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ACTIVATION OF CRUDE AND HOMOGENEOUS JUVENILE HORMONE ESTERASES BY ORGANIC SOLVENTS

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Abstract—A variety of water-miscible organic solvents caused profound activation of the homogeneous juvenile hormone esterase from *Manduca sexta*. Much lower activation was observed with other species. Methods for the handling of solutions of juvenile hormone are presented. The implications of solvent activation of juvenile hormone esterase activity are discussed, especially as they relate to analysis of the enzyme or titre determinations of juvenile hormone.

INTRODUCTION

In the Lepidoptera examined, a highly specialized group of enzymes termed juvenile hormone esterases (JHEs) appear to be involved in the regulation of juvenile hormone (JH) titres (Hammock, 1985). These enzymes have a low K_m and a relatively high k_{cat} indicating that they should be effective for the rapid clearance of JH at critical periods in development such as in the prepupa of species which show a high titre of JH during post-wandering development. The enzymes also should be capable, in conjunction with the haemolymph carrier protein, of extracting trace levels of JH from lipophilic depots, reducing levels of JH in specific tissues, and ensuring that JH titres are maintained at a low level even when biosynthesis is not totally halted. Recent data suggesting a possible biological role for JH acid may indicate that JHEs even have a biosynthetic role.

Since substrates and possible inhibitors are usually added in a water-miscible organic solvent to JHE, we examined the effects of ethanol of JHE activity. Surprisingly, it caused a profound increase in the activity of the pure JHE isolated from haemolymph of *Manduca sexta*. Such solvents are commonly present in enzyme incubations and are assumed to inactivate JH metabolism when insects are extracted for analysis of JH. Therefore, we surveyed the effects of a variety of organic solvents on the activity of JHE in several species.

MATERIALS AND METHODS

Enzyme preparation

Haemolymph was collected from the last stadium of *Bombyx mori*, *Heliothis virescens*, *Heliothis zea*, *M. sexta*, *Spodoptera exigua*, and *Trichoplusia ni* at a time of high JHE activity, centrifuged, diluted in buffer (sodium phosphate, pH 7.4, I = 0.2 M; 0.01% w/v phenylthiourea; 5% w/v sucrose), aliquoted and frozen at −70°C. For some experiments JHE was purified by an affinity chromatography procedure as described previously (Abdel-Aal and Hammock, 1985, 1986). Identical aliquots were thawed just before use.

Handling of low levels of JH

JH II and III [C-10 ^{3}H, approx. 11 Ci]/mmol) were from New England Nuclear while unlabeled geometrically pure materials were from Calbiochem. All materials were racemic. JH was handled in general as decribed previously (Hammock and Roe, 1985). Briefly, the chemical integrity of commercial materials is checked chromatographically and standard solutions are prepared in an aprotic, relatively non-volatile solvent such as isooctane. Concentrations can be verified radiochemically or by GLC or u.v. analysis for labeled and unlabeled materials, respectively. The concentration and purity of these stock solutions are constant for years if tightly closed and kept refrigerated. Appropriate levels of labeled and unlabeled materials are combined in a glass vial, and the isooctane removed at 35–40°C under a blanket of nitrogen ("blowing off" organic solvents can result in substantial loss of JH if carrier materials are absent). Ethanol is added to give the appropriate concentration of JH. These ethanol stock solutions also are kept at −20°C but warmed to room temperature before opening, and periodically examined for purity. Many non-volatile organic solvents and some polar impurities which may form in JH samples can be removed by diluting the sample in water or dilute base and extracting with a volatile solvent such as pentane or dichloromethane (must be acid free). Volatile solvents can be removed by warming under a blanket of nitrogen as above or by evaporation of the solvent in a Speedvac®. The use of a trapping solvent such as propane 1,2-diol (which can be added to tissue cultures, homogenates, or injected *in vivo*) ensures that losses of JH will be minimal. Treatment of glassware with siliconizing agents or polyethylene glycol can help to maintain JH in solution by masking binding sites on glass. The binding of JH to glass is most significant with higher homologs of JH, at very low concentration, and when only very small levels of protein and organic co-solvents are present. Some plastics may absorb tremendous amounts of JH while other are inert. Any plastic product used to handle aqueous solutions of JH should be examined periodically for binding to JH in the context of the experimental procedures used. Manufacturers may change the composition of their plastic product without notice.

