

COMPREHENSIVE INSECT PHYSIOLOGY BIOCHEMISTRY AND PHARMACOLOGY

Executive Editors

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12 Regulation of Juvenile Hormone Titer: Degradation

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1 JUVENILE HORMONE METABOLIC PATHWAYS

Before the metabolism of the juvenile hormones (JHs) was ever investigated, numerous scientists speculated that the JHs were metabolized by epoxide hydration and ester cleavage. Several

research groups even initiated synthetic programs aimed at replacing these supposedly labile functionalities to develop more stable JH mimics (juvenoids). Had people realized that both the epoxide and ester moieties of JH are much more chemically stable than most of the model epoxides

and esters studied, they possibly would not have jumped to the conclusion that hydrolysis of these two moieties represented the major routes of JH metabolism. Ignorance was on this occasion very useful because these hydrolytic pathways do account for the majority of JH metabolism in the insects so far studied. However, it is becoming more obvious that very specialized enzymes are responsible for this metabolism. The evidence to date strongly supports the hypothesis that all pathways of JH degradation represent loss of biological activity.

The basic scheme of JH metabolism proposed by Slade, M. and Zibitt, C. (1971, 1972) has been only slightly modified in the subsequent decade (Fig. 1). Several reviews have now appeared which cover in reasonable depth the metabolism of the JHs (Hammock, B. and Quistad, G. 1976, 1981; de Kort, C. and Granger, N. 1981; Richards, G. 1981). For those readers desiring such information, these reviews provide bibliographies and keys to much of the literature on JH metabolism, and still other reviews (referenced in Hammock, B. and Quistad, G. 1976, 1981) and other chapters in this series have considered JH metabolism in less detail. This chap-

ter covers the pathways of JH metabolism in insects from a more biochemical and physiological perspective. Then the regulation of JH degradation in the Coleoptera and Lepidoptera is considered in greater detail. In most endocrine systems so far studied, hormone action is regulated largely by biosynthesis and receptor presence, while hormone degradation remains rather constant. An argument is made in this chapter that in at least some insects hormone degradation plays a key role in JH regulation. Also discussed are some practical aspects of this work, first in relation to insecticide resistance and secondly as an avenue leading to new concepts in insect control.

In interpreting results in this field it is especially important to appreciate some of the difficulties in studying xenobiotic metabolism. *In vivo* studies are tedious, especially when using physiological levels of substrates. One can never be sure that the radiolabeled hormone to be used is entering the correct *in vivo* pool. *In vitro* studies are at best suspect and, in general, the more clearly defined the system, the further it is from biological reality. The controls necessary to integrate *in vivo* and *in vitro* data are not impossible, but they are numerous.

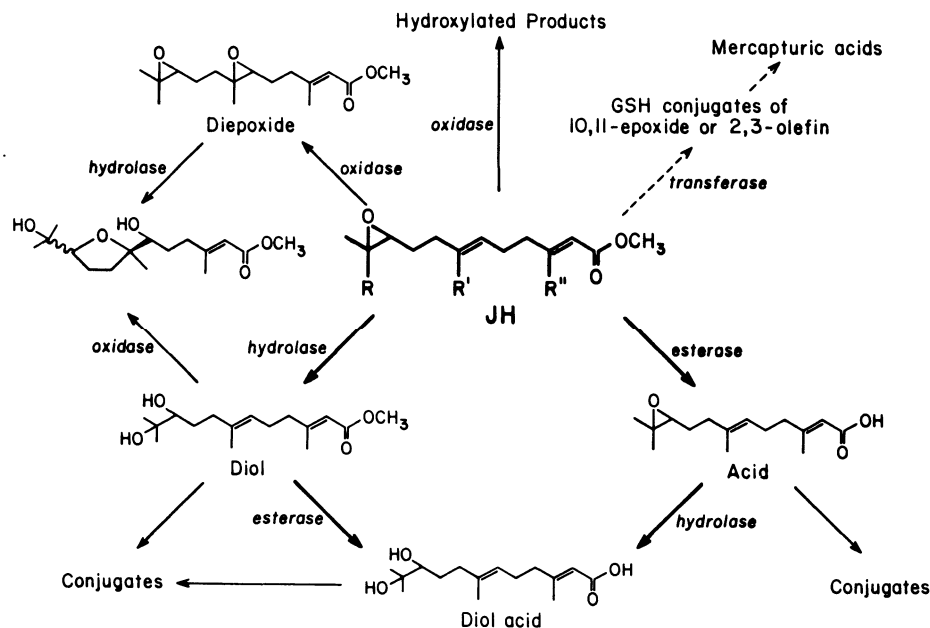


FIG. 1. Metabolic pathways and major metabolites of JH discussed in the text. JH 0, R = R' = R'' = C₂H₅; JH I, R = R' = C₂H₅, R'' = CH₃; JH II, R = C₂H₅, R' = R'' = CH₃; JH III, R = R' = R'' = CH₃. The major routes of metabolism are by epoxide hydration and/or ester cleavage (→) followed by conjugation. A variety of other oxidative pathways may be significant in some insects (→). The role of glutathione conjugation has not been established clearly in any species (--->).

Synthesis and analysis of JH metabolite standards are not nearly so trivial as one may glean from the literature. To further complicate matters, JH and its metabolites may stick to glass, dissolve in lipid micelles or plastics, be oxidized by solvent peroxides, or may hydrolyze or rearrange in the presence of acids, bases or certain ions. In light of these difficulties, it is surprising that such a large amount of excellent work has been accomplished.

1.1 Epoxide hydration

Ethers comprise a rather stable set of organic molecules with only a few exceptions. The three-membered ring of the epoxide moiety is, however, highly strained and possesses properties which are quite distinct from many of the usual properties attributed to ethers. Because of their highly strained nature many epoxides are very reactive, but it is important to remember that even here there is a continuum of reactivity from the very unstable to the environmentally persistent. For example, the aflatoxins are thought to be mutagenic and cytotoxic due to the presence of a metabolically formed epoxide ring. In fact, many of the most potent mutagens and carcinogens known act by alkylation through a very reactive epoxide. Slight changes in the structure surrounding the epoxide change the target for alkylation, and the allatotoxin, precocene (see Bowers, W., vol. 8), is also thought to act via a reactive epoxide. Somewhat less reactive epoxides have found industrial application, as evidenced by the epoxy plastics and cements. Still other epoxides, as represented by some of the sterically hindered cyclodiene insecticides, do not fit the unstable stereotypes and are, in fact, environmentally persistent. The JHs and related epoxy-containing juvenoids fall between the latter two groups on the reactivity scale. The epoxide moiety is certainly not environmentally persistent (outside of a hydrophobic microcosm), but neither is it likely to polymerize or be a potent alkylating agent. Even if the reactivity of the epoxide moiety were much greater, the JHs lack the planar structure needed to intercalate with DNA; and Ivie, G. *et al.* (1980) have shown that even reactive, trisubstituted epoxides are exceptionally poor mutagens when compared to their monosubstituted homologs. The structurally related squalene oxide does cyclize to yield the

steroid nucleus, and the carbonium ion formed when JH epoxide is exposed to mineral acids does invite cyclization and rearrangement. However, with the exception of some minor metabolites discussed in section 1.3, cyclization and rearrangement of the JH epoxide appear biologically unimportant.

Thus, the JHs of insects are unlikely to represent a mutagenic risk (should western societies shift to an insect diet) (Hsia, M. *et al.*, 1979), alkylation of an active site is an unlikely explanation of JH biological activity, and the insect will not be able to rely upon spontaneous hydrolysis or cyclization for rapid clearance of the JHs at critical times in its life. Two direct mechanisms are known which lead to metabolism of the epoxide moiety of JH: glutathione (GSH) conjugation (discussed in section 1.4) and epoxide hydration. Epoxide hydration involves addition of water to the epoxide moiety to yield 1,2-diols or glycols. The enzymes responsible for this reaction are known as epoxide ether hydrolases (E.C. 3.3.2.3, formerly known as hydrases or hydratases, E.C. 4.2.1.63) (Fig. 1). Interest first in the cyclodiene insecticides, and subsequently in mutagenicity, has led to much work on the epoxide hydrolases of mammals (Oesch, F. 1973; Hammock, B. *et al.*, 1980; Lu, A. and Miwa, G. 1980). Unfortunately, knowledge of insect epoxide hydrolases, related either to xenobiotic or JH metabolism, lags far behind.

