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Apparent Multiple Catalytic Sites Involved in the Ester Hydrolysis of Juvenile Hormones by the Hemolymph and by an Affinity-Purified Esterase from *Manduca sexta* Johannson (Lepidoptera: Sphingidae)[1]

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The esterases which metabolize juvenile hormone (JH) in some insects may be important in regulating the hormone titer. The JH ester-hydrolyzing activity (JHE) in the larval hemolymph of the tobacco hornworm (*Manduca sexta*) was found to be attributed to two forms of esterase with almost equivalent activity based on selectivity and kinetics of inhibition by two 3-substituted thio-1,1,1-trifluoropropan-2-ones and a phosphoramidothioate. Neither of the two forms were inhibited by diisopropyl phosphorofluoridate or iodoacetamide. Steady-state kinetics of JH II hydrolysis supported the inhibition studies and showed that the two forms were widely different in their affinity for JH II. The activity of the hemolymph was found to be bound selectively to an affinity column synthesized by the reaction of epoxy-activated Sepharose with 3-(4′-mercaptobutylthio)-1,1,1-trifluoropropan-2-one. This column offered a quantitative, one-step purification of JH esterase with a purification factor of ~800 and specific activity of ~573 nmol JH III hydrolyzed min^{-1} mg $protein^{-1}$. The purified protein showed only a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a molecular weight of ~65,000. However, the purified enzyme apparently revealed the same two kinetic forms as the native enzyme, which indicates that two sites of the same protein are likely to be involved in JH hydrolysis. © 1985 Academic Press, Inc.

During the early portion of the last larval stadium of numerous lepidopterous species, it has been demonstrated that a reduction in the titer of juvenile hormone (JH)[4] is a necessary prerequisite to the release of prothoracicotropic hormone (PTTH) which leads to a cascade of events, culminating in pupation and metamorphosis to the adult form (1). It was noticed by a variety of workers that this anticipated reduction in JH titer is associated with a dramatic rise in the rate of hydrolysis of the juvenile hormone methyl ester in hemolymph and other tissues (1-3).

The discovery that potent, selective inhibitors of this JH esterase activity (here-

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[4] Abbreviations used: JH, juvenile hormone; JHE, JH esterase(s); DFP, diisopropyl phosphorofluoridate; EPPAT, *O*-ethyl,*S*-phenylphosphoramidothioate; OTFP, 3-octylthio-1,1,1-trifluoropropan-2-one; BPTFP, 3-(4′-butylphenythio)-1,1,1-trifluoropropan-2-one; MBTFP, 3-[(4′-mercapto)butylthio)-1,1,1-trifluoropapan-2-one; MBTFP-Sepharose, the resin produced from reacting epoxy-activated Sepharose with MBTFP; PTU, phenylthiourea; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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after referred to as JHE) could disrupt metamorphosis led to the hypothesis that in some species an increase in catabolism, in addition to a decrease in biosynthesis, led to the reduction in JH titer (4–7). The evidence for this hypothesis recently has been reviewed (3).

It is thus important to examine the pharmacokinetics of JH degradation as well as the regulation of the JHE. To this end a detailed study of the kinetics of JHE as well as a method for its purification are requisite. Evidence is mounting that in the lepidopterous species examined, a single enzyme or a closely related group of highly specialized esterases are responsible for the majority of JH catabolism during the early part of the last larval stadium (8, 9). In the tobacco hornworm (*Manduca sexta* Johannson, Lepidoptera: Sphingidae), there is some confusion. Earlier studies indicated multiple enzymes involved in JH hydrolysis (10, 11), while later studies indicated a major involvement of a single blood protein (9, 12). This study was undertaken in an attempt to resolve these differences and to lay the foundation for further studies into the regulation and pharmacokinetics of the JH–JHE interaction.

EXPERIMENTAL PROCEDURES

Hemolymph preparation. Larvae of *M. sexta* and their artificial diet were provided through the courtesy of David A. Schooley and Mike Johnston (Zoecon Corporation, Palo Alto, Calif.). These larvae were obtained as late fourth instars, and they were reared in our laboratory until used. Hemolymph was from larvae having weights and developmental markers indicative of the prewandering peak of JHE activity (5, 13). Hemolymph was collected from the clipped anal horn and thoracic legs of the larvae into 15-ml centrifuge tubes which contained a few crystals of phenylthiourea (PTU) to inhibit tyrosinases. The hemolymph was mixed extensively and then centrifuged at 10,000*g* for 10 min at ~1°C, and the supernatant (plasma) was kept in aliquots at −70°C until used. JHE activity showed no change for several months under these conditions. The plasma was diluted with sodium phosphate buffer (pH 7.4, I = 0.2 M) containing 0.01% PTU, 0.05% Triton X-100, 0.02% sodium azide, 5% sucrose, and 10^{-3} M 2-mercaptoethanol (buffer cocktail). Preliminary experiments showed no effect of the latter four components on JHE activity. They were expected to stabilize the enzyme during the purification procedure.

Enzyme assays. JHE activity was assayed by the partition method of Hammock and Sparks (14). For routine analysis, 1 μl of 0.5 mM JH III or JH II was added to 100 μl of diluted plasma, and the reaction mixture was incubated for 5–20 min at 30 ± 0.5°C. In all cases the protein dilutions and the incubation times were selected to ensure that data were collected during the linear portion of the reaction (usually 10 min with 1% plasma in buffer). The racemic substrates [10-^{3}H]JH III (11 Ci/mmol) or [10-^{3}H]JH II (11.6 Ci/mmol) were from New England Nuclear, and unlabeled *2E,6E* substrates were from Calbiochem.

Inhibition. Five compounds were tested for their inhibitory potency against JHE of *M. sexta.* They included EPPAT, DFP, iodoacetamide, OTFP and BPTFP. The last two compounds were synthesized by Hammock *et al.* (7), and were checked for purity by thin-layer chromatography and nuclear magnetic resonance spectroscopy. These compounds were dissolved in ethanol and 1 μl of the inhibitor solutions was added to each reaction mixture. Two criteria were used for testing the potency of these compounds. The first was the I_{50} value [the concentration of the inhibitor required to reduce the activity of the enzyme by 50% after 10 min preincubation (7, 15)]. These compounds were added in 100 μl of 1% diluted plasma or 100 μl of the purified enzyme. The purified enzyme was diluted (~30 ng/assay) so that its esterase activity was very similar to that of 1% diluted plasma. Control experiments received only 1 μl ethanol. Semilog plots of percentage inhibition against inhibitor concentration were used to provide the inhibition curve. The second criterion was purely kinetic since it measured the rate of inhibition as a function of the preincubation time as has been described before (15, 16). It should be mentioned that the time scale for the preincubation was variable depending on the compound and the concentration. However, for each inhibitor concentration the time of incubation with the substrate was kept constant. The rate of inhibition for each inhibitor concentration was measured three times and the average percentage residual activity was plotted against preincubation time on semilog paper to test for pseudo-first-order kinetics. The data obtained from pseudo-first-order plots were found to be valid for two further kinetic treatments. These kinetic treatments were the first (17–19)- and second-order (20) inhibition reactions as will be presented under Results and Discussion.