Assay procedure

A previously described partition assay was used (Hammock and Roe, 1985; Hammock and Sparks, 1977) with slight modification. The organic co-solvent was added to

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buffer in a 10 × 75 mm glass tube to a total volume of 50 μl, followed by 50 μl of an appropriate enzyme dilution. The tubes were held at 30°C before addition of 1 μl of the substrate in ethanol. Incubation times of 5–10 min were selected to yield linear rates of hydrolysis at 30°C with longer times of incubation at 0°C. Slight exceptions to these protocols are indicated in figure legends. At least two independent replicates of triplicate determinations support each datum point with standard deviations representing well under 5% of the reported value.

Protein determination

Protein concentration for all studies were determined by a dye binding method (Bradford, 1976) as modified by Bio-Rad, using fraction V of BSA (Sigma) as the protein standard. The detailed procedure was reported previously (Abdel-Aal and Hammock, 1985).

RESULTS

A variety of organic solvents were tested for the activation of apparently homogeneous JHE from *M. sexta* as shown in Fig. 1A-D. All solvents tested gave some degree of activation with the apparent specific activity of the enzyme increasing linearly with solvent at low concentrations, a leveling off of activity, and then apparent inhibition of JHE activity at higher concentrations. Solvents varied in the degree of activation, the maximum activation achieved, and the concentration at which peak activation occurred. These data are summarized in Table 1 along with log *P* information (Hansch and Leo, 1979). Compounds such as ethane 1,2-diol, propane 1,2-diol, and diethanolamine are very polar having the most negative log *P*s of the compounds tested, and they gave low activation slopes (Fig. 1D, Table 1). In contrast *n*-butanol is relatively non-polar and yielded one of the highest initial activation slopes. In general, the less polar the compound, the greater the slope of initial activation. This trend is particularly clear for the straight chain alcohols (Fig. 1A).

As shown in Fig. 2A, addition of ethanol increased the rate of hydrolysis of both JH II and III by pure JHE. It also caused more rapid hydrolysis of JH III in diluted haemolymph. When hydrolysis of JH III in haemolymph from two different stages was monitored with varying concentrations of ethanol or propanol, no significant differences in the response of JHE were seen although the relative activities between the two stages varied by a factor of 18.3 when expressed on a rate/ml haemolymph basis.

The influence of three organic solvents on the activity of JHE at 0°C also was examined (Table 2). Obviously control rates were much lower than at 30°C, but at this temperature all solvent concentrations tested caused a dramatic increase in the rate of production of JH acid. For instance, 15% (v/v) of acetonitrile, ethanol and propanol increased the rate of production of JH acid 34-, 13- and 14-fold, respectively.

When the influence of varying concentrations of ethanol was examined on JHE activity in the plasma or pure JHE from several species, slight activation was observed with the JHE activities from *B. mori* and *T. ni*. The activities were decreased in *H. virescens*, *S. exigua* and most dramatically in *H. zea* (Fig. 2B). These differential effects also were dramatic when pure JHE activity from three species was