The early observations on JH metabolism by Slade, M. and Zibitt, C. (1971, 1972) were extended to other insects by White, A. (1972); Ajami, A. and Riddiford, L. (1973) and numerous other workers. It is clear from these studies that the contribution of epoxide hydration to JH metabolism varies dramatically in different insects. Although detailed data are lacking, epoxide hydration appears to vary from the major pathway of JH metabolism in *Sarcophaga bullata*, to a role of equal importance to JH esterase in *Musca domestica*, to a minor role in *Trichoplusia ni*, and *Manduca sexta* (Table 1). There also can be a tremendous variation within a single order as in the Diptera or Coleoptera where *Tribolium castaneum* produces largely the diol but *Sitophilus granarius* produces largely the acid and diol acid (Edwards, J. and Rowlands, D. 1977, 1978). Since it seems very likely that epoxide hydrolases play a major role in JH degradation in some insects, and

Table 1: Involvement of epoxide hydration in juvenile hormone metabolism

Order; Genus, species	References
Thysanura <i>Thermobia domestica</i> (firebrat)	Ajami, A. and Riddiford, L. (1973)
Orthoptera <i>Blattella germanica</i> (German cockroach) <i>Gromphadorhina portentosa</i> (Madagascar cockroach) <i>Locusta migratoria</i> (migratory locust) <i>Periplaneta americana</i> (American cockroach) <i>Schistocerca americana</i> (American grasshopper) <i>Schistocerca gregaria</i> (gregarious grasshopper) <i>Schistocerca vaga</i> (vagrant grasshopper)	Ajami, A. and Riddiford, L. (1973) Slade, M. and Wilkinson, C. (1974) Emmerich, H. and Hartmann, R. (1973); Erley, D. <i>et al.</i> (1978) Ajami, A. and Riddiford, L. (1973); Sams, G. <i>et al.</i> (1978) Gill, S. <i>et al.</i> (1972) White, A. (1972) Slade, M. and Zibitt, C. (1972)
Hemiptera <i>Oncopeltus fasciatus</i> (larger milkweed bug) <i>Pyrrhocoris apterus</i> (cotton stainer) <i>Rhodnius prolixus</i> (Assassin bug)	Ajami, A. and Riddiford, L. (1973) Ajami, A. and Riddiford, L. (1973) White, A. (1972)
Homoptera <i>Dactynotus ambrosiae</i>	Ajami, A. and Riddiford, L. (1973)
Lepidoptera <i>Antheraea pernyi</i> (antheraea moth) <i>Hyalophora cecropia</i> (cecropia moth) <i>Manduca sexta</i> (tobacco hornworm) <i>Samia cynthia</i> (cynthia moth) <i>Spodoptera eridania</i> (southern armyworm, formerly <i>Prodenia</i>) <i>Trichoplusia ni</i> (cabbage looper)	Ajami, A. and Riddiford, L. (1973) Slade, M. and Zibitt, C. (1971, 1972); Ajami, A. and Riddiford, L. (1973) Slade, M. and Zibitt, C. (1971, 1972); Hammock, B. <i>et al.</i> (1975b); Mitsui, T. <i>et al.</i> (1979) Ajami, A. and Riddiford, L. (1973) Slade, M. and Wilkinson, C. (1974); Slade, M. <i>et al.</i> (1976); Mullin, C. and Wilkinson, C. (1980a,b) Wing, K. <i>et al.</i> (1981)
Coleoptera <i>Hippodamia convergens</i> (convergent lady beetle) <i>Leptinotarsa decemlineata</i> (Colorado potato beetle) <i>Sitophilus granarius</i> (grain weevil) <i>Tenebrio molitor</i> (yellow mealworm) <i>Tribolium castaneum</i> (red flour beetle)	Ajami, A. and Riddiford, L. (1973) Ajami, A. and Riddiford, L. (1973) Edwards, J. and Rowlands, D. (1978) Ajami, A. and Riddiford, L. (1973); Hammock, B. <i>et al.</i> (1975a); Mumby, S. and Hammock, B. (1979) Edwards, J. and Rowlands, D. (1977)
Diptera <i>Chaoborus americanus</i> (phantom midge) <i>Calliphora erythrocephala</i> (blowfly) <i>Drosophila melanogaster</i> (vinegar fly) <i>Musca domestica</i> (house fly) <i>Phormia regina</i> (black blowfly) <i>Sarcophaga bullata</i> (flesh fly) <i>Stomoxys calcitrans</i> (stable fly)	Ajami, A. and Riddiford, L. (1973) Slade, M. and Wilkinson, C. (1974) Ajami, A. and Riddiford, L. (1973); Wilson, T. and Gilbert, L. (1978) Ajami, A. and Riddiford, L. (1973); Hammock, B. <i>et al.</i> (1974a,b, 1975a, 1977a); Yu, S. and Terriere, L. (1975, 1978a,b); Mumby, S. and Hammock, B. (1979) Yu, S. and Terriere, L. (1978a,b) Slade, M. and Zibitt, C. (1972); Yu, S. and Terriere, L. (1978a,b) Hammock, B. <i>et al.</i> (1975a)
Hymenoptera <i>Apis mellifera</i> (honey bee) <i>Atta texana</i> (Texas leaf cutting ant) <i>Solenopsis invicta</i> (red imported fire ant)	Ajami, A. and Riddiford, L. (1973); de Kort, F. <i>et al.</i> (1977) Ajami, A. and Riddiford, L. (1973) Ajami, A. and Riddiford, L. (1973)

probably in xenobiotic metabolism in many insects, it is unfortunate that so little is known about these enzymes. For more comprehensive discussions of epoxide hydration the reader should see Brooks, G. (1977); Mullin, C. and Wilkinson, C. (1980a,b), and

Hammock, B. and Quistad, G. (1981).

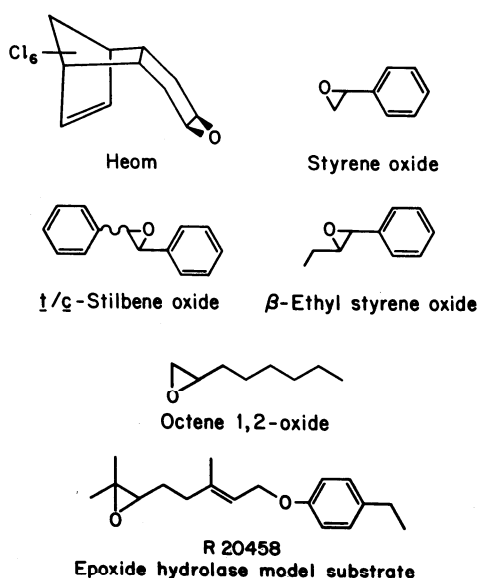
A discussion of epoxide hydration in insects is somewhat complicated by intraspecific and inter-specific variation, but of greater significance is the variation in the substrates used to measure epoxide

hydration. Since potent, selective inhibitors of ester hydrolysis have only recently become available, JH and JH acid have only occasionally been used as substrates. Commonly used substrates include the cyclodiene analog HEOM, styrene and octene 1,2-oxides, stilbene oxides, and epoxy-containing juvenoids. Reasonable circumstantial evidence supports the hypothesis that JH and epoxy juvenoids are degraded by the same enzyme(s); however, even in this case they may be metabolised by different enzymes than those which degrade JH acid. Evidence is building that the enzymes which metabolize monosubstituted epoxides, and possibly the cyclodienes, are quite different from those which hydrate JH.

With the substrates and insects so far studied, epoxide hydrolases of insects appear to be largely microsomal or at least membrane-bound. Such subcellular distribution was demonstrated using JH I and/or the juvenoid R 20458 in *M. domestica*, *P. regina*, *S. bullata*, *M. sexta*, and *T. ni*. As other substrates become routinely available, hydrolase activity may be found in other subcellular compartments. Epoxide hydrolase activity on JH and epoxy-containing juvenoids is maximal at a neutral pH, while the insect epoxide hydrolases acting on several other xenobiotics tend to have a more basic pH optimum in the several insects studied. This

situation is similar in mammals where both the microsomal and cytosolic epoxide hydrolases which metabolize JH and juvenoids have a neutral pH optimum, while those metabolizing epoxides such as benzopyrene oxide have a basic pH optimum. These data suggest the presence of different forms of the microsomal hydrolase acting on JH and xenobiotics such as HEOM and octene 1,2-oxide. It has been shown in mammals that the pH optimum of a single hydrolase can vary depending upon the substrate and the hydrophobic environment of the enzyme, but such large differences are unlikely (Brooks, G. *et al.*, 1970; Hammock, B. *et al.*, 1974a; Slade, M. and Wilkinson, C. 1974; Slade, M. *et al.*, 1975; Hammock, B. *et al.*, 1975a; Yu. S. and Terriere, L. 1978a,b; Wing, K. *et al.*, 1981; Mullin, C. and Wilkinson, C. 1980a,b).

A variety of compounds have been screened as potential inhibitors of epoxide hydration in insects, but as yet no potent inhibitors have been found. From the numerous compounds screened in *M. domestica* as inhibitors of the hydration of the epoxy-containing juvenoid R 20458, only the lack of inhibition caused by trichloropropene and cyclohexene oxides was noteworthy. These compounds are the best-known inhibitors of the microsomal epoxide hydrolase in mammals, but interestingly they fail to inhibit the hydration of R 20458 or JH hydration in mammalian liver cytosol or microsomes as well. Using the dieldrin analog HEOM as a substrate, a variety of compounds including JH, juvenoids, organophosphates, methylene dioxyphenyl and benzothiazole mixed function oxidase (MFO) inhibitors, trichloropropene oxide, and naphthalene, 1,2-epoxide all caused some inhibition of epoxide hydration. Interestingly, some glycidyl ethers proved to be quite potent inhibitors of HEOM hydration in *Calliphora erythrocephala* and *Spodoptera eridania* (previously *prodenia*). Unfortunately, only a few of these compounds have been screened (without success) as inhibitors of JH epoxide hydration. The action of several inhibitors of octene oxide hydration in *S. eridania* supports the presence of an essential lysine or histidine. However, no information is yet available on the mechanism of JH hydration. Preliminary studies, including the inhibition data discussed above, provide further evidence that JH is degraded in insects by a rather unique hydrolase(s).