Steady-state kinetics of JH II hydrolysis. Double-reciprocal plots for the JH II-hydrolyzing activity of 0.0625 and 0.125% of diluted plasma or diluted purified enzyme (~1.5 ng/assay) were examined using final substrate concentrations ranging from 8.85×10^{-9} to 1.09×10^{-6} M. In order to estimate the initial velocity accurately, the incubation time used was only 1 min at 30°C carried out in carbowax-treated glass tubes. The same kinetic treatment also was performed in

the presence of OTFP at two concentrations (5.47×10^{-8}, 4.38×10^{-7} M). The inhibitor was added in 1 μl ethanol solution to 100 μl of 0.25% diluted plasma, and the reaction was preincubated for 10 min at 30°C prior to addition of substrate and analysis of activity as discussed earlier.

Preparation of the affinity column (MBTFP-Sepharose). The affinity matrix was prepared by reacting epoxy-activated Sepharose with 3-[(4′-mercapto)butylthio]-1,1,1-trifluoropropan-2-one (MBTFP) (Fig. 1). Sepharose CL-6B was epoxy-activated according to literature procedures (21, 22). Thus, 40 ml of Pharmacia Sepharose CL-6B (washed with 10 vol of distilled H_2O) was added to 28 ml of 1 N NaOH containing 57 mg $NaBH_4$ and 5.7 ml of 1,4-butanediol diglycidyl ether (Aldrich). The slurry was swirled at room temperature for about 10 h using an orbital shaker. Then the epoxy-activated resin was washed extensively with water, water:methanol (1:1), and water, and then dried under suction. An aliquot of the resin was assayed for free epoxides by adding 1.5 ml of 1.3 M $Na_2S_2O_3$ (pH 7.0) to 0.3 g of the resin followed by back-titration of the resultant base using 0.01 N HCl. Values of ~10 μEq/g resin were obtained for these preparations. MBTFP was synthesized from the reaction of 1,4-dimercaptobutane (Aldrich Chemical Co.) with equimolar 3-bromo-1,1,1-trifluoroacetone (PCR Research Chemicals Inc., Gainesville, Fla.). The details of the synthetic procedure are covered elsewhere (6, 7, 23). To 10.0 g of moist epoxy-activated resin (~100 μEq) 10 ml of 1:1 methanol:0.1 M $NaHCO_3$ was added to give a pH of 8.7–8.9, followed by 10 ml of 40 mM MBTFP in methanol. The slurry was swirled for 24 h at room temperature, excess mercaptoethanol was added, and then the slurry was washed in a sintered-glass funnel with 20 vol of methanol:water (1:1) and methanol, alternatively. Then the gel was washed with 0.5 M NaCl, 1% Lubrol-Px, water, ethanol:water and ethanol, and was stored at 4°C in absolute ethanol containing a crystal of BHA (butylated hydroxanisole) as an antioxidant.

Affinity chromatography on MBTFP-Sepharose. Affinity purifications were performed at 2°C using a 1-ml tuberculin syringe. A 100-μl bed of the affinity gel was packed in the syringe over a thin layer of glass wool and then washed with 10 ml each of ethanol, ethanol:water (3:1), ethanol:water (1:1), ethanol:water (1:3), and water. Then the column was equilibrated with the buffer cocktail. The buffer was monitored to ensure that inhibitors were not leaching from the matrix. Plasma of *M. sexta* diluted to 20% in the above buffer was preincubated with 2×10^{-4} M DFP for 15 min at 30°C to inhibit general esterases (10, 11). The activity toward 5×10^{-6} M JH III was identical before and after adding DFP. The diluted plasma was then pumped onto the column at a flow rate of 3–5 ml/h using a peristaltic pump, and 2- to 3-ml fractions were collected automatically. A 100-ml sample of diluted plasma took ~24 h to pass through the column. Aliquots of the plasma taken before loading the column were checked repeatedly for their ability to hydrolyze JH III, and were used as a standard to monitor recovery. The ability of the diluted plasma to hydrolyze JH III was essentially stable for the duration of the experiment. The esterase activity on JH III and the protein concentrations of the loading fractions were assayed. No detectable JHE activity eluted from the column during loading, and the protein concentration in the loading fractions was indistinguishable from the original diluted plasma. The column was then washed with the aforementioned buffer for about 20 h at a flow rate of 5 ml/h. Except for the first 3–5 fractions (2 ml each) there was no measurable protein and no JHE activity was detected in any of the fractions. The column was then equilibrated with ~1.0 ml of a OTFP solution (10^{-3} M) in buffer for 6–12 h

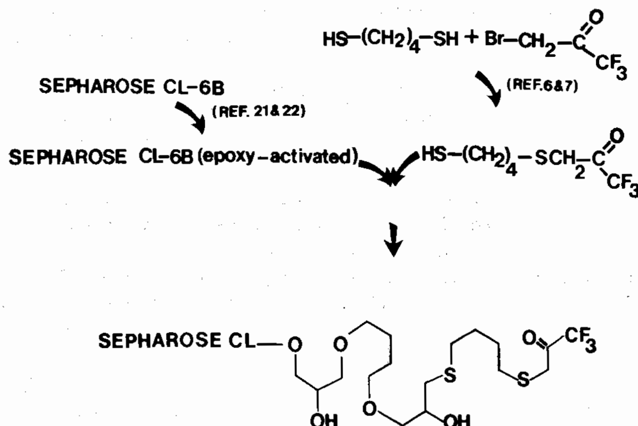


FIG. 1. Outline of the synthesis of MBTFP-Sepharose resin for affinity chromatography.

with shaking every 2 h. This procedure allowed the recovery of measurable protein in six fractions (0.95 ml each).

Reactivation of OTFP-inhibited JHE. To 20 μl from each of the above fractions was added 133 μl of a bovine serum albumin (BSA, Fraction V) solution at a concentration of 1 mg/ml, and the volume was adjusted to 2 ml using the buffer cocktail. No effect of the above BSA concentration on JH-hydrolyzing activity of the plasma was found. This condition gave a 100× dilution of both free and bound inhibitor, which was found to be critical for reactivation of JHE activity. These samples were dialyzed as has been described before (16) for 9 days against 1 liter of the buffer, and samples were withdrawn periodically and tested for JHE activity.