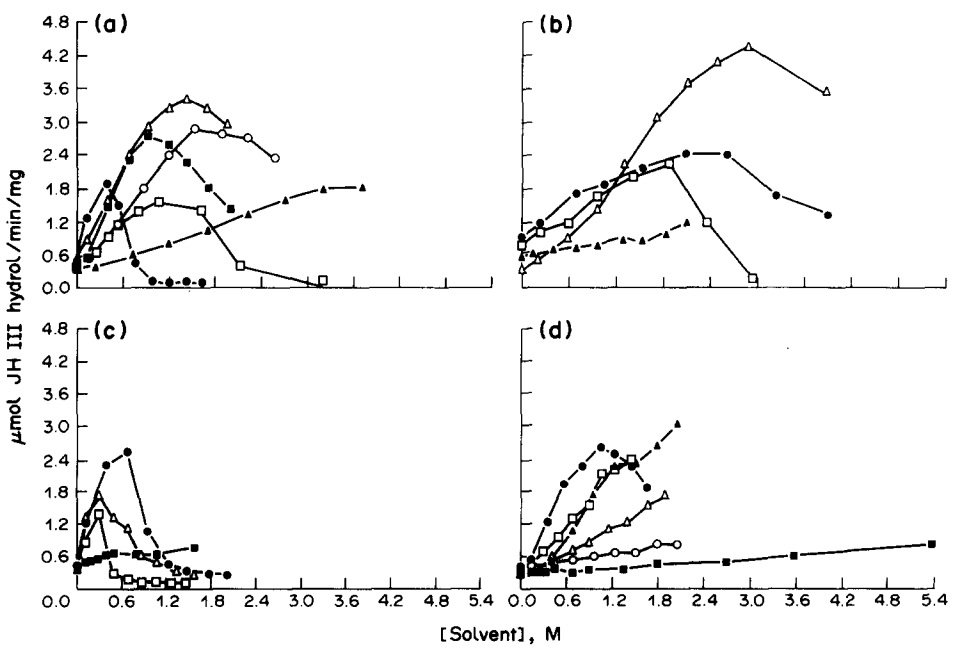

Fig. 1. Effect of organic co-solvents on JHE-catalyzed hydrolysis of JH III at 30°C. Homogeneous JHE from *M. sexta* was used for all studies. A. Alcohols: methanol (▲), ethanol (○), propanol (■), *i*-propanol (△), *n*-butanol (●) and *t*-butanol (□). B. Proteophilic solvents: dimethyl sulfoxide (▲), dimethyl formamide (●), 1,4-dioxane (□), acetonitrile (△). C. Amines: diethanolamine (■), ethylamine (●), diethylamine (□), dimethylamine (△). D. Ketones and glycols: acetone (▲), 2-butanone (●), 2-methoxyethanol (△), ethane 1,2-diol (■), propane 1,2-diol (○), ethylene glycol dimethylether (□).

Table 1. Activation by co-solvents of purified JHE from *M. sexta*

Solvent	log *P** (octanol)	Maximum activity (nmol/min/mg)	% Control	Solvent conc. at max activation (molar)	Slope† for activation
Acetone	−0.24	3084	1202	2.04	1477
Acetonitrile	−0.34	4264	1386	2.87	1851
n-Butanol	0.89	1891	450	0.33	7727
t-Butanol	0.35	1546	379	1.06	1561
2-Butanone	0.26	2675	828	1.01	3026
Diethanolamine	−1.43	NA‡	NA	>2.0	177
Diethylamine	0.43	1399	372	0.29	4930
1,2-Dimethoxy ethane	—	>2500	NA	>1.60	1549
Dimethylamine	—	1702	425	0.30	7767
Dioxane	−0.42	2182	286	1.88	780
Dimethyl formamide	−1.01	2367	264	2.60	943
Dimethyl sulfoxide	−1.35	NA	NA	>2.0	355
Ethanol	−0.32	2865	811	1.50	1733
Ethane 1,2-diol	−1.93	NA	NA	>2.50	98
Ethylamine	−0.13	2555	639	0.67	4628
Methanol	−0.64	1825	501	3.70	463
2-Methoxy ethanol	−0.77	NA	NA	NA	773
i-Propanol	0.05	3380	646	1.40	2658
n-Propanol	0.25	2599	952	0.90	3113
Propane 1,2-diol	−1.74	NA	NA	>2.0	212

*From Hansch and Leo (1979).
†Expressed as change in rate of hydrolysis per unit change in molarity for the apparently linear, ascending region of the curve.
‡NA = Maximum not attained.