SCHEME A

It should, however, be noted that such differential inhibition is likewise not definitive. In mammalian systems trichloropropene oxide has been found to be a moderate inhibitor of the hydration of some substrates which have a rapid turnover but low affinity for the enzyme. However, other substrates which have a higher affinity for the same enzyme suffer no inhibition with this material.

Just as selective inhibitors have proven useful in elucidating the role of esterases in JH regulation, selective, potent inhibitors of the epoxide hydrolases acting on JH could be very important in elucidating the role of these enzymes in insect development. Unfortunately such inhibitors are not yet known. Possibly the discovery of chalcone oxides as potent inhibitors of the mammalian cytosolic and mitochondrial epoxide hydrolases will lead to useful inhibitors of JH epoxide hydration in insect systems (Brooks, G. 1973, 1974; Hammock, B. *et al.*, 1974a; Slade, M. *et al.*, 1975; Craven, A. *et al.*, 1976; Yu, S. and Terriere, L. 1978a,b; Mullin, C. and Wilkinson, C. 1980b; Mullin, C. and Hammock, B. 1982). Phenobarbital has been reported to induce JH I epoxide hydrolase activity in *Musca domestica*, while hydrolase activity on octene oxide was induced in *S. eridania* with pentamethylbenzene. Potent inducers of JH epoxide hydrolase may place a strain on the mechanism regulating endocrine homeostasis (Yu, S. and Terriere, L. 1978b; Mullin, C. and Wilkinson, C. 1980a).

Several workers have reported the absence of epoxide hydrolase activity in the hemolymph from a variety of insects including *Periplaneta americana*, *Gromphadorhina portentosa*, *Trichoplusia ni*, *Spodoptera eridania*, *Manduca sexta*, *Hyalophora cecropia*, and *Tenebrio molitor*. Epoxide hydrolase activity is fairly widely distributed in other tissues, and in various insects it has been reported high in midguts, fat bodies, and imaginal disks (Hammock, B. *et al.*, 1974a; Slade, M. and Wilkinson, C. 1974; Hammock, B. *et al.*, 1975a; Slade, M. *et al.*, 1976; Wing, K. *et al.*, 1981).

Epoxide hydrolase activity has been titrated during the development of several insects. In *M. domestica* epoxide hydrolase activity, expressed in terms of metabolism per whole insect equivalent, increased to high levels towards the end of the last larval stadium, dropped in the pupa and increased

again during the first 2 weeks of adult life. Epoxide hydrolase activity on R 20458 was very high throughout the pupal stage of male and female *T. molitor*. Two separate studies using HEOM, JH I, and subsequently octene oxide led to the finding that the midgut epoxide hydrolase was most active during the middle or wandering-burrowing phase of the last larval instar. Similar peak activities of epoxide hydrolase acting on R 20458 were found in the fat body and midgut of last larval instar *T. ni*. However, midgut activity peaked 1 day earlier and fell off more rapidly than that in the fat body. Although detailed kinetic studies are lacking on the enzymes involved in JH metabolism in larval tissues, epoxide hydration appears relegated to a secondary role in the midguts, imaginal disks and fat bodies of *M. sexta* and *T. ni* larvae. At low substrate concentrations JH acid and JH acid diol are found as exclusive metabolites in homogenates or with organs held *in vitro*. Only at very high substrate concentrations is the diol found to be a significant metabolite. Several interpretations are possible from this observation, but one interpretation is consistent with other circumstantial evidence including *in vivo* data. JH ester cleavage appears to be the major route of initial metabolism and the hydrolase(s) involved appear to prefer JH acid over JH itself as a substrate. The situation appears different in *H. cecropia* and *S. eridania* where the diol is detected as a metabolite (Slade, M. and Zibitt, C. 1971, 1972; Slade, M. *et al.*, 1976; Yu, S. and Terriere, L. 1978a,b; Mullin, C. and Wilkinson, C. 1980a,b; Wing, K. *et al.*, 1981).

Only two comprehensive studies have been published on the biochemistry of any insect epoxide hydrolases. Mullin, C. and Wilkinson, C. (1980a,b) report the most catalytically active epoxide hydrolase ever purified from a eukaryotic source. By monitoring the relative activity on different substrates during purification, these workers clearly demonstrated the presence of multiple forms of microsomal epoxide hydrolases in midguts from *S. eridania*. Unfortunately for endocrinologists, the form purified had no hydrolytic activity on JH. Clearly, this excellent work needs to be duplicated on the hydrolase(s) metabolizing JH. The information needed to assess the role of epoxide hydrolases in insect endocrinology is illustrated by the more comprehensive information on JH esterases in

sections 1.2, 2, and 3. With improved assay methods such as the use of cellulose prelayer thin-layer plates, and with the availability of model substrates, hopefully this information will be available soon. Such expanded research on epoxide hydrolases could be justified, based upon the role of these enzymes in the degradation of both natural and man-made xenobiotics, intermediary metabolism such as prostaglandin or leukotriene metabolism, or the conversion of sitosterol into cholesterol (Morisaki, M. *et al.*, 1981), as well as insect endocrinology.

1.2 Ester hydrolysis

Ester hydrolysis appears to be the major route of JH metabolism in most insects so far studied, and by far the greatest amount of work has centered on this pathway (Table 2). It would be nice to think that this emphasis on esterase research stemmed from a knowledge of relative rates of metabolism by different pathways. Such is not the case. Most of the studies listed in Tables 1 and 2 focused immediately upon the biochemistry or physiology of the enzyme investigated, with total disregard of competing pathways and of *in vivo* studies. This lack of experimental information on *in vivo* pathways stems from two causes. First, although experiments on *in vivo* metabolism are conceptually simple, they are difficult to carry out. Second, JH esterase activity is much easier to measure than epoxide hydrolase activity. Several surveys of JH metabolism have been made, but these studies largely ignored the biology of the test organisms. Other studies have simply examined hemolymph metabolism, which would not detect epoxide hydration. Thus, the blanket statements in this chapter — as well as others — that ester hydrolysis is the major route of metabolism are based on a disappointingly small database.

There are numerous studies involving the monitoring of insect esterases, probably resulting from the ease by which esterase activity can be measured using esters of various phenols such as α and β -naphthol, nitrophenols, and β -methylumbelliferone or thiols such as thiocholine. It is tempting to extrapolate this information on esterases to JH esterases, but one should be wary of such extrapolation. These stains are likely to detect a variety of esterases, proteases, phosphatases and other hydrolytic enzymes. They may not, however, detect

JHE for three rather obvious reasons. First, the JHs are α,β -unsaturated and thus conjugated esters. Not only does this conjugation restrict rotation and lend planarity to this portion of the molecule; it also stabilizes the JH ester to chemical hydrolysis. Acid and base hydrolysis, as well as enzymatic hydrolysis, are thought to proceed through a tetrahedral intermediate at the carbonyl carbon as shown by Scheme C. As the trigonal carbon takes on tetrahedral character, it must move out of conjugation with the 2,3-olefin and thus is resistant to attack. Second, JH is an ester of a very small alcohol and a very large acid, while most of the model substrates are esters of very large alcohols such as naphthol and very small acids such as acetic acid. Several workers have found that even ethyl esters of JH and related juvenoids are very slow to hydrolyze in the presence of insect esterases, while isopropyl esters are still more stable. The concept of resonance stabilization of the ester and steric hindrance of JH esterases found practical utilization in development of the hydrolytically stable dodecadienoate esters hydroprene (ethyl ester) and methoprene (isopropyl ester). Ester hydrolysis must proceed through an intermediate in which the carbonyl group moves out of conjugation with two olefinic functionalities. It is unlikely that an enzyme which distinguishes between a methyl and ethyl ester will hydrolyze a naphthyl ester rapidly. In some species the 2*E* geometry is required for rapid enzymatic hydrolysis, and evidence is mounting in several species that the esterase's recognition of JH may be as specific as, or more specific than, that of the lepidopteran hemolymph carriers — or possibly some JH receptors. Finally, JH is a highly lipophilic molecule normally present at very low physiological concentrations. Although JH esterases may be very active, it is unlikely that they are present in high concentrations. Therefore, it is unlikely that JH esterases will be detected in the presence of more active enzymes using millimolar concentrations of spectrophotometric substrates. For any real chance of detecting JH esterases with classical substrates the enzyme(s) will probably have to be purified or at least enriched (Weirich, G. *et al.*, 1973; Weirich, G. and Wren, J., 1973, 1976; Yu, S. and Terriere, L., 1975; Hammock, B. and Quistad, G., 1976, 1981; Hammock, B. *et al.*, 1977a,b; Sparks, T. and Hammock, B., 1979a).