Protein assays. Protein concentrations for all studies were determined by a dye binding method (24), as modified by Bio-Rad, using BSA (fraction V, Sigma) as the protein standard. The method has been adapted to employ 100 μl of samples and 400 μl of 5x diluted dye (20% in distilled water). Ten minutes after the addition of the dye the absorbance was measured at 620 nm using a Gilford EIA manual ELISA reader interfaced with an Atari 400 microcomputer (programmed by R. Wixtrom, this laboratory).

SDS-polyacrylamide gel electrophoresis. Molecular weight determinations and homogeneity of JHE eluted with OTFP were assessed by denaturing polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS). A Hoefer SE600 vertical slab gel unit was employed to conduct discontinuous SDS-PAGE according to Laemmli (25) on 0.75-mm gels (12% acrylamide in separating gel, 5% in stacking gel).

RESULTS AND DISCUSSION

Selective inhibition. Iodoacetamide and DFP were found to be essentially inactive in that at 10^{-3} M the former gave no inhibition of JHE and the latter gave only 10% inhibition. However, EPPAT, OTFP, and BPTFP were found to be unusually potent which enabled the evaluation of their concentration-inhibition curves. In all cases, inhibitor concentrations were selected to give 0–95+% inhibition after a 10-min preincubation time. The residual enzyme activity was monitored using JH III at a final molar concentration of 5×10^{-6} M, and the percentage inhibition was plotted against log inhibitor concentration (Fig. 2). With the three inhibitors used, the inhibition pattern was not a simple one and in fact there is an apparent indication of the presence of two forms of JH-hydrolyzing activity with different sensitivity to these inhibitors. These forms are indicated by either a clear plateau in the inhibition curve or an inflection point near 50% inhibition. This behavior is quite different from that of JHE from larvae of the cabbage looper, *Trichoplusia ni,* where only a steep sigmoid curve was obtained with OTFP and EPPAT (7, 15), a characteristic of homogeneous enzyme activity. However, other inhibitors indicate the possibility that there are multiple forms of JHE in *T. ni* as well.

Equivalent activity was assumed for the two forms based on the shape of the inhibition curves, and percentage inhibition from each form was recalculated as follows. All the percentages of inhibition under 50% are expected to be from the sensitive form and should be doubled to be relative to that form since it contributes ~50% to the total activity. All the percentages of inhibition above 50 are expected to represent a complete inhibition of the sensitive form in addition to inhibition of a certain fraction of the less sensitive form. Therefore, 50 was subtracted from the percentage of inhibition and the residual was doubled to be relative to the less sensitive form. When the corrected percentage of inhibition was plotted against log inhibitor concentration, apparently straight lines were obtained for each form which resemble the inhibition of two homogenous catalytic sites. Regression analysis of these lines gave molar I_{50} values for OTFP of 1.2×10^{-9} and 3.8×10^{-6}; for BPTFP of 5.9×10^{-8} and 1.2×10^{-5}; and for EPPAT of 1.1×10^{-10} and 1.1×10^{-8}, respectively, for the sensitive and less sensitive forms. Solubility of the tested inhibitors seems not to be responsible for the behavior on the inhibition curves since the inflection point in each curve occurred at concentrations lower than 10^{-6} M. However, it was decided to use another line of evidence for the multiplicity of JHE from *M. sexta* which is the time course of inhibition.

Time course of inhibition. As has been reported before (15, 16) both OTFP and EPPAT inhibit JHE from *T. ni* in a time-dependent fashion. Therefore, these two compounds were used to evaluate the multiplicity of JH-hydrolyzing activity in *M.*

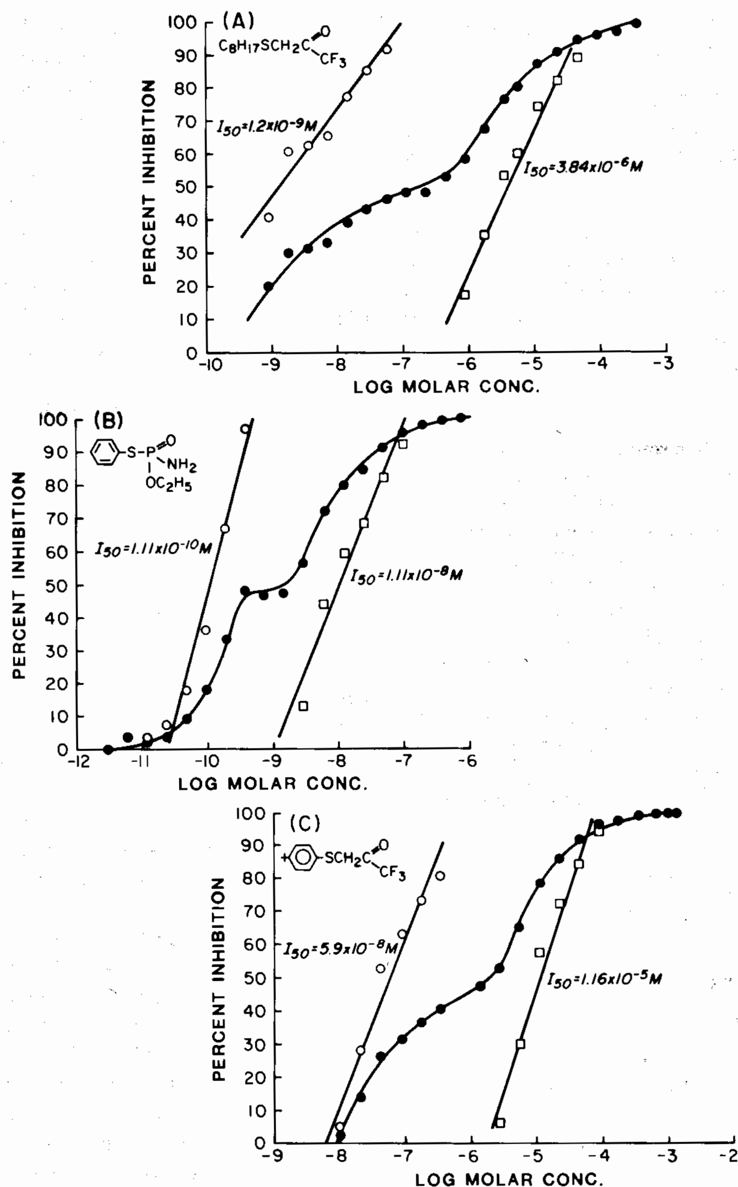


FIG. 2. Plots of percentage inhibition of JHE from larval hemolymph of *M. sexta* against log molar concentration of OTFP (A), EPPAT (B), and BPTFP (C). The inhibition curves showed the behavior of the sensitive and less sensitive forms of JHE with equivalent activity. Resolution of the inhibition for each form resulted in apparent straight lines for the sensitive (○) and less sensitive (□) forms. JH III at a final concentration of 5×10^{-6} M was added 10 min after the addition of the inhibitor. Each point on the curves was the average of at least four replicates with a standard deviation of less the 5% of the mean.

sexta using a time course to measure the rate of inhibition for each inhibitor concentration, a procedure that eliminates the solubility of the tested compound as a reason for the complex behavior observed. In the case of OTFP, four concentrations, ranging from 1.22×10^{-9} to 1.37×10^{-8} M, were preincubated with the enzyme for 0–

18 min and sampled at 0.5-min intervals, and the residual activity was then measured by adding JH III as substrate at a final concentration of 5×10^{-6} M. In each of the concentrations used, the residual activity decreased gradually until it reached about 50% of the original activity, and then stayed unchanged and independent of the preincubation time. Since the uninhibited residual activity is independent of the concentrations used, it is not likely that this plateau is due to the inhibited enzyme reaching a steady-state condition.

