and seven enzyme concentrations. The enzyme concentration–activity relationship is shown in Fig. 1. As seen in this Figure, higher esterolytic activity of JHE towards JH-I than towards JH-III was always observed with an average activity ratio of 1.55. The fact that JH-I is metabolized faster than JH-III under these conditions has been reported before not only in *T. ni*

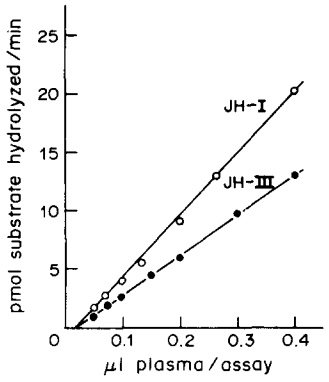


Fig. 1. The dependence of JH acid production on the concentration of plasma in the assay mixture. JH-I (○) and JH-III (●) were used as substrates with a final molar concentration of 5.0×10^{-6}. Each point represents the mean of four replicates with an average standard deviation of less than 5.0% of the mean.

(Sparks *et al.*, 1979; Wing *et al.*, 1984) but also in other lepidopterous larvae (Coudron *et al.*, 1981; Roe *et al.*, 1983). Although the JH-I/JH-III activity ratio under saturation conditions (high substrate concentration) is expected to be due mainly to differences in maximum velocity, this observation might reflect the specificity *in vivo* very poorly since JH titres in larvae are extremely low and generally range below 10 ng/ml (Schooley *et al.*, 1976; Mauchamp *et al.*, 1979; Jones, unpublished data). Therefore, we have attempted to determine the maximum velocity (V_{max}) and Michaelis constant (K_m) for JHE from *T. ni* plasma using JH-I and JH-III as substrates. These values were calculated from double reciprocal plots (Lineweaver and Burk, 1934) as shown in Fig. 2. The plots

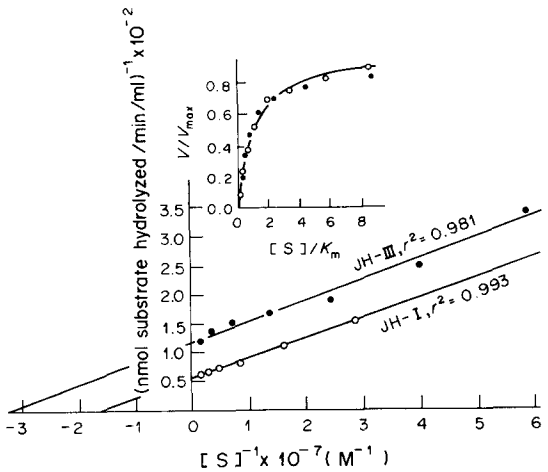


Fig. 2. Double reciprocal plot of substrate concentration against velocity for the hydrolysis of JH-I (○) and JH-III (●) by the haemolymph JHE of *T. ni*. The activity at each substrate concentration was the mean of five replicates with an average standard deviation of less than 5.0% of the mean. Inset shows a theoretical curve based on a modified equation ($V/V_{max} = [[S]/K_m]/[[S]/K_m + 1]$) of Michaelis–Menten equation. Included are experimental values for JH-I (○) and JH-III (●) that have been calculated from double reciprocal plots to show agreement with the theoretical values.

are sensibly linear with r^2 of more than 0.98. These plots were evaluated by linear regression analysis from which K_m and V_{max} were estimated to be 6.07×10^{-8} M; 177 nmol/min-ml and 3.05×10^{-8} M and 85.0 nmol/min-ml, respectively, for JH-I and JH-III. The JH-I/JH-III V_{max} ratio is 2.08 which agrees well with the activity ratio at a single substrate concentration. It is interesting that very similar V_{max} values for JH-I and JH-III have been reported for the same enzyme from the same insect species (Sparks and Rose, 1983), however, the K_m value was about one order of magnitude higher. The substrate concentrations used to generate the reciprocal plot in the present study were varied widely around the calculated K_m value for each substrate (Fig. 2, inset).

Kinetics of JHE inhibition

The inhibitory activity of OTFP on the hydrolysis of JH-I and JH-III by JHE was examined under the same experimental conditions. A 10 min pre-incubation time at 30°C was used, and the reaction was started by the addition of each substrate at a final concentration of 5.0×10^{-6} M. The inhibition pattern (Fig. 3) seems identical in spite of the substrate used indicating that the same enzyme system hydrolyzes both substrates.

Double reciprocal plot

As has been pointed out (Fig. 3) the enzyme sensitivity to inhibition of OTFP does not change by changing the substrate, thus most of the kinetic studies have been performed with JH-III as substrate. The first kinetic analysis was the classical treatment, i.e. the double reciprocal plot (Lineweaver and Burk, 1934). Shown in Fig. 4 are the plots of uninhibited, and OTFP-inhibited enzyme. These plots might readily be considered diagnostic of non-competitive inhibition. However, one may not conclude on the basis of these plots alone that true non-competitive inhibition occurred since the replot of the slope of double reciprocal plots is not a linear function of the

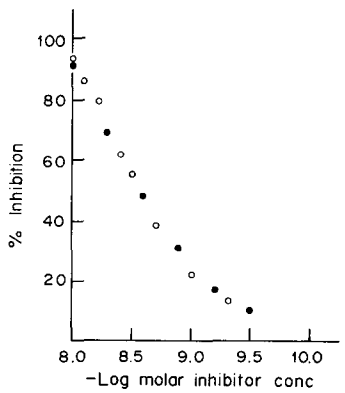


Fig. 3. Inhibition of JHE from *T. ni* by different OTFP concentrations. The enzyme was pre-incubated with the inhibitor for 10 min and the residual activity was measured using JH-I (○) or JH-III (●) at a final concentration of 5.0×10^{-6} M. Percentage inhibition was calculated from the average of four to five replicates of control and inhibited enzyme with an average standard deviation of less than 5.0% of the mean.

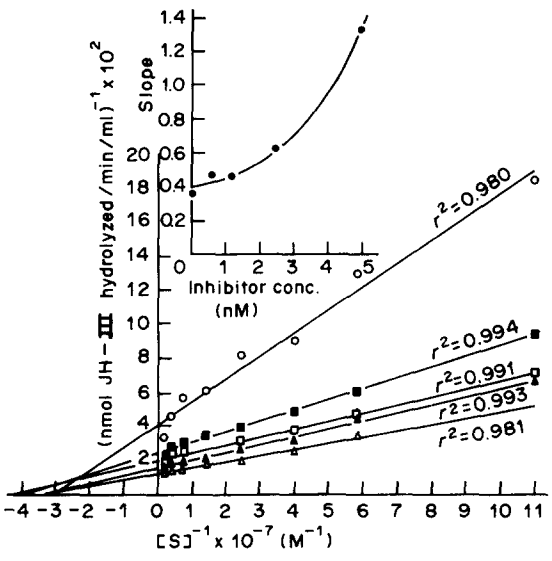


Fig. 4. Double reciprocal plot for the inhibition of JHE by OTFP after a 10 min pre-incubation using JH-III as substrate. The inhibitor concentrations used were: no inhibitor (△); 6.25×10^{-10} M (▲); 1.25×10^{-9} M (□); 2.50×10^{-9} M (■) and 5.00×10^{-9} M (○). Note the downward curve of the points at high substrate concentrations when inhibitor is present. The insert shows the relation between the slope of reciprocal plots and the inhibitor concentration.

inhibitor concentration (Fig. 4, inset). This replot should be linear for true, classical, non-competitive inhibition (Segel, 1975). However, the non linearity of such a replot has been explained as a characteristic of competitive tight binding inhibitors (Cha, 1975; Morrison, 1969, 1982). The possibility that OTFP might act as tight-binder of JHE from *T. ni* will be examined in the following section.