Table 2: Involvement of ester cleavage in juvenile hormone metabolism

Order; Genus, species	References
Thysanura <i>Thermobia domestica</i> (firebrat)	Ajami, A. and Riddiford, L. (1973)
Orthoptera <i>Blaberus giganteus</i>	Hammock, B. (1975); Hammock, B. <i>et al.</i> (1977b); Hammock, B. and Mumby, S. (1978); Mumby, S. <i>et al.</i> (1979)
<i>Blattella germanica</i> (German cockroach)	Ajami, A. and Riddiford, L. (1973)
<i>Diploptera punctata</i> (Pacific beetle cockroach)	Rotin, D. <i>et al.</i> , (1982); Szibbo, C. <i>et al.</i> (1982)
<i>Locusta migratoria</i> (migratory locust)	Emmerich, H. and Hartmann, R. (1973); Erley, D. <i>et al.</i> (1975); de Kort, C. <i>et al.</i> (1979); Peter, M. <i>et al.</i> (1979)
<i>Periplaneta americana</i> (American cockroach)	Ajami, A. and Riddiford, L. (1973); Sams, G. <i>et al.</i> (1978); de Kort, C. <i>et al.</i> (1979)
<i>Periplaneta brunnea</i> (brown cockroach)	Weirich, G. and Wren, J. (1976)
<i>Schistocerca gregaria</i> (gregarious grasshopper)	White, A. (1972); Pratt, G. (1975)
<i>Schistocerca vaga</i> (vagrant grasshopper)	Slade, M. and Zibitt, G. (1972); Weirich, G. and Wren, J. (1976)
Hemiptera <i>Dysdercus cingulatus</i>	Sláma K. and Jarolím, V. (1980)
<i>Oncopeltus fasciatus</i> (large milkweed bug)	Ajami, A. and Riddiford, L. (1973)
<i>Pyrrhocoris apterus</i> (cotton stainer)	Ajami, A. and Riddiford, L. (1973); Sláma, K. and Jarolím, V. (1980)
<i>Rhodnius prolixus</i> (assassin bug)	White, A. (1972)
Homoptera <i>Dactynotus ambrosiae</i>	Ajami, A. and Riddiford, L. (1973)
Lepidoptera <i>Anagasta kuehniella</i> (Mediterranean flour moth)	Kramer, K. and Childs, C. (1977)
<i>Antheraea pernyi</i> (antheraea moth)	Ajami, A. and Riddiford, L. (1973)
<i>Colias eurytheme</i> (alfalfa butterfly)	Wing, K. <i>et al.</i> (1982)
<i>Danus plexippus</i> (monarch butterfly)	Lessman, C. and Herman, W. (1981); Jones, D. <i>et al.</i> (1982); Wing, K. <i>et al.</i> (1982)
<i>Diatraea grandiosella</i> (Southwestern corn borer)	Brown, J. <i>et al.</i> (1977)
<i>Diatraea saccharalis</i> (sugarcane borer)	Roe, R. <i>et al.</i> (1983)
<i>Ephestia elutella</i> (tobacco moth)	Hammock, B. <i>et al.</i> (1981); Jones, D. <i>et al.</i> (1982); Wing, K. <i>et al.</i> (1982)
<i>Estigmene acraea</i> (salt marsh caterpillar)	Jones, D. <i>et al.</i> (1982); Hammock, B. <i>et al.</i> (1981)
<i>Galleria mellonella</i> (greater wax moth)	McCaleb, D. and Kumaran, A. (1980); McCaleb, D. <i>et al.</i> (1980); Reddy, G. <i>et al.</i> (1979, 1980); Rudnicka, M. <i>et al.</i> (1979) Sláma, K. and Jarolím, V. (1980); Hammock, B. <i>et al.</i> (1981); Kumaran, A. <i>et al.</i> (1981); Wing, K. <i>et al.</i> (1982); Jones, D. <i>et al.</i> (1982)
<i>Heliothis virescens</i> (tobacco budworm)	Hammock, B. <i>et al.</i> (1981); Wing, K. <i>et al.</i> (1982)
<i>Hemileuca nevadensis</i> (Nevada buck-moth)	Hammock, B. <i>et al.</i> (1981); Jones, D. <i>et al.</i> (1982); Wing, K. <i>et al.</i> (1982)

Table 2: Involvement of ester cleavage in juvenile hormone metabolism (Continued)

Order; Genus, species	References
<i>Hyalophora cecropia</i> (cecropia moth)	Slade, M. and Zibitt, C. (1971, 1972); Ajami, A. and Riddiford, L. (1973); Whitmore, D. <i>et al.</i> (1974)
<i>Hyalophora gloveri</i> (saturniid moth)	Whitmore, D. <i>et al.</i> (1972, 1974)
<i>Junonia coenia</i> (buckeye butterfly)	Hammock, B. <i>et al.</i> (1981); Jones, D. <i>et al.</i> (1982); Wing, K. <i>et al.</i> (1982)
<i>Manduca quinquemaculata</i> (tomato hornworm)	Kramer, K. and Childs, C. (1977)
<i>Manduca sexta</i> (tobacco hornworm)	Slade, M. and Zibitt, C. (1971, 1972); Ajami, A. and Riddiford, L. (1973); Weirich, G. and Wren, J. (1973); Weirich, G. <i>et al.</i> (1973); Ajami, A. (1975); Akamatsu, Y. <i>et al.</i> (1975); Hammock, B. <i>et al.</i> (1975b); Sanburg, L. <i>et al.</i> (1975a, b); Nowock, J. <i>et al.</i> (1976); Nowock, J. and Gilbert, L. (1976); Kramer, K. and Childs, C. (1977); Vince, R. and Gilbert, L. (1977); de Kort, C. <i>et al.</i> (1979); Mitsui, T. <i>et al.</i> (1979); Kramer, S. and Law, J. (1980); Coudron, T. <i>et al.</i> (1981); Hammock, B. <i>et al.</i> (1981); Wing, K. <i>et al.</i> (1982); Jones, D. <i>et al.</i> (1982); Sparks, T. <i>et al.</i> (1983)
<i>Orgia vetusta</i> (western tussock moth)	Hammock, B. <i>et al.</i> (1981); Jones, D. <i>et al.</i> (1982); Wing, K. <i>et al.</i> (1982)
<i>Pectinophora gossypiella</i> (pink bollworm)	Hammock, B. <i>et al.</i> (1981); Jones, D. <i>et al.</i> (1982); Wing, K. <i>et al.</i> (1982)
<i>Pieris rapae</i> (cabbage butterfly)	Jones, D. <i>et al.</i> (1982); Wing, K. <i>et al.</i> (1982)
<i>Plodia interpunctella</i> (Indian meal moth)	Oberlander, H. and Silhacek, D. (1976)
<i>Pseudoplusia includens</i> (soybean looper)	Sparks, T. <i>et al.</i> (1982)
<i>Samia cynthia</i> (cynthia moth)	Ajami, A. and Riddiford, L. (1973); Weirich, G. and Wren, J. (1976)
<i>Sphinx chersis</i> (great ash sphinx)	Kramer, K. and Childs, C. (1977)
<i>Spodoptera eridania</i> (southern armyworm, formerly <i>Prodenia</i>)	Slade, M. and Wilkinson, C. (1974)
<i>Spodoptera exigua</i> (beet armyworm)	Hammock, B. <i>et al.</i> (1981); Jones, D. <i>et al.</i> (1982); Wing, K. <i>et al.</i> (1982)
<i>Spodoptera littoralis</i> (army worm)	Sláma, K. and Jarolím, V. (1980)
<i>Trichoplusia ni</i> (cabbage looper)	Hammock, B. <i>et al.</i> (1977a,b); Hammock, B. and Sparks, T. (1977); Mumby, S. <i>et al.</i> (1979); Sparks, T. <i>et al.</i> (1979a,b); Sparks, T. and Hammock, B. (1979a,b); Hammock, B. <i>et al.</i> (1981); Sparks, T. and Hammock, B. (1980); Jones, G. <i>et al.</i> (1981); Jones, D. <i>et al.</i> (1982); Wing, K. <i>et al.</i> (1981, 1982) Rudnicka, M. and Hammock, B. (1981a,b); Yuhás, D. <i>et al.</i> (1983)
Coleoptera	
<i>Hippodamia convergens</i> (convergent lady beetle)	Ajami, A. and Riddiford, L. (1973)
<i>Leptinotarsa decemlineata</i> (Colorado potato beetle)	Ajami, A. and Riddiford, L. (1973); Kramer, S. and de Kort, C. (1976a,b); Kramer, S. (1978a,b); de Kort, C. <i>et al.</i> (1978, 1979); Kramer, S. and Law, J. (1980); de Kort, C. <i>et al.</i> (1981); de Kort, C. and Granger, N. (1981)
<i>Leptinotarsa haldemani</i>	de Kort, C. <i>et al.</i> (1979)
<i>Tenebrio molitor</i> (yellow mealworm)	Ajami, A. and Riddiford, L. (1973); Weirich, G. and Wren, J. (1976); McCaleb, D. <i>et al.</i> (1980); Sláma, A. and Jarolím, V. (1980); Sparks, T. and Hammock, B. (1980)
<i>Tribolium castaneum</i> (red flour beetle)	Edwards, J. and Rolands, D. (1977)