Figure 3 shows an example of the pseudo-first-order plots of JHE inhibition by two inhibitor concentrations using JH III as substrate. Also, with one of these concentrations JH II was used instead. It is interesting that although the proportion of the less sensitive form is different based on the substrate used, the corrected percentage residual activity for the sensitive form was almost identical for the two substrates. Pseudo-first-order plots for the inhibition of JH III and JH II hydrolysis by 1.37×10^{-8} M OTFP gave second-order rate constants (k_i) of 5.43×10^7 and 5.73×10^7 M^{-1} min^{-1}, (Fig. 3, inset), respectively.

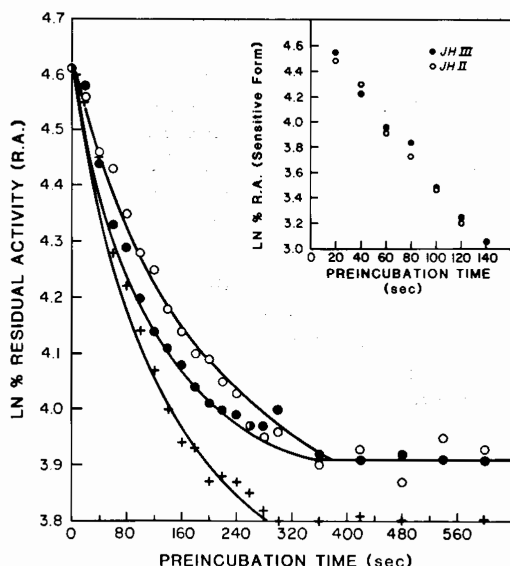


FIG. 3. Pseudo-first-order plots for the inhibition of JHE from larval hemolymph of *M. sexta* by OTFP at final molar concentrations of 6.84×10^{-9} M (○), 1.37 $\times 10^{-8}$ M (●) using JH III as a substrate, and 1.37 $\times 10^{-8}$ M (+) using JH II as a substrate. Inset shows pseudo-first-order plots of the inhibition of the sensitive form by 1.37×10^{-8} M OTFP after correction for the less sensitive form. Each point represents the mean of three separate determinations.

An extensive effort has been devoted to study the mode of inhibition of JHE from *T. ni* by OTFP (15, 16). In these studies, the compound was shown to act as a slow, tight-binding inhibitor, which seems to be the case with the sensitive form of JHE from *M. sexta*. If this is the case, one would expect a relationship between I_{50} and k_i, according to the equation (26)

$$k_i = 0.693/I_{50}(t). \quad [1]$$

It is worth noting that substitution for the 10-min preincubation I_{50} value in the above equation resulted in a calculated k_i value of 5.68×10^7 M^{-1} min^{-1}, which is almost identical to the experimental value obtained from the pseudo-first-order kinetics. This reproducibility not only indicates that the reaction of the sensitive form with OTFP is of second order kinetics and demonstrates the characteristics of slow tight binding inhibition (27), but also gives additional supportive evidence for the presence of two forms of JHE in the hemolymph of *M. sexta*.

In order to test the time course of inhibition of the less sensitive form, higher concentrations of OTFP (2.2×10^{-7}–5.5×10^{-6} M) were used to ensure complete inhibition of the sensitive form. Figure 4 shows that the less sensitive form reacts with OTFP in a time-independent manner for preincubation times greater than 2 min. This behavior opens the question of why some trifluoromethylketones behave as pseudoirreversible inhibitors while others act as classical competitive inhibitors of esterases. The dehydration of the corresponding hydrate back to the active keto form as a rate-limiting step, a hypothesis that was suggested by Brodbeck *et al.* (28), cannot explain time-dependent and time-independent courses of inhibition by the same compound, OTFP. However, as has been hypothesized before, this difference in the behavior of OTFP is likely to be due to the nature of its binding to the enzyme

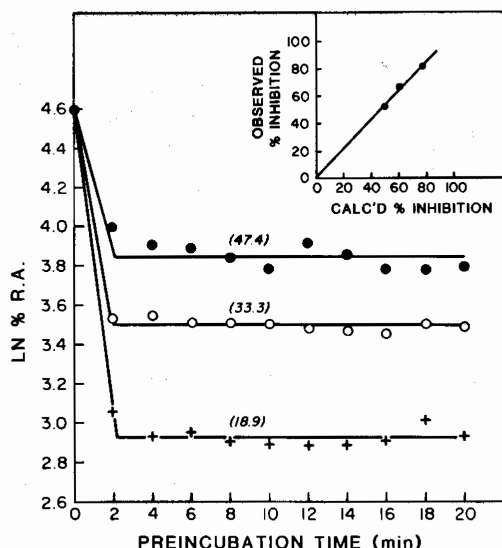


FIG. 4. Relationship between ln percentage residual activity (RA) of JHE from larval hemolymph of *M. sexta* and preincubation time using 2.19×10^{-7} (●), 8.75×10^{-7} (○), and 5.47×10^{-6} M (+) OTFP. JH III at a final concentration of 5×10^{-6} M was used as substrate. Numbers in parentheses represent the average percentage RA for the time-independent phase of inhibition. Inset shows a relationship between maximum observed percentage inhibition (from Fig. 4) and the percentage inhibition calculated from Eq. [3] in the text.

rather than to its dehydration back to the keto form. OTFP is expected to react with the sensitive form in roughly equimolar amounts, and it behaves as a slow, tight-binding inhibitor (27, 29–31). In contrast, the same compound interacts with the less sensitive form at higher concentrations, a condition required to ensure the classical behavior of competitive inhibition for this type of compound under steady-state conditions.