Ackermann–Potter plot

In order to test whether OTFP acts as a tight binding inhibitor of JHE from *T. ni*, varying amounts of the enzyme were incubated with several concentrations of the inhibitor for 30 min at 30°C, after which residual enzymic activity was assayed at 5.0×10^{-6} M of JH-III (Fig. 5) or JH-I (data not shown). These data were plotted by the method of Ackermann and Potter (1949) according to their modified equation (Cha, 1975):

$$V = \frac{k_3 S}{2(K_m + S)}\left[-(K_i + I_t - E_t) + \sqrt{\{(K_i + I_t + E_t)^2 - 4I_t E_t\}}\right] \quad (1)$$

where k_3 is the first order rate constant of the degradation of the enzyme substrate complex to products, S, I_t, and E_t are the initial concentrations of substrate, inhibitor and enzyme, respectively, and K_i is the equilibrium dissociation constant for the reaction of the enzyme with the inhibitor. The above equation indicates that the plot of V against E_t at various levels of I_t must be a curve having an asymptote (linear portion) for a tight binding inhibitor. As seen in Fig. 5, and based on the above assumption, the reaction of OTFP with the JHE from *T. ni* can be accepted as tight binding inhibition. Furthermore, the linear portion for each inhibitor concentration is described by the equation of Cha (1975):

$$V = \left(\frac{k_3 S}{K_m + S}\right) E_t - \left(\frac{k_3 S}{K_m + S}\right) I_t \quad (2)$$

When $[I_t] = 0$ the above equation reduces to the Michaelis–Menten equation. As the substrate concentration used (5.0×10^{-6} M) is much higher than K_m values for both substrates, the above equation can be reduced to a simpler form:

$$V = k_3 (E_t - I_t) \quad (3)$$

In the Michaelis–Menten equation, V_{max} equals $k_3 E_t$. Since a tight binding inhibitor approaches stoichiometric titration of the enzyme, the total enzyme available is approximated by $E_t - I_t$. Therefore, equation (3) indicates that under conditions of tight binding inhibition the asymptote in Fig. 5 intersects the E_t-axis (μl plasma/assay) at $E_t = I_t$ and the V-axis (pmol JH-III hydrolyzed/min) at $-k_3 I_t$. This approach simplifies the evaluation of the enzyme molar equivalency and its catalytic number, k_3. A replot of the E_t-intercepts against the molar inhibitor concentrations (which in this case equal the molar equivalencies of the enzyme) gives an apparent straight line

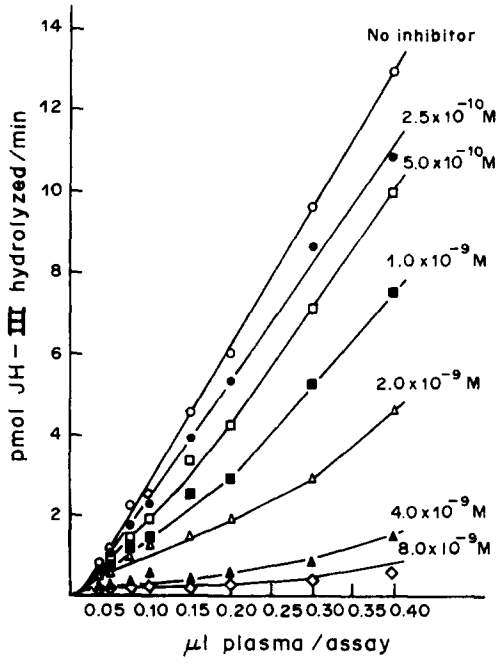


Fig. 5. Ackermann–Potter plot of the reaction of JHE with OTFP. Various amounts of JHE were pre-incubated with a reaction mixture that contained various concentrations of OTFP. After a 30 min pre-incubation the reaction was started by the addition of JH-III at a final concentration of 5.0×10^{-6} M. Each datum point represents the mean of four replicates with an average standard deviation of less than 5.0% of the mean. The straight line portions of the curves of the inhibited enzyme from the lowest four concentrations of OTFP were extended to the X- and Y-intercepts by linear regression analysis to calculate the enzyme molar equivalency and its catalytic number according to equation (3) in the text.

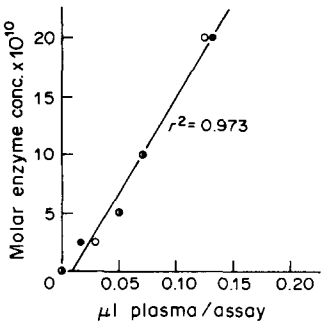


Fig. 6. Plot of the amount of enzyme (μl plasma/assay) against the expected molar equivalency of JHE. The results of this Figure were taken from Fig. 5 using JH-III as substrate (●) and a similar treatment using JH-I as substrate (○) according to equation (3) in the text.

with $r^2 = 0.973$ (Fig. 6). The data for this plot were calculated from Fig. 5 (JH-III as substrate) and from a similar treatment (data not shown) using JH-I as substrate. The intercepts used for drawing Fig. 6 and those for calculating k_3 were measured from regression analysis of the linear portion of the enzyme concentration velocity relationship (Fig. 5 for JH-III). From Fig. 6 the molar equivalency of JHE comparable to 1 μl plasma per 100 μl reaction mixture was readily measured from the slope of the line to be 1.6×10^{-8} M for both JH-I and JH-III. The catalytic number, k_3, i.e. the number of moles of substrate converted to product per one mole of the active centre per minute was calculated and averaged to be 19 and 37 min^{-1} respectively for JH-III and JH-I. The data that support these estimates were acquired using substrate concentrations much higher than K_m and rates which approached those of V_{max}.

Thus, it has been demonstrated that the Ackermann–Potter plot is applicable for inhibition of JHE from *T. ni* by OTFP as diagnostic of tight-binding inhibition as well as the estimation of the molar enzyme equivalency and its catalytic number towards JH-I and JH-III. However, it should be pointed out that the value of K_i cannot be estimated readily from this plot. Therefore, a non steady state kinetic approach (Cha, 1975; Morrison, 1982) has been applied for K_i determination.