Table 2: Involvement of ester cleavage in juvenile hormone metabolism (Continued)

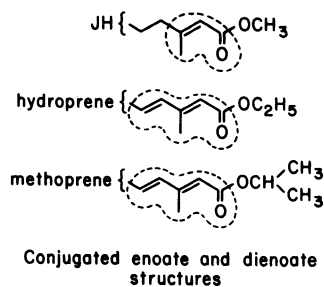
Order; Genus, species	References
Diptera	
<i>Culex pipiens pipiens</i> (northern house mosquito)	Hooper, G. (1976); Brown, T. and Hooper, G. (1979)
<i>Chaoborus americanus</i> (phantom midge)	Ajami, A. and Riddiford, L. (1973)
<i>Drosophila hydei</i> (fruit fly)	Klages, G. and Emmerich, H., (1979a,b); Bisser, A. and Emmerich, H. (1981)
<i>Drosophila melanogaster</i> (vinegar fly)	Chihara, A. <i>et al.</i> (1972); Ajami, A. and Riddiford, L. (1973); Wilson, T. and Gilbert, L. (1978)
<i>Musca domestica</i> (house fly)	Ajami, A. and Riddiford, L. (1973); Hammock, B. <i>et al.</i> (1977a); Yu, S. and Terriere, L. (1978a); de Kort, C. <i>et al.</i> (1979); Mumby, S. <i>et al.</i> (1979)
<i>Phormia regina</i> (black blowfly)	Yu, S. and Terriere, L. (1978a)
<i>Sarcophaga bullata</i> (flesh fly)	Slade, M. and Zibitt, C. (1972); Weirich, G. and Wren, J. (1976); Yu, S. and Terriere, L. (1978a)
Hymenoptera	
<i>Apis mellifera</i> (honey bee)	Ajami, A. and Riddiford, L. (1973); de Kort, C. <i>et al.</i> (1977, 1979); Mane, S. and Rembold, H. (1977)
<i>Atta texana</i> (Texas leaf cutting ant)	Ajami, A. and Riddiford, L. (1973)
<i>Bombus terrestris</i> (Bumble bee)	Röseler, P. and Röseler, I. (1978)
<i>Solenopsis invicta</i> (Red imported fire ant)	Ajami, A. and Riddiford, L. (1973)

Any definitive detection of JH esterase activity must employ radioactive JH as a substrate, and a number of analytical methods, as well as radiosynthesis of the JHs, have been reviewed by Hammock, B. and Quistad, G. (1981). If JH acid is not clearly the sole metabolite, chromatographic methods are required such as thin-layer, high-performance liquid, or gas-liquid chromatography. The most rapid chromatographic technique now employed involves direct spotting of biological extracts on the cellulose pre-layer of silica gel thin-layer plates, visualization of suspected metabolites by co-chromatography with ultraviolet dense standards, and radiotracer analysis. When JH acid is the only metabolite, two methods have proven very useful. The first method involves release of radioactive methanol from methoxy-labeled JH. Unfortunately the methyl-labeled JHs are not commercially available, although several published procedures describe their preparation. The second method involves partition of chain-radiolabeled JH and JH acid between a basic aqueous phase and a hydrocarbon organic phase. Iso-octane or dodecane are often used to retard volatilization, but thorough mixing is necessary (Sanburg, L. *et al.*, 1975a;

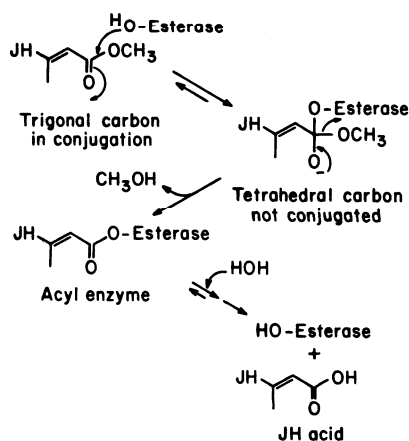
Nowock, J. *et al.*, 1976; Hammock, B. and Quistad, G., 1976, 1981; Hammock, B. *et al.*, 1977b; Hammock, B. and Sparks, T., 1977; Vince, R. and Gilbert, L., 1977; Hwang-Hsu, K. *et al.*, 1979; Klages, G. and Emmerich, H., 1979a,b; Sparks, T. *et al.*, 1979b; Peter, M. 1981).

1.3 Oxidation

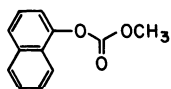
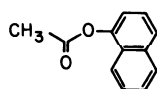
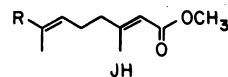
A student of xenobiotic metabolism could look at the JH structure and envision numerous possible oxidative metabolites involving epoxidation, hydroxylation alpha to the olefins or epoxide, hydroxylation on methyl or ethyl side-chains, and other such pathways. Surprisingly, oxidative pathways of metabolism appear unimportant in most of the insects examined. However, in only a few cases, such as the investigation of JH metabolism in *M. sexta* fat body and wing disks, have techniques been rigorous enough to exclude moderate levels of oxidative metabolism. Ajami, A. and Riddiford, L. (1973) examined JH metabolism in an impressive number of species and report some evidence for oxidative degradation. This work cannot be taken too seriously because the analytical method used



SCHEME B



SCHEME C



Comparison of JH ester with model substrates

SCHEME D

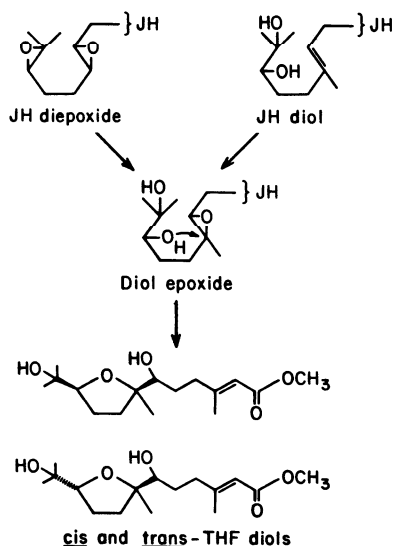
yields poor resolution. The solvent system employed did not resolve JH diepoxide from JH, and the authenticity of their standards is equivocal. However, there is some evidence from this study, as well as from earlier work by Slade, M. and Zibitt, C.

(1972), that oxidase activity may contribute to metabolism.

The best demonstration of oxidative metabolism of JH comes from studies involving the flies *Phormia regina*, *Sarcophaga bullata* and especially *Musca domestica*. The initial studies by Hammock, B. *et al.* (1977a) and the more comprehensive work by Yu, S. and Terriere, L. (1978a) are surprisingly consistent considering the disparate strains and analytical techniques. The autoradiographic method of Hammock, B. *et al.* (1977a) facilitated the detection of minor discrete metabolites as distinct spots, while the work of Yu, S. and Terriere, L. (1978a) was able to discern lower levels of oxidase-involved metabolism.

Hammock, B. *et al.* (1977a) found that roughly equal amounts of JH acid and diol, with traces of JH diol acid, were produced from JH when microsomes from both susceptible (NAIDM) and resistant (methoprene-R and dimethoate-R-unpublished) *M. domestica* were incubated without NADPH. When NADPH was added, little change was noted with the susceptible strains, but extensive metabolism was noted with the resistant strains. This observation indicates that some insecticide-resistant *M. domestica* have an enhanced ability to metabolize JH. Most oxidative metabolites were unidentified but discrete radioactive spots co-chromatographed with the *cis*- and *trans*-tetrahydrofuran diol acids (Fig. 1 and below). The tetrahydrofuran diol esters were also present. However, in the solvent system used they chromatographed with JH acid and were only detected after the metabolite band was reacted with diazomethane. Although oxidative metabolism of JH I was very minor in microsomes from susceptible flies, metabolism of the juvenoid R 20458 by oxidative pathways was extensive and rapid. Since these compounds are of similar structure and polarity, this discrepancy is interesting.

Yu, S. and Terriere, L. (1978a) reported metabolic pathways similar to those above involving 6, 7-epoxidation. Using a variety of techniques they provided good evidence that an NADPH-dependent, CO-sensitive microsomal oxidase was involved in the metabolism of JH I in *M. domestica*. They also found a large increase in oxidase activity in two resistant strains of *M. domestica*, and this increase could even be underestimated due to



SCHEME E

co-chromatography of esterase and oxidase bands. In one of the two susceptible strains examined, oxidative metabolism of JH I was also found to be a major route of metabolism. Oxidative as well as hydrolytic metabolism was much slower in *P. regina* and *S. bullata* than in *M. domestica*. In an excellent subsequent experiment, oxidation as well as conjugation were demonstrated *in vivo* (Yu, S. and Terriere, L., 1978b).