The data in Fig. 4 also support the reproducibility of the overall percentage inhibition based on the assumption that two forms of JHE with equivalent activity are present in the hemolymph of *M. sexta*. At the concentrations used to generate that figure, the sensitive form is expected to be completely inhibited, which represents 50% of the total activity. However, the regression equation for the inhibition line of the less sensitive form (Fig. 2A),

Percentage inhibition (less sensitive)

$$= 279.7 + 42.4 \log[\text{OTFP}], \quad [2]$$

can be used to calculate the total percentage inhibition as follows:

Total percentage inhibition

$$= (279.7 + 42.4 \log[\text{OTFP}])/2 + 50. \quad [3]$$

The calculated values from the above equation at 2.19×10^{-7}, 8.75×10^{-7}, and 5.47×10^{-6} M OTFP are, respectively, 50, 61.4, and 78.3, which are in a good agreement with average maximal percentage of inhibition (Fig. 4). The correlation between expected and experimental values is higher ($r^2 = 0.99$, Fig. 4 inset); better yet, the slope of the line is very close to unity (0.995), which gives additional evidence for the consideration of two forms with equivalent JHE activity in the hemolymph of *M. sexta*.

As expected, EPPAT would be a good candidate to be tested as a time-dependent inhibitor since it acts as a phosphorylating (irreversible) inhibitor of JHE (16, 32). With the use of EPPAT, different rates of inhibition were obtained, first fast and then slow (Fig. 5). Extending the same argument for the presence of two forms of JH-hydrolyzing activity, it was suggested that the data points that fit a straight line in the slow phase of inhibition represent a pseudo-first-order plot for the inhibition of the less sensitive form in a time scale where the sensitive form was completely inhibited. These data points for each inhibitor concentration were subjected to linear regression analysis. The regression equations of the pseudo-first-order plots gave intercepts on the y axis equivalent to percentage residual activities ranging from 46.4 to 51.0, with an average of 49.5.

This percentage represents the proportion of the less sensitive form in the overall JHE activity, and supports the conclusion drawn from the inhibition curves and the time-course inhibition by OFTP. The equations were also used to extrapolate these lines back in order to calculate the expected percentage residual activity of the less sensitive form on the time scale of the fast phase of inhibition. This calculation permitted the resolution of the percentage

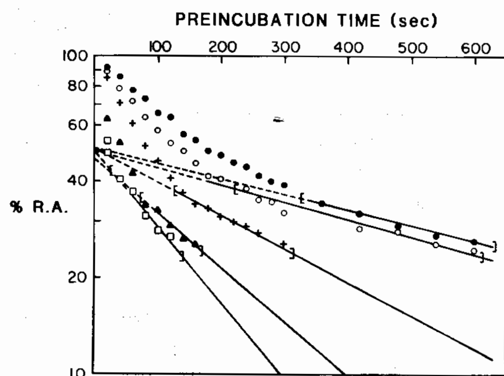
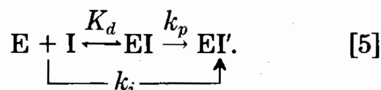


FIG. 5. Pseudo-first-order plots of the inhibition of JHE from larval hemolymph of *M. sexta* by EPPAT at final concentrations of 6.1×10^{-9} (●), 1.22×10^{-8} (○), 2.44×10^{-8} (+), 4.88×10^{-8} (▲), and 9.76×10^{-8} M (□). JH III at a final concentration of 5×10^{-6} M was used as a substrate. Notice that there is a fast and then a slow rate of inhibition. The slow rate of inhibition was considered to be that of the less sensitive form, and the data that fit a straight line in this phase were subjected to regression analysis ($r^2 \geq 0.96$) except for the highest concentration ($r^2 = 0.91$). Each point on these plots was the mean of at least three separate determinations.

residual activity of the sensitive form since the data points in this phase represent the percentage residual activity of both forms. A semilog plot of the corrected percentage residual activity of the sensitive form plotted against preincubation time (Fig. 6) seem to fit a straight line for each inhibitor concentration. The slopes of these regression lines ($\Delta \log v/\Delta t$) as well as those for the less sensitive form (Fig. 5) were evaluated by the first-order kinetic approach developed by Main and his co-workers (17–19) for the inactivation of esterases by organophosphates according to the following equation:

$$i\Delta t/2.303\Delta \log v = i/k_p + 1/k_i, \quad [4]$$

where i is the inhibitor concentration, k_p is the phosphorylation rate constant, and k_i is the overall bimolecular rate constant which equals k_p/K_d, where K_d is the equilibrium dissociation constant. The steps in the reaction of the enzyme (E) and the inhibitor (I) that are controlled by the aforementioned constants can be depicted as



$$E + I \overset{K_d}{\longleftrightarrow} EI \overset{k_p}{\rightarrow} EI'. \quad (E + I \xrightarrow{k_i} EI') \quad [5]$$

A plot of $i\Delta t/2.303\ \Delta \log v$ against i gave a straight line for each form (Fig. 7), from which the above kinetic constants were calculated to be $k_p = 0.65$ and $3.65\ \text{min}^{-1}$; $k_i = 7.05 \times 10^6$ and $8.79 \times 10^7\ \text{M}^{-1}\ \text{min}^{-1}$; and $K_d = 9.25 \times 10^{-8}$ and 4.16×10^{-8} M, respectively, for the less sensitive and sensitive forms.

The data for the sensitive form should be taken with caution since the second-order reaction constant is expected to be even higher than the calculated value as there is an upper limit for this rate constant, especially when the diffusion-controlled encounter of the enzyme and inhibitor is the rate-limiting step. Furthermore, the concentrations of EPPAT used are far higher than the I_{50} value of the sensitive form, a condition which requires very short preincubation times or simultaneous inhibition techniques in the presence of the

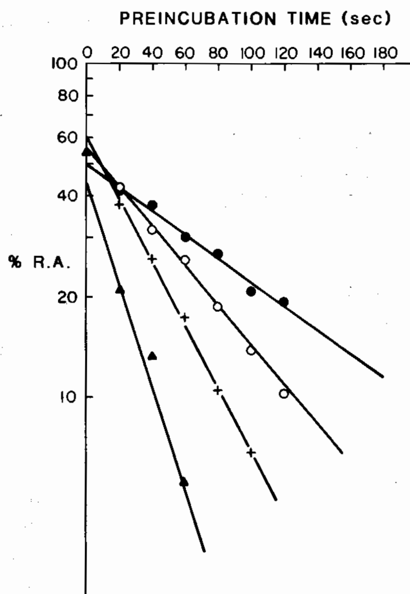


FIG. 6. Pseudo-first-order plots for the inhibition of the sensitive form of JHE by 6.1×10^{-9} (●), 1.22×10^{-8} (○), 2.44×10^{-8} (+), and 4.88×10^{-8} M (▲) EPPAT. Data on these plots were calculated from Fig. 5 after correction for the less sensitive form, and were subjected to linear regression analysis ($r^2 \geq 0.97$).