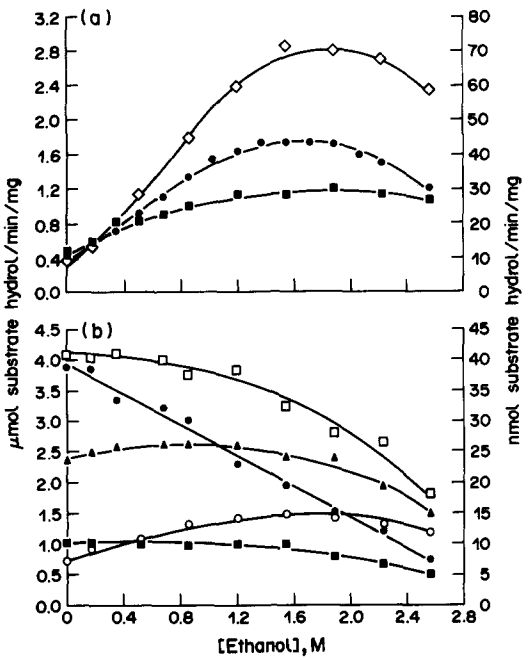

Fig. 2. Effect of ethanol on JHE-catalyzed hydrolysis of JH II and JH III in phosphate buffer, pH 7.4 at 30°C: 1A, the esterase from *M. sexta* was used either in the crude form towards 5 μM JH III (■) or in the affinity purified form towards 5 μM JH III (◇) or 0.9 μM JH II (●); 1B, the enzyme from the plasma of *S. exigua* (□) and *T. ni* (▲) and the affinity purified enzyme from the plasma of *B. mori* (○), *H. virescens* (■) and *H. zea* (●) were used with 5 μM JH III as a substrate. Activities of crude enzymes are expressed in nmol JH acid formed/min/mg protein and activities of pure enzymes are expressed in μmol JH acid formed/min/mg protein. Using the same procedure and 5% (v/v) methanol, ethanol, or propanol concentrations, respectively, JHE activities were 216, 478 and 971% of control activity for *M. sexta*; 146, 193, and 186% for *B. mori*; and 92, 96, and 88% for *H. virescens*.

Table 2. Formation of JH acid at 0°C by JHE from *M. sexta* in the presence of three solvents

Concentration (% v/v)	Percent control activity* Acetonitrile	Ethanol	Propanol
15	3353	1342	1388
25	719	1031	193
40	399	377	383

*Control activity was 6.48 nmol/min/mg for the pure enzyme at 0°C. Activity at 0°C was approximately 1.0% of that observed at 30°C.

examined in the presence of a 5% (v/v) concentration of three alcohols (legend Fig. 2).

DISCUSSION

The apparent activation of enzymes such as estradiol 17-β-dehydrogenase (Lubbert, 1982) or even esterases (Ocken and Levy, 1970; Lombardo and Guy, 1981) is common and will be reviewed in greater detail in a subsequent report. However, the dramatic activation observed with the JHE of *M. sexta* is exceptional.

The mechanism by which JHE is activated is not understood. Since JH is lipophilic and some JHEs catalyze JH hydrolysis near the diffusion controlled limit, changes in JH solubility or diffusion rate could be involved. However, the effects were observed with JH in true solution in the presence or absence of binding proteins and lipid micelles. In some cases the effect of nucleophilic co-solvents can be interpreted in terms of competitive partitioning of the acyl intermediate between water–solvent mixtures (Lombardo and Guy, 1981 and included references). However, the clear structure/activity relationships indicate an effect directly on the enzyme involving a relatively hydrophobic modifier site. The apparent inactivation of JHEs could occur by several mechanisms. At the solvent concentrations used, perturbation of the behavior of JH and JH acid in the partition assay is