Yu, S. and Terriere, L. (1978a) also followed the relative contribution of oxidation, epoxide hydration, and ester cleavage throughout the development of *M. domestica*. Hydrolase and esterase levels are high in the early larval instars and drop as pupation approaches, while oxidase levels increase before pupation. All three activities are low during the early pupal stage, and later increase. Epoxide hydrolase activity peaks shortly after eclosion, while esterase and oxidase activities continue to increase for 5 or more days into the adult stage. Possibly all three pathways are involved in JH regulation.

The origin of the tetrahydrofuran diols may seem complex, but it is actually rather straightforward as illustrated by some earlier work with 1,5-diepoxyhexane and the juvenoid R 20458. When one of the two epoxides of the corresponding JH diepoxide is opened to the diol by acid-catalyzed hydrolysis, one of the alcohols can attack the

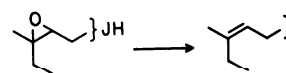
remaining epoxide to give a 5-, 6- or 7-membered ring or a bicyclic ether. By far the major products are the two tetrahydrofuran diols which are *cis* or *trans* across the ring. When the JH diol is oxidized under neutral conditions, the JH diol epoxide is an intermediate that can be isolated. This compound will slowly cyclize to yield almost exclusively the tetrahydrofuran diols. Similar reactions can occur enzymatically (Gill, S. *et al.*, 1974; Hammock, B. *et al.*, 1974a,b; 1975b; 1977a; Hammock, B. and Quistad, G. 1976, 1981; Yu, S. and Terriere, L., 1978a; Hammock, B., 1981).

With the juvenoid R 20458 reduction of the epoxide to the corresponding olefin was found to occur rapidly in the gut contents of rats and steers (Hoffman, L. *et al.*, 1973; Ivie, G. *et al.*, 1976; Ivie, G., 1976). This reaction has been demonstrated to occur in the gut contents of man and several other mammals with several compounds including JH (Hammock, unpublished). The pathway has not been detected in insects, but the search has not been exhaustive, and it is complicated by the facile reoxidation of the 10,11 olefin by solvent peroxides. However, this pathway may be of use to entomologists for providing radiolabeled precursors including methyl farnesoate to study JH biosynthesis.

1.4 Conjugation

Compared to research on the microsomal oxidases, very little research on conjugating reactions has been reported. This disparity is disappointing because conjugating reactions are likely to play a major role in the metabolism of a variety of xenobiotics. It is thus not surprising that there is still less information on conjugating reactions in insects than in vertebrates (Yang, R., 1976) and almost none on the conjugation reactions involving JH.

Slade, M. and Zibitt, C. (1972) found that apparent conjugates were produced from JH I *in vivo* in *Manduca sexta*, *Hyalophora cecropia*, *Schistocerca vaga* and *Sacrophaga bullata*. For example, in 2 h, 45% of the hormone injected into *S. vaga* appeared as conjugated material. Cleavage with Glusulase



SCHEME F

and rechromatography resulted in firm evidence for conjugation of JH diol and possibly JH acid and diol acid. Reasonable evidence was also presented for sulfate conjugation of JH diol in *S. bullata*. In his study of JH III metabolism in *Schistocerca gregaria* and *Rhodnius prolixus*, White, A. (1972) demonstrated the presence of JH diol, JH acid, and JH diol acid as sulfate and/or glucose conjugates. The most detailed study of the conjugation of JH to date was by Slade, M. and Wilkinson, C. (1974) in *Spodoptera eridania*. Using sulfatase cleavage and subsequent enzymatic synthesis of a sulfate conjugate of the diol acid, they presented good evidence that sulfate conjugation of the diol acid was the major, if not the only, route of conjugation.

If the presence of highly polar metabolites is taken as evidence of conjugation, then essentially every *in vivo* metabolism study involving JH has resulted in detection of conjugates. It is possible that some of the highly polar *in vivo* metabolites of JH result from extensive metabolism of the JH backbone rather than from conjugation of a primary metabolite. In most studies the conjugates have not been hydrolyzed to the aglycones. Even when hydrolytic enzymes are used, one must be very careful about their integrity. In no study has the structure of a JH conjugate been established or the mechanism of conjugation studied in detail.

In their 1973 survey Ajami, A. and Riddiford, L. found the appearance of water-soluble metabolites to be very common. Three water-soluble products were obtained from extracts of *H. cecropia* and *M. sexta*, and when hydrolyzed about 60% of the radioactivity co-chromatographed with JH acid and JH acid diol. Apparent conjugation has also been found in *Drosophila melanogaster*, *Leptinotarsa decemlineata*, *Locusta migratoria*, *Musca domestica*, *Tribolium castaneum*, and *Trichoplusia ni* (Edwards, J. and Rowlands, D. 1977; Erley, D. *et al.*, 1975; Hammock, B. *et al.*, 1981; Kramer, S. *et al.*, 1977; Sparks, T. and Hammock, B. 1980; Wilson, T. and Gilbert, L. 1978; Yu, S. and Terriere, L., 1978a).

Thus, there is excellent evidence for sulfate conjugation of JH diol and/or diol acid, and less evidence for other possible routes of conjugation.

Glutathione conjugation may superficially appear to be a likely route of metabolism, but conclusive evidence for this route is so far lacking in

insects. Glutathione is known to attack epoxides both spontaneously and enzymatically. However, using the juvenoid epoxide R 20458, Gill, S. *et al.* (1974) were unable to find evidence for glutathione conjugation catalyzed by mammalian liver homogenates. Analogous studies failed to show R 20458 conjugation with insect systems, which is not surprising since conjugation is usually very slow with polysubstituted epoxides (Chasseaud, L., 1979). However, Morello, A. and Agosin, M. (1979) report that the glutathione conjugate leading to the subsequent formation of the mercapturate at the 10 position of JH is the major metabolite in rat hepatocyte suspensions. Another possible route of metabolism is a Michael-like attack of GSH at the conjugated 2,3-olefin. Even a reversible attack at this position could result in partial loss of biological activity due to *E/Z* isomerization. This reaction between glutathione and JH was only demonstrated to occur with JH I at 37° and higher. Addition of *M. domestica* 100,000 g supernatant to JH caused a negligible increase in the production of water-soluble radioactivity or 2*E/Z* isomerization as monitored by liquid chromatography (Hammock, B. *et al.*, 1977a; Hammock, B. and Mumby, S. unpublished). Possibly the degradation of 2*Z* juvenoids in deproteinated insect hemolymph observed by Sláma, K. and Jarolím, V. (1981) was due to glutathione. Thus, there are no firm data supporting glutathione conjugation of JH as an important route of metabolism in insects (Fig. 1). However, reaction between JH and glutathione or another sulfhydryl group could lead to primary metabolism, especially in the presence of an effective catalyst. Also, artifacts could arise if high levels of glutathione are used to inhibit tyrosinase action.

Thus, the few *in vivo* studies performed indicate that conjugation (probably sulfate conjugation) is important in the ultimate conversion of JH into water-soluble metabolites. Although this question has not been addressed, it is unlikely that conjugation is the rate-limiting step in JH inactivation. However, further research is needed on the mechanisms of JH conjugation if we wish to glean an appreciation of its ultimate disposition.

1.5 JH dynamics

An appreciation of the dynamics of JH biosynthesis,

distribution, action, metabolism and elimination is ultimately very important to our understanding of insect endocrinology. Yet there is no study even on the kinetics of JH degradation. Hammock, B. *et al.* (1974b, 1975a) employed simplistic kinetic arguments to explain the action of some juvenoids. The best study of the *in vivo* kinetics of JH degradation was reported by Erley, D. *et al.* (1975) in *L. migratoria*. These workers found very rapid elimination of JH from the hemolymph and appearance of radioactivity in the feces. There was little difference in the rates of elimination of JH in males or females of either sex. It was interesting that the Malpighian tubules appeared to be the site of most JH excretion. A similar approach by Edwards, J. and Rowlands, D. (1977) correlated JH metabolism with development in *T. castaneum*, while Kramer, S. (1978b) attempted to summarize his extensive investigation of JH metabolism in *L. decemlineata* with a balance sheet of biosynthesis and degradation.

Several approaches need to be taken in the investigation of the kinetics of JH metabolism and excretion. One approach involves careful *in vivo* studies such as those performed by Erley, D. *et al.* (1975) or Edwards, J. and Rowlands, D. (1978). Such studies could indicate the relative importance of metabolism in the regulation of JH, yet one must consider the effects of *in vivo* pools. Appropriate models could involve a comparison of species where JH metabolism appears slow, such as in *D. melanogaster* or *Apis mellifera*, with species where JH metabolism is rapid and appears important in endocrine regulation such as *M. sexta* or *T. ni*. Such studies become even more crucial in insects falling in an intermediate class. A second approach is to correlate *in vivo* metabolism with *in vitro* metabolism by organ culture and tissue homogenates. For instance, Wing, K. *et al.* (1981) have found in *T. ni* that ester hydrolysis by a single enzyme in the fat body and hemolymph accounts for most of the *in vivo* degradation of JH. From this information simplistic models of the kinetics of JH degradation can be developed. Such models can then be coupled with binding data and partition characteristics of JH as first approached by Nowock, J. *et al.* (1976). As additional information becomes available, such as JH metabolism by the small but very active imaginal disks, a model of increasing complexity can be devised.