Non-steady state kinetic analysis

The Ackermann–Potter plot (Fig. 5) in addition to the replot of the slope of double reciprocal plots against the inhibitor concentrations (Fig. 4, inset) gave evidence from two different kinetic treatments for tight binding inhibition; however, it does not exclude the possibility of slow tight binding inhibition since a progressive time-dependent inhibition pattern has been noted for inhibition of JHE from *T. ni* by OTFP (Abdel-Aal *et al.*, 1984). Cha (1975) derived an equation (4) for slow binding inhibition which is based theoretically on non-steady state or transient state conditions between the inhibitor and the enzyme:

$$P = V_s t + (V_0 - V_s)(1 - e^{-kt})/k \qquad (4)$$

where P is the amount of product formed, V_0 and V_s are the velocity of uninhibited enzyme and steady state velocity of the inhibited enzyme, t is time, k is an apparent first order rate constant whose meaning varies with the mechanism of inhibition. In order to apply the above equation experimentally, the activity of JHE from *T. ni* to 5.0×10^{-6} M of JH-III in the absence or the presence of various concentrations of OTFP was traced for at least 72 min (Fig. 7). In these cases the substrate and either ethanol or ethanol-inhibitor solutions were added to the reaction mixture and the reactions were started by the addition of enzyme. It should be noted that the same equation can be applied also to reactions started by the addition of substrate. Since no pre-incubation of enzyme and inhibitor occurred, high inhibitor concentrations could be used, and the depletion of free inhibitor due to binding with the enzyme would be negligible. This condition makes a slow tight binding inhibitor behave as a slow binding inhibitor. This type of experimental procedure is usually satisfactory for initial velocity determination of weak and reversible classical inhibitors. In the present case, however, there is negligible difference in the very early reaction velocities, regardless of the inhibitor concentration despite the fact that profound differences in the velocities become obvious after a few minutes (Fig.7). This biphasic reaction pattern is in good agreement with the velocity equation (equation 4) and fits all the assumptions for slow binding inhibition (Cha, 1975; Williams and Morrison, 1979; Morrison, 1982). Although the above equation is quite valid for determining k from which K_i can be evaluated, it is difficult to measure accurately the steady state velocity of

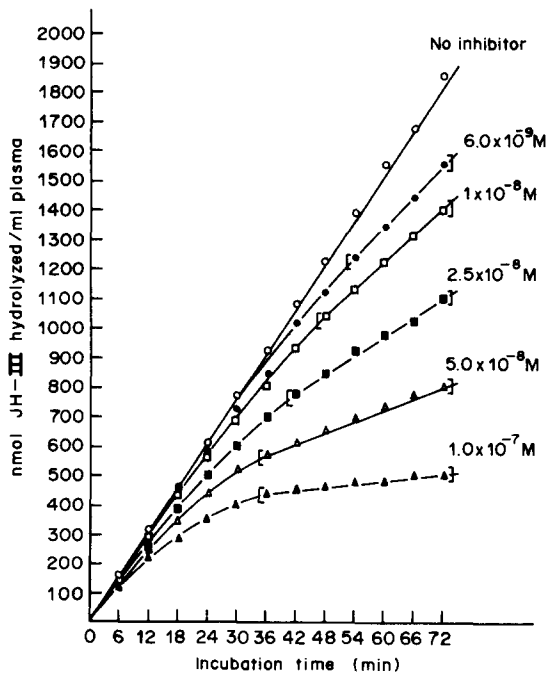


Fig. 7. Time course of JH-III (5.0×10^{-6} M) hydrolysis by JHE in the absence and presence of different concentrations of OTFP. In each case the reaction was started by the addition of the enzyme (no pre-incubation) and run four times. Notice the concave downward curves in the presence of the inhibitor which show that the lag period of enzyme inhibition is consistent with equation (4) for slow binding in the text.

inhibited enzyme which might not be reached before all of the substrate is depleted. However, the velocity of the enzyme in the presence of a slow binding inhibitor (V) can be related to that in the absence of the inhibitor (V_0) according to the following equation (Morrison, 1982).

$$V = V_0/(1 + I/K_{iapp}) \quad (5)$$

Equation (5) can be converted to a linear form as follows:

$$V_0/V = 1 + \frac{I}{K_{iapp}} \quad (6)$$

where I is the inhibitor concentration and K_{iapp} is the apparent equilibrium dissociation constant between the enzyme and inhibitor which equals $K_i[1 + (S/K_m)]$ The above equation is quite valid for determining K_{iapp} if the steady state inhibited rates are acquired at a constant substrate concentration. However, in the present study (Fig. 7) these steady state rates started at different substrate concentrations. Therefore K_{iapp} in equation (6) was substituted by $K_i[1 + (S/K_m)]$ to give equation (7) which can be used to hold different values

$$V_0/V = 1 + \frac{I}{K_i(1 + \frac{S}{K_m})} \quad (7)$$

of I and S. The value of S for each steady state inhibited rate was calculated by taking the value of S remaining after the pre-steady state phase. These values in addition to the known value of K_m (3.1×10^{-8} M) were used to calculate $I/[1 + (S/K_m)]$. A more sophisticated mutual depletion analysis does not appear warranted. The values of V have been calculated from the linear portions (in brackets) of the curves in Fig. 7 by the means of regression analysis. A plot of V_0/V against $I/[1 + (S/K_m)]$ according to equation (7) gave a straight line (Fig. 8) with $r^2 = 0.987$ and slope of $1/K_i$ from which K_i was calculated to be 1.2×10^{-10} M. Similar K_i value (9.7×10^{-11} M) was obtained when the data were subjected to equation (6) assuming substrate concentration remained constant at 5.0×10^{-6} M.

DISCUSSION

The activity and kinetic analysis of the JHE from *Trichoplusia ni* hydrolyzing JH-I and JH-III and the kinetics of the enzyme inhibition by 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP) have been examined. The sensitivity of the enzyme to the inhibition by OTFP is identical when either JH-I or JH-III is used as substrate (Fig. 3). This indicates that the same enzyme system is responsible for hydrolyzing both JH homologues. In agreement with the results reported earlier, based on analytical isoelectric focusing and other data (Sparks and Hammock, 1979; Wing *et al.*, 1981, 1984) kinetic evidence indicates that a single enzyme is responsible for the majority of JH hydrolysis in the haemolymph of prewandering *T. ni* larvae.

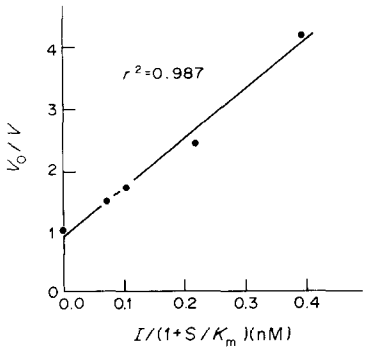


Fig. 8. Replot of v_0/v against $I/(1 + S/K_m)$ according to equation (7) in the text. The initial velocity of the uninhibited reaction (V_0) and the apparent steady state velocity of the inhibited reaction were calculated from the control enzyme and the straight line portions of inhibited enzyme (Fig. 7) respectively by means of linear regression analysis.