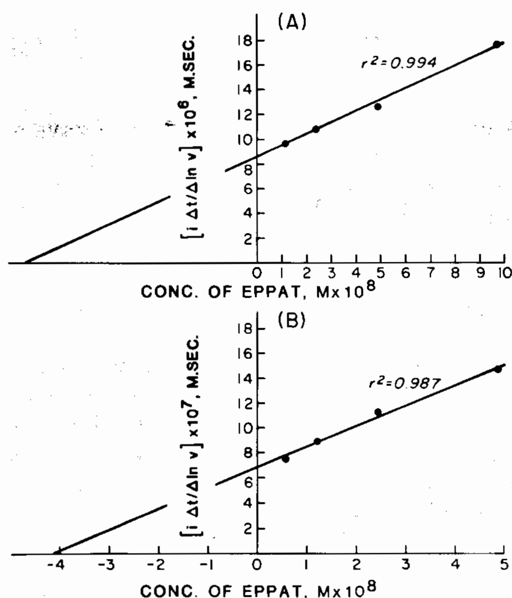


FIG. 7. Main inhibition kinetic treatment (17–19) for the inhibition of the less sensitive (A) and sensitive (B) forms according to Eq. [4] in the text. The data were calculated from Figs. 5 and 6, respectively.

not indicate the presence of multiple forms exclusively since one could observe a similar behavior if there were enzyme activation at high substrate concentrations.

In order to exclude the possibility of substrate activation before discussing the complex behavior of JH II hydrolysis by JHE(s) from *M. sexta*, two concentrations of OTFP were preincubated with the enzyme for 10 min before adding the same substrate concentrations, and the double-reciprocal plots from these experiments are shown in Fig. 8B. In the presence of the low concentration of OTFP (5.47×10^{-8} M), two distinctive lines were also obtained; however, with 4.38×10^{-7} M of this compound (~40× the I_{50} value of the sensitive form) the line became straight, indicating that substrate activation is not the reason behind the complex behavior obtained in the absence of the inhibitor.

Although it is hard and sometimes even impossible to calculate the K_m value of the substrate for higher accuracy (33). However, the kinetic constants for the less sensitive form seem more accurate since there is a good agreement between the experimental I_{50} value calculated from the inhibition curve (1.1×10^{-8} M) and that calculated from the k_i value and a 10-min preincubation time (9.8×10^{-9} M) according to Eq. [1].

Substrate specificity. The substrate used in most of the inhibition experiments was JH III since it is the most water soluble of the JH homologs. Since JH II appears to be more predominant than JH III in larvae of *M. sexta* (34), it was used for the substrate specificity studies.

The data for the double-reciprocal plot (35) were generated from 12 concentrations of JH II, with the activity being measured after a 1-min incubation using two plasma dilutions. Figure 8A represents a typical plot from this experiment, which shows a straight line at the lowest seven concentrations and then a downward curvature making a steeper line at the highest five concentrations. This complex behavior does

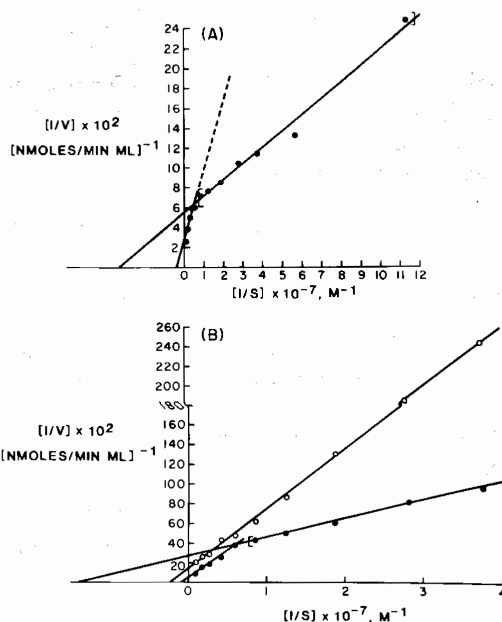


FIG. 8. Double-reciprocal plots of JH II hydrolysis by JHE from larval hemolymph of *M. sexta* in the absence (A) or the presence (B) of 4.38×10^{-7} (○) or 5.47×10^{-8} M (●) of OTFP for a 10-min preincubation time. Each point was from the average velocity of four to five replicates (A and B) and three determinations (A).

low-affinity form especially if they are two sites of unknown cooperative behavior, the high-affinity form is expected to behave independently of such cooperation. It has a K_m value of 3.07×10^{-8} M. More important are the V_{max} calculated from the two lines (18.53 nmol min^{-1} ml^{-1} for the high-affinity form and 38.75 nmol min^{-1} ml^{-1} for the total activity) which indicate that the high-affinity form contributes ~48% of the total activity, in excellent agreement with the proportion of the two forms obtained from the inhibition kinetic data.

Purification of JHE. To the best of our knowledge only four publications have dealt with the purification of JHE from *M. sexta* (10–13). In those studies, classical purification techniques were used. Although affinity chromatography offers rapid and quantitative procedures for the purification of enzymes, it has never been applied to JHE for any insect species. The fundamental reasons behind the delay in using this powerful and specific technique is usually the absence of a specific reversible inhibitor that can be attached to an insoluble support and used as a ligand for affinity chromatography. The unusually high inhibitory potency and selectivity of trifluoroketones to JHE indicated that these compounds might be good affinity ligands (6, 7, 36). Thus, MBTFP was reacted with epoxy-activated Sepharose (MBTFP–Sepharose) to yield the column used in this study.

Several trials to test for the efficiency and consistency of this column indicated that the JHE activity in ~20 ml of hemolymph (~400 mg protein) was retained almost completely upon loading 100 μl of MBTFP–Sepharose in a 1-ml tuberculin syringe, run at a flow rate of 3–5 ml/h of 1:5 diluted plasma. Interestingly, there was no detectable change in the protein content before and after passing through the column which, in addition to the fact that there was a drastic decrease (98+%) in JHE activity upon passing the column, indicates the efficiency and selectivity of the ligand used. However, the enzyme was bound so tightly to the column that elution was exceptionally difficult. This observation is not surprising since these compounds were found to act as slow, tight-binding inhibitors (15, 16), and sometimes to behave as pseudoirreversible inhibitors. Eluting the column for 24 h with ~100 ml of the buffer failed to regenerate the enzyme bound to the column. However, only the first 3–5 fractions exhibited measurable protein. Furthermore, OTFP at 10^{-3} M in buffer was not able to displace the enzyme upon regular washing. Therefore, it was decided to equilibrate the column with ~9 vol of OTFP solution for 6–12 h with shaking every 2 h. This procedure was successful in eluting most of the activity in six fractions of 0.95 ml each. The data collected from one of several columns run are presented in Table I, and the specific activity and the behavior of the purified protein(s) on SDS–PAGE are consistent with the other columns.