2 JH ESTERASES

As indicated in section 1.2, much more is known about JH ester hydrolysis than about other pathways of metabolism. Thus, in this section the biochemical properties of JH esterase are discussed in more detail.

2.1 JH specific esterases, possible definitions

Sanburg, L. *et al.* (1975) coined the term JH specific esterase to refer to those esterases in *M. sexta* hemolymph which are difficult to inhibit with the organophosphate di-isopropylphosphofluoridate (DFP), show marginal hydrolytic activity on α -naphthyl acetate, and hydrolyze JH on or off the carrier protein. At the onset, it must be remembered that in this context specific means at most highly selective. As discussed in more detail below, one of the key experiments which Sanburg, L. *et al.* (1975a) used to distinguish general and specific JH esterases cannot be duplicated in the same or several other laboratories (Coudron, T. *et al.*, 1981). More alarming has been the widespread and indiscriminate application of the term JH specific esterase to a variety of insects. In general the workers who have applied this terminology have failed to experimentally verify Sanburg's criteria for a JH specific esterase. In spite of these problems, the concept that JH specific esterases exist is extremely useful, but the term should be defined clearly in the publication using it. To this end several possible definitions of varying biological and biochemical rigor are discussed below and in Table 3.

Sanburg, L. *et al.* (1975a) indicated the presence of two predominant peaks of JH esterase activity. The major peak of JH hydrolytic activity also hydrolyzed α -naphthyl acetate. This peak of activity failed to hydrolyze JH bound to the carrier protein and was DFP-sensitive. The smaller peak of JH hydrolytic activity had very low activity on α -naphthyl acetate, was DFP-resistant, and metabolized JH in the presence of carrier protein. One could thus have the impression that insect hemolymph is full of very active general esterases which will rapidly hydrolyze any ester, including JH. Only the carrier protein then saves JH from rapid catabolism. At critical stages a DFP-resistant JH specific esterase degrades JH regardless of the

presence of carrier protein. The attractiveness of this hypothesis has led to its being applied to numerous insect systems, and in some it may be true. At least in *Diatraea saccharalis*, *Manduca sexta* and *Trichoplusia ni* the hypothesis is quantitatively misleading. The predominant high molecular weight activity peak or JH general esterase of Sanburg, L. *et al.* (1975a) has not been found by subsequent workers. JH is undoubtedly degraded by general esterases, proteases, lipases, phosphatases and other enzymes in insect tissue, but in *D. saccharalis*, *M. sexta* and *T. ni* these enzymes are orders of magnitude less active, on a tissue wet weight basis, than the JH specific esterase. That is not to say these enzymes are unimportant, and the carrier protein may be important in protecting JH from these enzymes. After all, JH hydrolysis is usually assayed in hemolymph diluted 10–1000-fold with incubation times of only a few minutes, while JH must survive in the presence of general esterases for long enough to exert its action. An example of the potential role of general esterases in degrading JH was obtained using hemolymph from fourth-instar *T. ni*. The JH specific esterase from the fifth instar rapidly hydrolyzes both JH I and III, but the carrier protein has a K_d of 4.2×10^{-7} M for JH I but only 3.7×10^{-6} for JH III (Hammock, B. *et al.*, 1977b). Thus, in concentrated fourth-instar hemolymph JH I was relatively stable (largely protected by binding to the carrier protein) while JH III was hydrolyzed very quickly to the free acid, presumably by general or DFP-sensitive esterases. Thus, even if the corpora allata produced relatively high ratios of JH III to JH I and II, the circulating titer of III would be much lower than that expected from the rate of biosynthesis. This observation could explain some of the differences observed between the JH homolog ratios determined *in vivo* and *in vitro*.

In a variety of last-stadium lepidopterous larvae, a single enzyme or a closely related group of enzymes appear responsible for most JH degradation. In general, these enzymes appear DFP-resistant and have low activity on α -naphthyl acetate. However, these two characteristics of the JH esterase are not essential to the definition of JH specific esterase. It should also be recalled that the ester bond of α -naphthyl acetate is not stabilized by conjugation. Thus, if it can fit into the active site of

Table 3: Possible definitions (criteria) for the term "JH specific esterase"

Biological	
(1)	A JH hydrolyzing esterase whose role has been shown to be essential for the clearance of JH from an insect's body.
(2)	A JH hydrolyzing esterase whose titer correlates with suspected declines in JH titer.
Biochemical	
(3)	An esterase capable of rapidly hydrolyzing JH in the presence or absence of carrier protein.
(4)	An enzyme which hydrolyzes JH with a high k_{cat}/K_m ratio.
(5)	An enzyme which hydrolyzes JH with a low apparent K_m .

a JH esterase, it would not be surprising if it were hydrolyzed more quickly than JH. One would not exclude an enzyme from biological relevance simply because it metabolized both JH and α -naphthyl acetate.

The arguments put forward in section 1.2 support the concept that in many insects specific enzymes are involved in JH metabolism. The definitive proof that an esterase is important in endocrine regulation rests upon the biological effects observed when that enzyme is chemically or biochemically eliminated. Such information is likely to be slow in coming (definition 1, Table 3). Circumstantial evidence, as first presented by Weirich, G. *et al.* (1973), that JH esterase levels correlated with declines in JH titers is also reasonable (definition 2, Table 3). One can also make biochemical arguments that an enzyme capable of hydrolyzing a lipophilic, stable, planar methyl ester must be somewhat special. For the Lepidoptera the definition that a JH specific esterase will degrade JH in the presence of carrier protein seems very reasonable (Fig. 2) (definition 3, Sanburg, L. *et al.*, 1975a). However, many insects lack a carrier protein. It is possible to define JH specific esterase more biochemically. The term specificity is greatly overused in biochemistry, but conservatively it is based on a ratio of k_{cat}/K_m (Fersht, A., 1977). Thus, one could rigorously define a JH specific esterase as an enzyme which shows a higher k_{cat}/K_m ratio for JH than for an alternative substrate such as α -naphthyl acetate. Such a definition requires separation of the JH esterase from other esterases if not in fact purification to homogeneity (definition 4).

Based upon knowledge of JH titers a less rigorous definition could also be useful. Since circulating JH

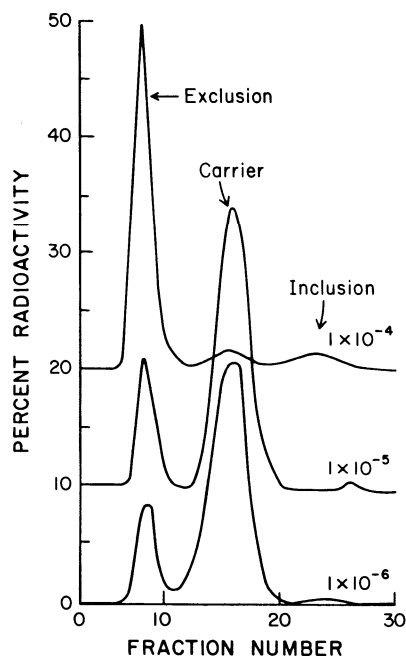


FIG. 2. Binding of varying concentrations of $[^3\text{H}]\text{JH I}$ to hemolymph proteins from *Manduca sexta*. Hemolymph from day 4 last-instar larvae was collected and treated with 2×10^{-3} M DFP under N_2 to inhibit esterase activity. Varying concentrations of JH I were incubated with 1 ml of undiluted hemolymph (1×10^{-4} [top] to 1×10^{-6} M [bottom]) at 30° and the hemolymph passed rapidly through a Sephadex G-100 column held at 1° . Equilibrium was apparently reached almost immediately. The radioactivity in each fraction was determined and expressed as a percentage of the amount applied to the column. This figure shows qualitatively how the JH carrier can be overlooked at unphysiologically high JH concentrations. It also indicates that the equilibrium between the specific carrier and lipid depots (represented by the lipoprotein in the exclusion volume) may be significant.

titers are orders of magnitude lower than the K_m values of JH esterases, it is unlikely that a JH molecule will encounter a JH esterase which is saturated with hormone or in the acyl form. Thus, k_{cat} has less relevance than in the classical definition of enzyme specificity. Arbitrarily one could define a JH specific esterase as an esterase hydrolyzing JH with a low apparent K_m (for instance, 1×10^{-6} M or below) (definition 4). A JH specific esterase is not, however, an esterase that happens to be resistant to DFP inhibition. In spite of the widespread usage of the term JH specific esterase, very few workers have defined the term in their publications, and the data presented do not justify using JH specific esterase by any of the four definitions discussed. Any enzyme

which degrades JH is of biological significance, and thus should be examined experimentally regardless of its ability to hydrolyze other substrates, or of its susceptibility to DFP. In some insects it is appropriate to further consider the influence of a carrier protein, and the original concept of Sanburg, L. *et al.* (1975a) may, in fact, be applicable to some, but certainly not to all, insects.