It must be noted in this study that racemic substrates were used for all experiments and the reader should keep this fact in mind when evaluating the results. As shown in Fig. 7 the rate of hydrolysis of JH-III (and also JH-I) by JHE is apparently linear even after 75% metabolism had occurred. Thus, either JHE demonstrates only a minor degree of enantioselectivity or the enantioselectivity for K_m and k_3 tend to cancel each other out. Further consideration of the stereo and enantioselectivity of JHE from *T. ni* is beyond the scope of this manuscript.

The JH-I/JH-III activity ratio at a single substrate concentration (5.0×10^{-6} M) and different enzyme concentrations (Fig. 1) shows higher specificity toward JH-I over JH-III. This observation was consistent with the specificity of JHE from other lepidopterous larvae (Coudron *et al.*, 1981; Roe *et al.*, 1983). In many cases, workers have compared the specificity of the enzyme by determining the activities toward a number of substrates at an arbitrarily fixed constant and high concentration of each of these substrates. Such values can be very confusing, for when *in vivo* concentrations are low they may predict the true specificity quite poorly. Therefore some other kinetic parameters were determined in order to discuss the relation between different estimates of specificity that have been used in the literature, and their biological significance.

The K_m values for JH-I and JH-III calculated from double reciprocal plots (Fig. 2) are one order of magnitude lower than those reported before for the same insect species (Sparks and Rose, 1983; Wing *et al.*, 1984). A similar K_m was obtained using the integrated form of the Michaelis–Menten equation as done previously with *Galleria mellonella* by Rudnicka and Kochman (1984). Possibly higher K_m values were previously reported for JHE of *T. ni* because those workers used higher plasma concentrations than in the present study and all substrate concentrations were above the K_m.

Previous studies in this laboratory showed the potency of trifluoromethylketones as JHE inhibitors (Hammock *et al.*, 1982, 1984; Prestwich *et al.*, 1984) and that compounds having a sulphide bond beta to the carbonyl group are highly active. OTFP was the most active compound in the sulphide series (Hammock *et al.*, 1984) and about 44 times more active than TFT (1,1,1-trifluorotetradecan-2-one, the most active compound in the series lacking the sulphide bond) (Hammock *et al.*, 1982). TFT was found to be a reversible competitive inhibitor based on its behaviour on Lineweaver and Burk analysis (Hammock *et al.*, 1982), and its time independent pattern of in-

hibition (Abdel-Aal *et al.*, 1984). In the latter study, however, OTFP showed time dependent and progressive inhibition. A time dependent course of inhibition is usually known to be a characteristic of an irreversible type of inhibition (Aldridge, 1950; Main, 1964; Smissaert, 1970). Recent evidence, however, has shown that reversible competitive inhibitors can be time dependent and progressive inhibitors if they are slow or slow tight binders (Cha, 1975; Cha *et al.*, 1975). The possibility that OTFP might act as slow tight binder rather than an irreversible inhibitor of JHE was first considered for at least two reasons. First, TFT as an analogue of OTFP acts as a competitive inhibitor and second, the formation of covalently inhibited JHE by a ketone inhibitor is not consistent with our knowledge of the inhibition of serine hydrolases by substrate-like compounds (Aldridge and Reinder, 1975) or with the stability of the trifluoromethylketone moiety. Before starting with the kinetic analysis of slow tight binding inhibition, Lineweaver-Burk analysis for the inhibition of JHE by OTFP was first performed (Fig. 4). As seen in this figure the double reciprocal plots might be considered to support non-competitive inhibition. However, one may not conclude on the basis of these plots alone that "true" non-competitive inhibition occurred. This misinterpretation can be predicted by the replot of the slope of double reciprocal plots against the inhibitor concentration which shows the appearance of a non-linear replot (Fig. 4, inset). This replot must be linear for classical non-competitive inhibition. Furthermore, careful examination of Lineweaver and Burk plots reveals that the lines are concave downward, non rectangular hyperbolas whose initial slopes are greater than those at low substrate concentration. This downward curve is very pronounced particularly at high inhibitor concentrations and shows clearly the nature of competitive inhibition since in contrast to the regression lines, the activity of the inhibited enzyme tends to be practically the same as the control enzyme at high substrate concentrations. This clearly shows the difficulties that can be associated with graphical determination of the true slopes and intercepts particularly if a limited range of substrate concentrations are used to generate simple computer fitted plots. The behaviour of OTFP inhibition of JHE on Lineweaver–Burk analysis is typical of a tight binding inhibitor based on the assumptions of Morrison (1969). In order to understand directly the nature of the OTFP reaction with JHE, two different kinetic treatments for tight and slow binding inhibition were applied. The first one was the so-called Ackermann–Potter plot (Fig. 5). This plot clearly indicates that OTFP has characteristics of a stoichiometric inhibitor, however, as emphasized before (Cha, 1975; Cha *et al.*, 1975) apparent stoichiometric or titrating inhibition does not necesarily indicate the formation of a covalent bond (irreversible inhibition) between the enzyme and the inhibitor, but it may be explained by a very much slower equilibrium as compared to the enzyme–substrate reaction. Although the Ackermann–Potter plot allows one to show the tightness of the OTFP–JHE complex, measure the enzyme molar equivalency and calculate its catalytic numbers to JH-**I** and JH-**III**, it has the disadvantage of being invalid for measuring the K_i value for the inhibition reaction. The kinetics of slow binding inhibition (Cha, 1975; Morrison, 1982) were applied under zero time pre-incubation conditions. These conditions allowed the use of high concentrations so that the depletion in the inhibitor concentration due to tight binding to the enzyme would be negligible. It should be noted that when the reaction was started with addition of the enzyme (no pre-incubation), the degree of inhibition was negligible, but the enzymic reaction velocity decreased with time (Fig. 7). This finding indicates that the formation and dissociation of the EI complex is markedly slower than the reaction between the enzyme and substrate which usually takes place within a fraction of a second. The tendency of trifluoromethyl ketones to hydrate in water due to the electronic induction of the fluorine atoms as compared to hydrogen made Brodbeck *et al.* (1979) suggest that the rate limiting step in acetylcholinesterase inhibition by these compounds is dehydration back to the keto form. However, this hypothesis seems unlikely to explain fully the nature of the slow and tight binding inhibition of JHE by OTFP for a least three reasons. First, the trifluoromethyl ketone analogue (TFT) behaves completely as a classical competitive inhibitor based on a double reciprocal plot (Hammock *et al.*, 1982). Second, supporting the rapid equilibrium between TFT and JHE is the apparent time independent pattern of inhibition shown by Abdel-Aal *et al.* (1984) based on a time course of 1–10 min. Third, four more analogues that have a beta-thioether group were found to act as classical competitive inhibitors (Abdel-Aal and Hammock, unpublished data). Therefore these biphasic reaction tracings obtained, i.e. initially fast, then slow might be due to the nature of OTFP reaction with the enzyme and show one of the major theoretical points developed for slow, tight binding inhibition (Cha, 1975). The K_i calculated from this kinetic treatment is 1.2×10^{-10} M.

There are two basic mechanisms which can be described by equation (4). The first mechanism assumes that the interaction of a competitive inhibitor with the enzyme to form an enzyme–inhibitor complex (EI) is slow and simple. For the second mechanism, it is assumed that there is an initial rapid interaction between enzyme and inhibitor to form EI which then undergoes a slow isomerization reaction to form another complex (see Morrison, 1982). The first mechanism (equation 8), is felt best to describe the reaction of OTFP with JHE for at least four reasons.