Six fractions of OTFP-inhibited enzyme were obtained and dialyzed independently against phosphate buffer for 9 days. The activity of inhibited enzyme in each fraction was regenerated gradually, and peaked after dialysis for 7 days (Fig. 9). Control experiments with 1% plasma preincubated with ethanol or ethanol–OTFP solution at a final concentration of 1.0×10^{-5} M were dialyzed identically but separately. These experiments indicated, respectively, that the enzyme was stable at least for 7 days under the dialysis conditions and that the inhibited enzyme followed exactly the same patterns of regeneration as the enzyme eluted from the column. The activity 7 days after dialysis was subjected to further evaluation (Table I). As seen in this table, the specific activity for the six fractions ranged from 441.5 to 702.8 with an average of 573 nmol JH III hydrolyzed min^{-1} mg $protein^{-1}$. The corresponding purification factors were 604.8–962.8 with an average of 785.9.

In attempting to compare the purification of JHE by the affinity column with the classical purification by Coudron *et al.* (12) a minor error was found due to their assuming the specific activity of the hemolymph was 0.05 instead of 0.19 nmol min^{-1} mg^{-1}. Thus, an actual purification factor of 442 was obtained in four steps to give 7.5% yield of apparently homogenous JHE with a specific activity of 84 nmol min^{-1} mg^{-1} which, in comparison with the data ob-

TABLE I

PURIFICATION OF JHE FROM THE HEMOLYMPH OF *M. sexta* BY AFFINITY CHROMATOGRAPHY

Material assayed	Volume (ml)	Mg protein/ml (±SD)	Specific activity (nmol min^{-1} mg^{-1} ± SD)	Purification factor	Total activity (nmol/min)	Percentage recovery
Hemolymph	19.00	20.14 (1.6)	0.73 (0.012)	—	278.92	—
OTFP fractions						
Fractions						
1	0.95	0.085 (0.006)	589.15 (21.87)	807.1	47.58	17.06
2	0.95	0.082 (0.003)	503.90 (8.88)	690.3	39.25	14.07
3	0.95	0.075 (0.008)	630.13 (8.43)	863.2	44.90	16.10
4	0.95	0.041 (0.003)	702.8 (21.12)	962.8	27.38	9.80
5	0.95	0.071 (0.008)	441.51 (27.61)	604.8	29.78	10.68
6	0.95	0.056 (0.005)	574.69 (32.61)	787.24	30.57	11.00

tained from the MBTFP–Sepharose column, clearly illustrates the advantage of the affinity procedure.

The six fractions obtained from the column showed a single band upon SDS–PAGE (Fig. 10) with a calculated molecular weight of ~65,000. Previous studies showed a molecular weight of 68,000 (12) using both SDS-gel and gel-permeation chromatography, and 67,000 (10) using sedimentation and diffusion techniques. Comparing our data with those of Coudron *et al.* (12) was not meant to underestimate the quality of their classical purification approach of

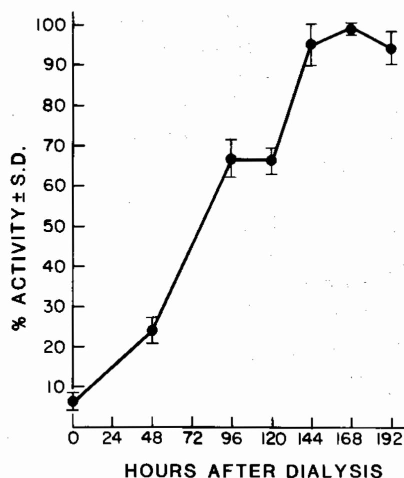


FIG. 9. Data for the dialysis of OTFP-inhibited activity from six fractions of the affinity column. The dialyzed activity for each fraction followed the same regeneration pattern, with maximum activity 168 h after dialysis. The activity of dialyzed enzyme was calculated as the percentage of the maximum activity, and the average percentage activity from all fractions was plotted against dialysis time. JH III was used at final concentration of 5 × 10^{-6} M as a substrate.

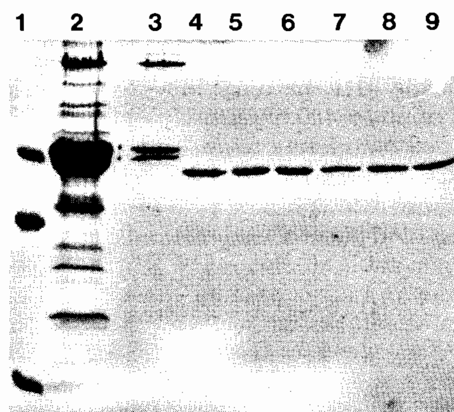


FIG. 10. SDS–PAGE of standard proteins of M_r 68,000, 43,000, and 12,000 (1); plasma of *M. sexta* (2); buffer wash of the affinity column (3); and OTFP fractions (4–9). The buffer wash did not show any JHE activity.

JHE from *M. sexta* since it was the first study to give homogenous enzyme. However, we offer in the present study a more specific, quantitative, and rapid technique. Therefore, we had to compare the affinity column with the classical purification that gave the highest purification factor and specific activity (12) among all the published work.

Kinetics of purified JHE. Although it has been shown that the hemolymph of *M. sexta* exhibits two separable kinetic forms of JH-hydrolyzing activity, several physicochemical techniques (5, 9, 12, 13) indicated the presence of a single JHE in the hemolymph of the same species. The above information, in addition to the fact that the hemolymph used in the present work showed only one peak of JH-hydrolyzing activity upon gel-permeation chromatography (data not shown), might indicate that the two forms are, in fact, two catalytic sites on the same protein. This seems likely since only one major band of protein of the purified enzyme was shown on SDS-PAGE gels [Fig. 10 and Ref. (12)]. Therefore, it was decided to apply some of the kinetic approaches used with the hemolymph to the purified enzyme. Inhibition of the purified enzyme by EPPAT (Fig. 11) indicates the presence of two forms of JH-hydrolyzing activity toward JH III. Qualitative comparison of Figs. 11 and 2B indicates that the inhibition pattern is the same for both purified and crude enzyme except that the proportion of the sensitive form was only 30% of the total activity of the purified enzyme instead of 50% of the activity of the crude enzyme. Quantitative resolution of the inhibition of each form, as explained before for the crude enzyme, resulted in I_{50} values of 6.0×10^{-11} and 1.5×10^{-8} M, respectively, for the sensitive and less sensitive form, as compared with 1.1×10^{-10} and 1.1×10^{-8} M for the crude enzyme. The higher I_{50} value for the less sensitive form (1.4×) and the lower value for the sensitive form (1.8×) from the purified enzyme as compared with the crude enzyme is not surprising since the I_{50} value of irreversible inhibitors is dependent on the molar equivalency of the available active sites for the inhibitor (27, 38, 39).