minimal. Some apparent inactivation at higher concentrations of organic solvents certainly is associated with both reversible and irreversible denaturation of the protein. In general this effect is more dramatic with relatively non-polar materials while polyhydroxy compounds are far less potent (Tanford, 1968). However, the partition assay cannot detect transesterification. With high concentrations of some alcohols, transesterification may become significant (McDonald and Balls, 1956). This hypothesis is provocative especially in the case of the JHE from *H. zea* and with the low temperature data in Table 2. This hypothesis might explain the detection of the ethyl homologs of JH when larvae of *M. sexta* were bled directly into cold ethanol (Bergot and Schooley, personal communication).

There are numerous aspects to the possible significance of these data. The most provocative speculation is that the greater than 10-fold activation of JHE observed could explain a significant part of the dramatic changes in JHE activity observed during insect development. Thus, an endogenous modifier working in concert with biosynthesis, activation, inactivation and other mechanisms of protein regulation may have an *in vivo* role. The high concentrations of solvents used and the failure to observe dramatic activation in other species discredit but do not disprove this hypothesis. Since other data indicate that JHE is conserved during evolution, the dramatic differences among species is surprising. Most kinetic experiments are performed with dilute enzyme solutions. These data raise the concern that the true JHE activity may be dramatically different from that observed *in vitro* due to interaction with other molecules in tissues or haemolymph.

From a more practical standpoint, organic solvents may have an effect on measurements of JHE activity. The low levels of ethanol commonly used in assays for JHE have only marginal effects on rates even in *M. sexta*, but the effects could be profound in other species. The solvents also could alter the enzyme's substrate specificity. A mixture of propane 1,2-diol and acetontrile of acetonitrile and water could be a more appropriate co-solvent than ethanol even with *M. sexta*.

For routine assays the partition method for monitoring JHE should be applicable. However, for precise measurements, especially with short incubation times, errors will occur if the reaction is not terminated at an exact time. Simply the addition of basic methanol even with tubes in an ice bath will not halt the reaction. Rapid extraction of the unreacted substrate with isooctane and/or placement of assay tubes in an ice/salt bath will halt the reaction more quickly.

For analysis of JH by chromatographic, immunochemical or biological means, it is important that JH is not degraded during homogenization of tissue. Dilution of biological material during homogenization will reduce enzymatic degradation. Since denaturation of enzymes by organic solvents increases with temperature, homogenization of tissues at room temperature in acetonitrile or other solvents followed by subsequent storage at low temperature to minimize chemical decomposition should be an effective procedure even in worst case scenarios. However, the possibility of enzymatic formation of JH metabolites during homogenization should be considered. The use of ethyl esters of JH homologs as internal standards may only partially control for JH decomposition during work-up in species which metabolize methyl and ethyl esters differentially.

The stability of some JHEs to organic solvents and their apparent propensity to carry out transesterification may allow them to be used to label natural and unnatural homologs with stable or radioactive isotopes. The stability of some JHEs to solvents, especially at reduced temperatures, will allow one to use techniques of cryoenzymology to investigate the catalytic mechanism (Fink and Geeves, 1979) as well as kinetic approaches to evaluate the role of water in hydrolysis (Koshland and Herr, 1957) and nucleophilic water analogs in transacylation (Lombardo and Guy, 1981).

Biochemical and physiological investigations on JHEs from Lepidoptera have helped to explain the role of catabolism in the regulation of JH titres. The profound *in vivo* effects of injected JHE indicate that further work on the enzyme may provide insect control agents based on molecular approaches. Data presented in this manuscript now indicate that JHEs may be useful models to probe the mechanisms of enzyme catalysis. The unique properties of the lepidopteran JHEs so far studied certainly raise questions regarding their complete role in insect development.

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