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \qquad (8)$$

First, the initial velocity is independent of the OTFP concentration (Fig. 7). Second, following dialysis, spontaneous reactivation of OTFP-inhibited enzyme was monophasic through total regeneration (Abdel-Aal *et al.*, 1984). Third, the kinetic data that were generated in the above reference failed to fit the Main and Iverson (1966) kinetic treatment which is based on assumptions similar to those outlined in the second mechanism. Fourth, the apparent (pseudo) first order rate constants (k′) for the reaction of OTFP with JHE from *T. ni* were calculated from the time-course inhibition data of Abdel-Aal *et al.* (1984)

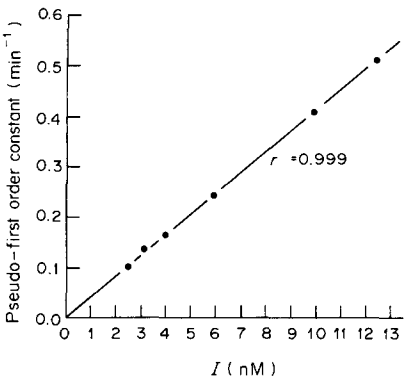


Fig. 9. Relation between the apparent (pseudo) first order rate constant and the concentration of OTFP. The values on this plot were calculated from the time course of inhibition of JHE from *T. ni* by OTFP (Abdel-Aal *et al.*, 1984).

and found to vary as a linear function of OTFP concentration (Fig. 9); a condition that is restricted to the first mechanism.

If one assumes that the inhibition reaction is a simple one represented by equation (8) and if the K_i estimated above is correct, the dissociation constant of the EI complex (k_2) should follow logarithmic decay kinetics with a first order rate constant = $K_i \times k_1$. Taking into consideration the value of k_1 as calculated before ($3.37 \times 10^7\,M^{-1}\,min^{-1}$, Abdel-Aal *et al.*, 1984), then k_2 must equal $4.1 \times 10^{-3}\,min^{-1}$. To illustrate the significance of these parameters let us calculate the half time ($t_{0.5}$) for the dissociation which is $0.693/k_2$ or 169 min. On the other hand, the rate of association may be considered as pseudo-first order with regard to E with a rate constant of $k_1 I$. Assume I is $5.0 \times 10^{-9}\,M$ which is about two times the I_{50} value (Hammock *et al.*, 1984), then the pseudo-first order constant is $(5.0 \times 10^{-9}\,M) \times (3.37 \times 10^7\,M^{-1}\,min^{-1}) = 0.17\,min^{-1}$. Therefore the $t_{0.5}$ value for the association reaction is 4.08 min. The comparative half times for association and dissociation of the EI complex explains the progressive inhibition of JHE by OTFP (Abdel-Aal *et al.*, 1984). Furthermore, if the enzyme is pre-incubated with the inhibitor before adding the substrate, the observed velocity will be due to the unbound free enzyme alone and any contribution by enzymes in the EI complex to the reaction velocity will be negligible because the rate of the dissociation of EI complex is too slow. Therefore OTFP would appear to completely inactivate a certain fraction of the enzyme and resemble a non-competitive inhibitor on Lineweaver–Burk plots (Fig. 4) as defined by Dixon and Webb (1964). On the other hand, when the reaction starts with addition of the enzyme (Fig. 7), the initial velocity approaches that of the uninhibited enzyme since no significant inactive EI complex forms due to a slow rate of association. However, as the reaction progresses, the inhibited velocity starts to decrease as more EI complex is formed. It is worth noting that Fig. 9 resulted in values of $7.1 \times 10^{-11}\,M$, $4.1 \times 10^7\,M^{-1}\,min^{-1}$ and $2.9 \times 10^{-3}\,min^{-1}$ respectively for K_i, k_1 and k_2 in a good agreement with the aforementioned values. Furthermore, k_1, $3.4–4.1 \times 10^7\,M^{-1}\,min^{-1}$ can be used to calculate the expected I_{50} value for OTFP from the following relationship.

$$I_{50} = \frac{0.693}{k_1 t} \quad (9)$$

The expected I_{50} for 10 min pre-incubation time is $1.7–2.1 \times 10^{-9}\,M$ which is almost identical with the experimental value, $2.3 \times 10^{-9}\,M$, which was found by Hammock *et al.* (1984).

In conclusion OTFP acts as a slow tight binding inhibitor of the JHE from *T. ni* with a K_i value of more than two orders of magnitude lower than the K_m values for JH-I and JH-III. This difference compares favourably with many of the most potent transition state analogues (Wolfenden, 1976). Based on the belief that transition state analogues must function as tight binding inhibitors (Williams and Morrison, 1979), the slow binding inhibition of JHE by OTFP can be explained since the forward reaction would then be slow because the inhibitor would lack many of the essential structural features of the substrate or its transition state, while the reverse isomerization reaction would be even slower because that rate is not enhanced by product formation. The stability of the JHE–OTFP complex may help gain insight into the transition state of the JHE–JH complex using fluorine nuclear magnetic resonance. Furthermore, a comparison of the three dimensional structures of classical inhibitors, slow tight binding inhibitors and acylating inhibitors might be a good starting point to shed some light on the detailed mechanism of JH hydrolysis by JHE and the configuration of the JHE active site(s) under activated conditions.

An appropriate model of the dynamic regulation of JH titre in *T. ni* requires information regarding JH biosynthesis, distribution, metabolism and elimination. However, the information generated above allows us to begin to construct a more limited model describing the capability of the haemolymph to hydrolyze JH. During the prewandering peak of JHE in the haemolymph and other tissues, JHE may be considered the key factor in determining the rate of JH catabolism. Although JHE has been studied extensively in different insect species, very little information is known about its substrate specificity (Hammock, 1984) and no information is at present available on the JHE molarity. The present study considers the use of enzyme inhibitors for the determination of the molar equivalency of JHE based on kinetic treatments. The molar equivalency of JHE was found to be $1.6 \times 10^{-6}\,M$ in *T. ni* haemolymph. This estimate of molarity came from a pool of haemolymph collected from larvae between 3 and 5 hr after lights on 2 days after the emergence of larvae in their last stadium and metabolized JH-I and JH-III at 45 and 34 nmol/min-ml respectively when assayed at $5.0 \times 10^{-6}\,M$ with a 500-fold dilution. Assuming that JHE from *T. ni* has only one catalytic site, one can calculate the molarity of the purified enzyme. In their study Yuhas *et al.* (1983) purified JHE from *T. ni* haemolymph about 436-fold with a recovery of 28%. They finally obtained 0.03 mg protein/ml which gave a single band when electrophoresed on a SDS–polyacrylamide gel and had a molecular weight of 48,000. After correcting for the

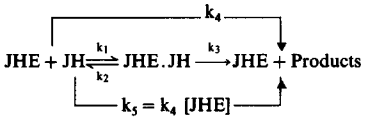
percentage recovered, this amount of protein is equivalent to 2.2×10^{-6} M JHE in the haemolymph. This calculated value is in good agreement with the value calculated from the haemolymph preparation used in this study (1.6×10^{-6} M). The same calculations applied to the 2630-fold purified JHE from *Galleria mellonella* haemolymph (Rudnicka and Kochman, 1984) with a molecular weight of 60,000 (McCaleb *et al.*, 1980) resulted in an estimate of 9.5×10^{-7} M JHE in the haemolymph. It is worth noting that the calculated molarity based on the kinetic treatments is the same for the same enzyme preparation and is indendent of the substrate used (Fig.6). These data support the concept that in *T. ni* one major enzyme hydrolyzes both JH-I and JH-III during the last larval stadium.