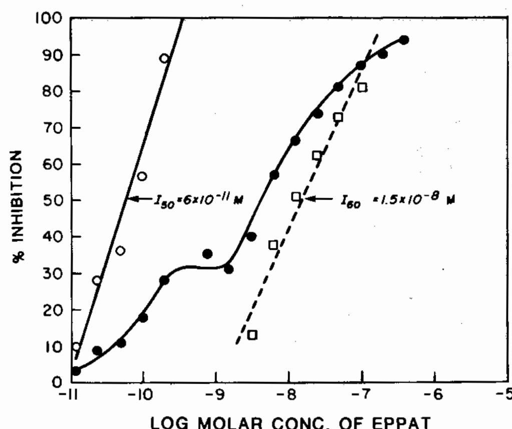


FIG. 11. Plots of percentage inhibition of the affinity-purified JHE of *M. sexta* against log molar concentration of EPPAT. The inhibition curve showed a behavior similar to crude enzyme (Fig. 2B). Resolution of the inhibition for the sensitive (○) and less sensitive (□) forms resulted in apparent straight lines. JH III at a final concentration of 5×10^{-6} M was added 10 min after the addition of the inhibitor. Each point on the curve was the average of three replicates and two separate determinations.

A double-reciprocal plot for the activity of the purified enzyme against the same concentrations used with the hemolymph is shown in Fig. 12. This figure shows that the data points fit two lines, as is the case with the crude hemolymph (Fig. 8A), with a V_{max} of 320.2 for the high-affinity form and a total V_{max} of 1079.9 nmol min^{-1} mg protein^{-1}. In support of the inhibition data, this gives a proportion of 30:70 for the two forms. It is now clear that the high-affinity form is the same as the EPPAT-sensitive form, a conclusion that could not be drawn from the kinetic studies on the crude enzyme since both forms contribute equally to the overall activity of the hemolymph enzyme. It is rather interesting that the high-affinity form from the purified enzyme has a K_m value of 2.3×10^{-8} M, which is almost identical to the K_m of this form from the crude enzyme. The data which fit the high-affinity form from Figs. 8A and 12 were subjected to a weighed least-squares analysis (40) of velocity against substrate concentration. The analysis revealed K_m values of $2.56 \pm 0.21 \times 10^{-8}$ and $2.40 \pm 0.09 \times 10^{-8}$ M, respectively. The total V_{max} value calculated for the crude enzyme was 38.75 nmol min^{-1} ml plasma^{-1}. Recal-

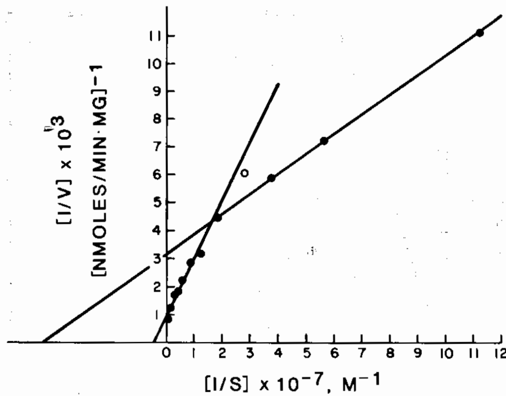


FIG. 12. Double-reciprocal plots of JH II hydrolysis by the affinity-purified JHE of *M. sexta*. Each point was from the average velocity of four replicates.

culation of this V_{max} value as per milligram protein of the hemolymph (20.14 mg/ml) resulted in a purification factor of 561×, a value that is only 0.71 the average purification factor calculated from the activity at a single JH III concentration (Table I). This difference might explain the lower proportion of the EPPAT-sensitive and JH II high-affinity form in the purified enzyme as compared with the crude hemolymph. The lower proportion might be due to incomplete recovery of the sensitive form when the OTFP-inhibited enzyme was dialyzed for the kinetic studies. If this is true, one would expect the purification factor of the less sensitive form to be close to the average purification factor (Table I). This explanation seems to be acceptable since the purification factor of the less sensitive form was 751.5×, which is almost identical to the average purification factor (Table I) calculated from totally regenerated activity (see Fig. 9).

CONCLUSION

Three different approaches were used independently to indicate the presence of two catalytic sites involved in JH hydrolysis by the hemolymph and by an affinity purified esterase from the larvae of the tobacco hornworm. In two of these approaches, three inhibitors were used either in an empirical approach (inhibition curves) or in a pure kinetic treatment of the inhibition reaction. The third approach involved studying the steady-state kinetics of JH II hydrolysis. The two sites were catalytically equivalent at substrate saturation and exhibited selectivity toward the inhibitors and specificity toward the substrate. The equivalent activity of these sites was consistent in at least five different hemolymph pools, and was independent of the number of larvae used in each pool. The purification technique used failed to prove that these two sites were on two separate proteins. Only one band was detected upon electrophoresis of the active JHE fractions from the affinity column. This information also fails to indicate that these two sites are on separate proteins. However, there is the possibility that either two JHEs cochromatograph on both affinity chromatography and electrophoresis or that one form with a low turnover number obscures the presence of a second form with a very high turnover number.

The K_m exhibited by the hemolymph JHE of *M. sexta* clearly is low enough to remove JH from the carrier protein by mass action as previously discussed for *T. ni* (15). The discovery of two sites on the enzyme with different apparent K_m's indicates that a careful examination of the kinetics of ester hydrolysis in other lepidopterous species will yield K_m's lower than those reported to date [see Ref. (3) for review]. However, other biological implications of this work are not fully clear. The K_m of the JHE from *M. sexta* certainly is low enough to make it an effective scavenger for a substrate existing at very low concentrations. For many enzymes involved in the metabolism of endogenous substrates, the physiological concentration of the substrate approaches the K_m of the enzyme which metabolizes it. However, the concentration of JH by the time JHE reaches its maximum titer in the early last larval stadium is far below the molar concentration of catalytic sites and below the apparent K_m of JHE as well. Thus, when only a very small proportion of the available JHE molecules will be interacting with even a single substrate, it is difficult to envision a biological role for two nonequivalent catalytic sites. This observation again raises the possibility that JHE may have other as yet undetermined roles.

It is clear that the transition state mimics discussed in this manuscript will be very useful in research on JH catabolism. They have been used in the past to demonstrate a biological role for JHE (6, 7), and in this study were used as an affinity ligand to purify JHE rapidly. A rapid, high-yield method for the purification of the enzyme will speed studies on the kinetics of the enzyme in several laboratories. Also, it will facilitate the production of probes needed for further studies into the regulation of the enzyme. Preliminary studies indicate that these transition state mimics will be similarly applicable to the study of other esterases of biological and toxicological interest.

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