JHE activity in the haemolymph of *T. ni* increases over a dynamic range of more than 10-fold from approximately the time of ecdysis to the last larval stadium until the activity peaks early on day two. It then decreases over 100-fold by late day 3 only to increase again to yield a peak of JHE in post-wandering larvae. The absolute activity of JHE in a single developmental stage of *T. ni* (and some other Lepidoptera) shifts during the year, although the JHE pattern during development remains the same. However, using the data of Wing *et al.* (1981) molarity varies from undetectable levels to $\sim 2.0 \times 10^{-6}$ M during the last larval stadium. Even near the time of ecdysis to the last stadium, the molarity is over 1.0×10^{-7}, and as discussed later, the presence of such an active enzyme even at this low titre will play a significant role in the catabolism of JH.

Besides the molarity of the enzyme, the catalytic number has been calculated from the Ackermann–Potter plot to be 19.4 and 37.1 min^{-1} respectively for JH-III and JH-I (Table 1). Also listed in this Table are some other kinetic parameters that have been generated directly from kinetic treatments or from mathematical relations of the enzyme substrate reactions.

The JH-I/JH-III specificity ratios have been calculated from different parameters (Table 1) to show their relation, meaning and biological significance. First, as described before, the specificity was calculated from the activity ratio at high and saturating concentration (5.0×10^{-6} M) of each substrate and resulted in a higher specificity (1.6 times) for JH-I than JH-III. The activity at such high substrate concentrations which are about 82 and 164 times the K_m values for JH-I and JH-III, respectively, showed approximate V_{max} as the velocity equation ($V = V_{max}S/K_m + S$) reduces to $V = V_{max}$. Therefore, the activity ratio at a single constant substrate concentration closely approaches the V_{max} ratio which is 2.1 in favour of JH-I hydrolysis. Furthermore, as the velocity is proportional to the concentration of the enzyme substrate complex, $V = k_3[ES]$, and at an extremely high substrate concentration ($V = V_{max}$), all the enzyme should be in the form of an enzyme substrate complex and then $V_{max} = k_3[E_t]$. This situation indicates that the ratios of the V_{max}'s using JH-I and JH-III as substrates must be equal to the ratios of k_3's. Interestingly, both ratios are very close to 2.0 and agree very well with the theoretical interpretation. On the other hand the activity ratio at 5.0×10^{-6} M of each substrate (1.6) approaches the k_3 and V_{max} ratios. It is quite clear that this specificity is real under zero order kinetics ($S \gg K_m$) where all the enzyme is saturated or oversaturated with the substrate. The question at issue is to what extent, if any, these specificity ratios express the specificity under *in vivo* conditions. Although there are large differences in the absolute concentration of circulating hormones between different insect species (deKort and Granger, 1981) even at the same developmental stage (Trautmann *et al.*, 1976), reported JH titres in the last larval stadium of lepidopterous larvae are generally below 10 ng/ml (Schooley *et al.*, 1976; Mauchamp *et al.*, 1979; Schooley, personal communication) and range from about 10–400 pg/g of tissue in *T. ni* (Jones, unpublished). Assuming homogeneous distribution of JH in tissues of *T. ni* and an average unit density, a molar concentration of about 3.4×10^{-11}–1.4×10^{-9} equivalents of JH-I, which is far below the K_m value of JHE to JH-I. Under such conditions, the velocity equation reduces to the form ($V = V_{max}[S]/K_m$) in which V_{max}/K_m represents a pseudo-first order rate constant (k_5) for the overall reaction which follows first order kinetics with regard to [S]. Therefore, the best measure for substrate specificity for an enzyme that is known to be at high concentration in comparison to its substrate(s) under

Table 1. Kinetic parameters and substrate specificity of juvenile hormone esterase (JHE) from *Trichoplusia ni* hydrolyzing JH-I and JH-III



Kinetic parameter	JH-I	JH-III	JH-I/JH-III
K_m (M)	6.1×10^{-8}	3.1×10^{-8}	2.0
V_{max} (nmol/min-ml)	177	85.0	2.1
k_3 (min^{-1})	37.1	19.4	1.9
$k_4 = \frac{k_3}{K_m}$ ($M^{-1}\,min^{-1}$)	6.1×10^{8}	6.4×10^{8}	1.0
$k_5 = \frac{V_{max}}{K_m}$ (min^{-1})	2.9×10^{3}	2.8×10^{3}	1.0
$t_{0.5} = \frac{0.693}{k_5}$ (min)	2.4×10^{-4}	2.5×10^{-4}	1.0

in vivo conditions is the first order rate constant ($k_5 = V_{max}/K_m$) that governs the reaction under such conditions. As has been discussed earlier the k_3/K_m can be used instead which represents the second order rate constant (k_4) for the overall reaction when both enzyme and substrate concentrations are comparable and much lower than K_m values. The advantage of using k_3/K_m as a specificity measure is that it is independent of the enzyme concentration and preparation. Interestingly, in contrast to the specificity value measured from the activity ratio, V_{max} ratio, and k_3 ratio which all showed higher preference for JH-I than for JH-III, the specificity calculated from the first or second order rate constants showed equal specificity of the enzyme to both substrates, which indeed expresses the substrate specificity under *in vivo* conditions where the reaction mechanism is bimolecular and follows pseudo-first order kinetics with regard to the substrate.

There appears to be a single low molecular weight JH carrier molecule in the haemolymph of *T. ni* (Sparks and Hammock, 1979; Wing *et al.*, 1984). The molarity of binding sites was estimated previously as 2.9 and 3.0×10^{-6} M for JH-III and JH-I with K_d's of 3.7×10^{-6} M and 4.2×10^{-7} M, respectively (Hammock *et al.*, 1977). Wing *et al.* (1981) reported that the dynamic range of fluctuation of binding sites is rather limited in the last larval stadium and using a pool of haemolymph Wing *et al.* (1984) estimate a K_d of 6.9×10^{-7} M for JH-I with an association constant (k_a) of $4.5 \times 10^5\,M^{-1}\,min^{-1}$ and a dissociation constant (k_d) of $\sim 0.3\,min^{-1}$ using excess JH to displace the label from the carrier molecule. Since a large excess of hormone was used for these studies, the racemic nature of the hormone should not influence the above estimates of equilibrium and kinetic parameters greatly.

Although the JH binding protein is about twice the maximal concentration of JHE, calculated from the present work, the 10-fold higher affinity of JH-I to JHE than that of JH-I to the binding protein favours the interaction with the enzyme. K_m was taken as a measure of affinity since k_3 is not high so that $K_m \simeq K_s$. If $K_m \neq K_s$ and even if the real affinity of the enzyme–JH complex were lower than that of the binding protein–JH complex, the former species would be enhanced through product formation. This condition can be explained by calculating the half time ($t_{0.5}$) for JH-I hydrolysis which equals $0.693/k_5$ or $0.693K_m/V_{max}$, which is only a fraction of second (0.01). The extremely high and constant concentration of the binding protein as compared with the concentrations of JH homologues under *in vivo* conditions suggests that the binding process is likely a pseudo-first order reaction as well. The pseudo-first order rate constant equals the second order rate constant of the association times the binding protein concentration. A calculation from the data of Wing *et al.* (1984) resulted in a pseudo-first order rate constant of $1.59\,min^{-1}$ and half time for the association reaction of 0.44 min or 26.4 sec. Comparing this value with the half time of JH hyrolysis (0.01 sec) indicates clearly that in the presence of JHE, the whole reaction would be shifted aggressively toward JH hydrolysis particularly if we consider the relatively high rate of dissociation between the binding protein and JH-I. Therefore K_s is useful for comparing substrate affinity with an enzyme, but the real measure of the ability of an enzyme to clear a substrate from a pool is a function of its K_m and k_3 for the overall reaction is irreversible with JHE yet reversible with the binding protein.

There is some question regarding the true k_d of the JH carrier protein under *in vivo* conditions (Wing *et al.*, 1984; Law, personal communication), but even if the k_d is much slower one can argue that JHE will still be a very effective scavenger of JH. JHE and the carrier protein obviously interact to control the titre of JH in the tissues. Since the binding activity of JH in haemolymph varies over a limited range while JHE activity varies dramatically during the last larval stadium, the latter changes are more likely to lead to large changes in JH titre. However, since JHE shows equal specificity for JH-I and JH-III under physiological conditions while the carrier protein binds JH-III very poorly, one can anticipate that the relative speed of hydrolysis of JH homologues in the haemolymph will be determined by the JH carrier rather than JHE.

There is a limited range over which the kinetic properties of a biological catalyst can vary. Those of JHE (apparent high affinity for the substrate, but a low to moderate turnover) are appropriate for a very effective scavenger of JH at physiological concentrations. These properties explain why almost total inhibition of JHE is required before *in vivo* effects are observed (Sparks and Hammock, 1980; Abdel-Aal *et al.*, 1984). Thus, JHE does not appear to be a likely target for inhibitors if one wishes to disrupt insect development in the field. However, precocious induction of JHE could lead to profound developmental effects. One can further argue that even less specialized esterases are likely to have an important role in JH catabolism, although their role is insignificant by comparison when JHE is present.

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REFERENCES

Abdel-Aal Y. A. I., Roe R. M. and Hammock B. D. (1984) Kinetic properties of the inhibition of juvenile hormone esterase by two trifluoromethylketones and *O*-ethyl, *S*-phenyl phosphoramidothioate. *Pestic. Biochem. Physiol.* **21,** 232–241.

Ackermann W. W. and Potter V. R. (1949) Enzyme inhibition in relation to chemotherapy. *Proc. Soc. exp. Biol. Med.* **72,** 1–9.

Aldridge W. N. (1950) Some properties of specific cholinesterase with particular reference to the mechanism of inhibition by diethyl *p*-nitrophenyl thiophosphate (E605) and analogues. *Biochem. J.* **46,** 451–458.

Aldridge W. N. and Reiner E. (1975) Enzyme inhibitors as substrates. In *Interaction of Esterases with Ester of Organophosphorous and Carbamic Acids*, p. 197. North Holland, Amsterdam.

Brodbeck U., Schweikert K., Gentinetta R. and Rottenberg M. (1979) Fluorinated aldehydes and ketones acting as quasi-substrate inhibitors of acetylcholinesterase. *Biochim. biophys. Acta* **567,** 357–369.

Cha S. (1975) Tight-binding inhibitors—I. Kinetic behavior. *Biochem. Pharmac.* **24,** 2177–2185.

Cha S., Agarwal R. P. and Parks R. E. Jr (1975) Tight-binding inhibitors—II. Non-steady state nature of inhibition of milk xanthine oxidase by allopurinol and alloxanthine and of human erythrocytic adenosine deaminase by coformycin. *Biochem. Pharmac.* **24,** 2187–2197.

Coudron T. A., Dunn P. E., Seballos H. L., Wharen R. E., Sanburg L. L. and Law J. H. (1981) Preparation of homogeneous juvenile hormone specific esterase from the haemolymph of the tobacco hornworm, *Manduca sexta. Insect Biochem.* **11,** 453–461.

Dixon M. and Webb E. C. (1964) *Enzymes*, 2nd edn, p. 316. Academic Press, New York.

Granger N. A., Niemiec S. M., Gilbert L. I. and Bollenbacher W. E. (1982) Juvenile hormone III biosynthesis by the larval corpora allata of *Manduca sexta. J. Insect Physiol.* **28,** 385–391.

Hammock B. D., Nowock J., Goodman W., Stamoudis V. and Gilbert L. I. (1975) The influence of hemolymph-binding protein on juvenile hormone stability and distribution in *Manduca sexta* fat body and imaginal disks *in vitro. Molec. cell. Endocr.* **3,** 167–184.

Hammock B. D. and Sparks T. C. (1977) A rapid assay for insect juvenile hormone esterase activity. *Analyt. Biochem.* **82,** 573–579.

Hammock B. D., Sparks T. C. and Mumby S. M. (1977) Selective inhibition of JH esterases from cockroach hemolymph. *Pestic. Biochem. Physiol.* **7,** 517–530.

Hammock B. D. and Quistad G. B. (1981) Metabolism and mode of action of juvenile hormone, juvenoids and other insect growth regulators. In *Progress in Pesticide Biochemistry* (Edited by Hutson D. H. and Roberts T. R.), Vol. 1, pp. 1–83. Wiley, New York.

Hammock B. D., Wing K. D., McLaughlin J., Lovell V. M. and Sparks T. C. (1982) Trifluoromethylketones as possible transition state analog inhibitors of juvenile hormone esterase. *Pestic. Biochem. Physiol.* **17,** 76–88.

Hammock B. D. (1984) Regulation of juvenile hormone titers: Degradation. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Edited by Kerkut G. A. and Gilbert L. I.). Pergamon Press, Oxford. In press.

Hammock B. D., Abdel-Aal Y. A. I., Mullin C. A., Hanzlik T. N. and Roe R. M. (1984) Substituted thiotrifluoropropanones as potent selective inhibitors of juvenile hormone esterase. *Pestic. Biochem. Physiol.* In press.

Jones D., Jones G. and Hammock B. D. (1981) Growth parameters associated with endocrine events in the larval *Trichoplusia ni* (Hübner) and timing of these events with developmental markers. *J. Insect Physiol.* **27,** 779–788.

Jones D., Jones G., Wing K. D., Rudnicka M. and Hammock B. D. (1982) Juvenile hormone esterases of Lepidoptera. I. Activity in the hemolymph during the last larval instar of 11 species. *J. comp. Physiol.* **148,** 1–10.

deKort C. A. D. and Granger N. A. (1981) Regulation of the juvenile hormone titer. *A. Rev. Ent.* **26,** 1–28.

Kramer K. J., Dunn P. E., Peterson R. C. and Law J. H. (1976) Interaction of juvenile hormone with binding proteins in insect hemolymph. In *The Juvenile Hormones* (Edited by Gilbert L. I.), pp. 327-341. Plenum Press, New York.

Lineweaver H. and Burk D. (1934) The determination of enzyme dissocation constants. *J. Am. chem. Soc.* **56,** 658–666.

Main A. R. (1964) Affinity and phosphorylation constants for the inhibition of esterases by organophosphates. *Science* **144,** 992–993.

Main A. R. and Iverson F. (1966) Measurement of the affinity and phosphorylation constants governing irreversible inhibition of cholinesterases by di-isopropyl phosphorofluoridate. *Biochem. J.* **100,** 525–531.

Mauchamp B., Lafont R. and Jourdain D. (1979) Mass fragmentographic analysis of juvenile hormone I levels during the last larval instar of *Pieris brassicae. J. Insect Physiol.* **25,** 545–550.

McCaleb D. C., Reddy G. and Kumaran A. K. (1980) Some properties of the haemolymph juvenile hormone esterases in *Galleria mellonella* larvae and *Tenebrio molitor* pupae. *Insect Biochem.* **10,** 273–277.

Morrison J. F. (1969) Kinetics of the reversible inhibition of enzyme-catalysed reactions by tight-binding inhibitors. *Biochim. biophys. Acta* **185,** 269–286.

Morrison J. F. (1982) The slow-binding and slow, tight-binding inhibition of enzyme catalysed reactions. *TIBS, March,* 102–105.

Nijhout H. F. and Williams C. M. (1974) Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L): cessation of juvenile hormone secretion as a trigger for pupation. *J. exp. Biol.* **61,** 493–501.

Nijhout H. F. (1975) Dynamics of juvenile hormone action in the larvae of the tobacco hornworm, *Manduca sexta* (L.). *Biol. Bull. Woods Hole* **149,** 568–579.

Prestwich G. D., Wai-Si E., Roe R. M. and Hammock B. D. (1984) Synthesis and bioassay of isoprenoid 3-alkylthio-1,1,1-trifluroro-2-propanones: Potent selective inhibitors of juvenile hormone esterase. *Archs Biochem. Biophys.* **228,** 639–645.

Roe R. M., Hammond A. M. Jr and Sparks T. C. (1982) Growth and development of larval *Diatraea saccharalis* on an artificial diet and synchronization of the last larval stadium. *Ann. ent. Soc. Am.* **75,** 421–429.

Roe R. M., Hammond A. M. Jr and Sparks T. C. (1983) Characterization of the plasma juvenile hormone esterase in synchronous last stadium female larvae of the sugar cane borer, *Diatraea saccharalis* (F.). *Insect Biochem.* **13,** 163–170.

Rudnicka M. and Kochman M. (1984) Purification of the juvenile hormone esterase from the haemolymph of the wax moth *Galleria mellonella*, Lepidoptera. *Insect Biochem.* **14,** 189–198.

Schooley D. A., Judy K. J., Bergot B. J., Hall M. S. and Jennings R. C. (1976) Determination of the physiological levels of juvenile hormones in several insects and biosynthesis of the carbon skeletons of the juvenile hormones. In *The Juvenile Hormones* (Edited by Gilbert L. I.), pp. 101-117. Plenum Press, New York.

Segel I. H. (1975) *Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp. 100–226. Wiley, New York.

Shorey H. H. and Hale R. L. (1965) Mass-rearing of the larvae of nine noctuid species on a simple artificial medium. *J. econ. Ent.* **58,** 522–524.

Smissaert H. R. (1970) Application of first-order kinetics in irreversible inhibition of acetylcholinesterase. In *Toxicology, Biodegradation and Efficacy of Livestock Pesticides* (Edited by Khan M. A. and Haufe W. O.), pp. 43–72. Swets & Zeitlinger, Amsterdam.

Sparks T. C., Willis W. S., Shorey H. H. and Hammock B. D. (1979) Haemolymph juvenile hormone esterase activity in synchronous last instar larvae of the cabbage looper, *Trichoplusia ni. J. Insect Physiol.* **25,** 125–132.

Sparks T. C. and Hammock B. D. (1979) A comparison of the induced and naturally occurring juvenile hormone esterases from the last instar larvae of *Trichoplusia ni. Insect Biochem.* **9,** 411–421.

Sparks T. C. and Hammock B. D. (1980) Comparative inhibition of the juvenile hormone esterases from *Trichoplusia ni, Tenebrio molitor* and *Musca domestica. Pestic. Biochem. Physiol.* **14,** 290–302.

Sparks T. C., Hammock B. D. and Riddiford L. M. (1983) The haemolymph juvenile hormone esterase of *Manduca sexta* (L.): Inhibition and regulation. *Insect Biochem.* **13,** 529–541.

Sparks T. C. and Rose R. L. (1983) Inhibition and substrate specificity of the haemolymph juvenile hormone esterase of the cabbage looper, *Trichoplusia ni* (Hübner). *Insect Biochem.* **13,** 633–640.

Trautmann K. H., Suchy M., Masner P., Wipf H.-K. and Schuler A. (1976) Isolation and identification of juvenile hormones by means of a radioactive isotope dilution method: Evidence for JH III in eight species from four orders. In *The Juvenile Hormones* (Edited by Gilbert L. I.), pp. 118–130. Plenum Press, New York.

Williams C. M. (1961) The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the cecropia silkworm. *Biol. Bull. Woods Hole* **121,** 572–585.

Williams J. W. and Morrison J. F. (1979) The kinetics of reversible tight-binding inhibition. *Meth. Enzym.* **63,** 437–467.

Wing K. D., Sparks T. C., Lovell V. M., Levinson S. O. and Hammock B. D. (1981) The distribution of juvenile hormone esterase and its interrelationship with other proteins influencing juvenile hormone metabolism in the cabbage looper, *Trichoplusia ni. Insect Biochem.* **11,** 473–485.

Wing K. D., Rudnicka M., Jones G., Jones D. and Hammock B. D. (1984) Juvenile hormone esterases of Lepidoptera. II. Isoelectric points and binding affinities of hemolymph juvenile hormone esterase and binding protein activities. *J. comp. Physiol.* **154,** 213–223.

Wolfenden R. (1976) Transition state analog inhibitors and enzyme catalysis. *A. Rev. Biophys. Bioeng.* **5,** 271–306.

Yuhas D. A., Roe R. M., Sparks T. C. and Hammond A. M. Jr (1983) Purification and kinetics of juvenile hormone esterase from the cabbage looper, *Trichoplusia ni* (Hübner). *Insect Biochem.* **13,** 129–136.