2.2 JH esterase tissue and subcellular distribution

Much of the work on JH ester hydrolysis has been concentrated on the hemolymph. However, work which defines the tissue(s) responsible for most JH metabolism in the whole animal, as well as experiments which define the JH metabolism in target tissues, is wanting. A nice approach was used with *Leptinotarsa decemlineata* where it was first found that the majority of the metabolism occurred in hemolymph and that contributions from the fat body were minor by comparison. Subsequent work concentrated on the hemolymph (Kramer, S. and de Kort, C., 1976a,b; Kramer, S. *et al.*, 1977; de Kort, C. *et al.*, 1978). In *Tenebrio molitor* larvae and pupae over 95% of the JH esterase activity was found to be in the cytosolic fraction (Sparks, T. and Hammock, B., 1980).

Although the thrust of the work concentrated on other topics, the subcellular distribution of JH esterase activity in *Musca domestica* larvae, pupae and adults was monitored in two laboratories. The highest JH esterase activity was found in mature larvae where over 80% of the activity appeared to be microsomal. The relative amounts in the microsomal and cytosolic fractions appeared rather constant at several developmental stages. Although the JH esterase activities in the two fractions appear similar, they can be differentially inhibited. The microsomal JH esterase activity was even less stable than the cytosolic activity. Possibly this instability has led workers to overlook esterase activity in the Diptera and Hymenoptera, especially in whole-body homogenates (Hammock, B. *et al.*, 1977a; Yu, S. and Terriere, L., 1978a,b; Mumby, S. *et al.*, 1979; Sparks, T. and Hammock, B., 1980). In an early study using *Drosophila melanogaster*, Chihara, C. *et al.* (1972) first reported the ability of imaginal disks to inactivate JH very quickly. The metabolism of JH in target tissues has still not been well studied

although Chihara's work illustrates the importance of such an approach. Klages, G. and Emmerich, H. (1979b) performed the more aesthetically pleasing study of monitoring subcellular distribution in isolated tissues of *Drosophila hydei*. The fat body contains high levels of both esterases and epoxide hydrolases, while the body wall metabolizes JH, largely by ester hydrolysis. Slightly higher activity was reported in the cytosolic fraction. It is not known if the JH esterase activity in the two fractions is identical. The subsequent work of Bisser, A. and Emmerich, H. (1981) raises the possibility that large aggregates of a cytosolic enzyme are pelleted with the microsomal fraction, or that a lipophilic microsomal enzyme is dislodged during tissue preparation. The hemolymph of *D. hydei* (along with a number of other dipterous and hymenopterous species) appeared remarkably inactive in metabolizing JH esterase. An exception was the hemolymph and body fluid of the prepupa and pupa. High levels of JH esterase activity have also been found in the molting fluid of *Manduca sexta*. Possibly an examination of molting fluids could provide a clue to the evolution of JH esterase.

Day 4 last-instar larvae of *Galleria mellonella* have very high JH esterase activity in the midgut and fat body when expressed as activity per unit protein. In fact, both the gonads and wing disks had higher specific activity than the hemolymph. The JH esterase activity in the wing disks was shown to be dependent upon developmental stage and peaked before the hemolymph esterase activity, then rapidly fell to negligible levels. Thus, JH metabolism could play a role in reducing the sensitivity of the disks to both endogenous and exogenous JH (Reddy, G. *et al.*, 1980).

High esterase activity occurs in the epidermis, fat body, hemolymph, and imaginal disks of *M. sexta*, and in the fat body the activity is largely cytosolic. The JH esterase activity in the epidermis, fat body and hemolymph all increased at similar times for the pre-wandering peak (Fig. 3 e,g) (Hammock, B. *et al.*, 1975b; Nowock, J. and Gilbert, L. 1976; Vince, R. and Gilbert, L. 1977; Mitsui, T. *et al.*, 1979), possibly indicating similar regulatory mechanisms.

In terms of total *in vivo* activity, the fat body and hemolymph account for the majority of the JH esterase activity. For instance, during the pre-wandering peak of JH esterase activity (Fig. 3 e,g)

37% of the total body activity is in the fat body and 50% is in the hemolymph; 90% of activity in the fat body is cytosolic and due to the same enzyme which is present in the hemolymph. This work illustrates the importance of expressing metabolism on a larval equivalent basis if the data are to be useful in constructing pharmacokinetic models of JH (Wing, K. *et al.*, 1981).

2.3 JH esterase substrate selectivity

Very little unequivocal information is available on JH esterase substrate specificity since the pure enzymes have not been well studied. It is possible that some of the enzymatic activities attributed to JH esterases are due to other enzymes which co-purify

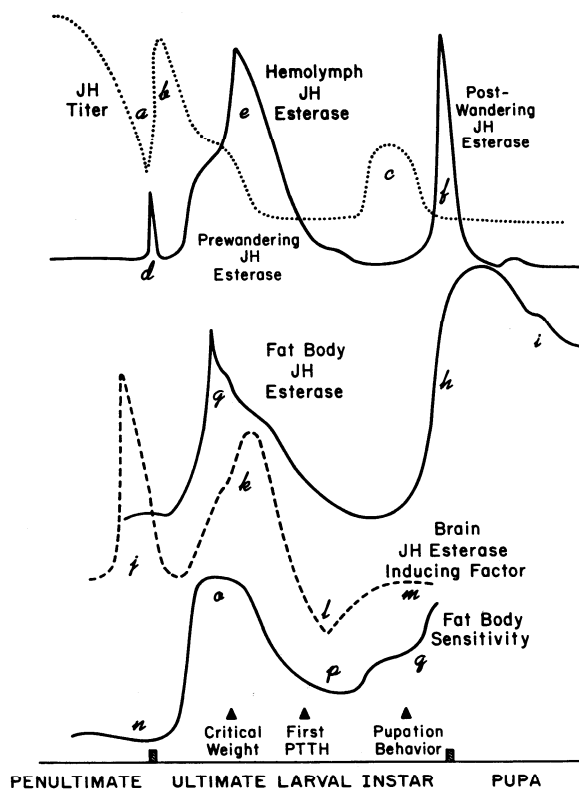


FIG. 3. Temporal variations in JH and factors influencing its metabolism during the larval-pupal transformation in a generalized lepidopterous larva. Data are largely from *Manduca sexta* and *Trichoplusia ni*. Relative JH titer (.....), hemolymph JH esterase activity (—), fat body JH esterase activity (---), brain JH esterase-inducing factor (-----), and sensitivity of the fat body to the brain factor (—). The lower-case letters refer to events discussed in more detail in the text.

with them. In some cases selective inhibitors have been used to eliminate the activity of other enzymes. More commonly, the substrate specificity of crude hemolymph was examined. The resulting information is only valuable in saying certain structures cannot be hydrolyzed by the crude enzyme mixtures. It must again be noted that true substrate specificity can only be obtained from the ratio k_{cat}/K_m or by mixing two potential substrates and monitoring enzymatic hydrolysis when combined.

Hemolymph from the cockroach *Blaberus giganteus* hydrolyzed both JH I and III, but as observed with numerous other species, was unable to hydrolyze ethyl or isopropyl esters of several juvenoids and model substrates such as esters of 3,3-dimethyl acrylic and cinnamic acid (Hammock, B. *et al.*, 1977b). With crude hemolymph from *Locusta migratoria*, JH I is hydrolyzed faster than III and the unnatural enantiomer of III is hydrolyzed faster than the natural enantiomer. These results may reflect a combined interaction of carrier protein(s) (see Goodman, W. and Chang, E., this volume) and esterases, but also reflect true substrate specificity of the crude hemolymph since JH I and III were combined in a single incubation (Peter, M. *et al.*, 1979). K_m values of 10.6×10^{-6} M and similar rates of hydrolysis were found for JH I and III in *Lepidoptarsa decemlineata* hemolymph. The ethyl ester of hydroprene and isopropyl ester of methoprene were not hydrolyzed under these conditions (Kramer, S. and de Kort, C., 1976a,b). Weirich, G. and Wren, J. (1976) found that *Tenebrio molitor* hemolymph rapidly hydrolyzed natural 2*E* but not 2*Z* JH I or its isopropyl ester. In this and most reported experiments, high protein concentrations preclude experimental detection of carrier interactions. Larval homogenates of *Culex pipiens pipiens* were similarly unable to degrade hydroprene and methoprene (Hooper, G., 1976; Brown, T. and Hooper, G., 1979). *Musca domestica* are also unable to hydrolyze methoprene, but can slowly hydrolyze hydroprene (Hammock, B. *et al.*, 1977a; Terriere, L. and Yu, S., 1977). Thus, the results of Bissler, A. and Emmerich, H. (1981) that ethyl 10,11-epoxy-farnesoate is an excellent competitive inhibitor of JH hydrolysis by *Drosophila hydei* epidermis are not surprising.

Weirich, G. and Wren, J. (1973, 1976) reported that last-instar *Manduca sexta* hemolymph could

hydrolyze the 2*E* but not the 2*Z* isomers of JH or the isopropyl ester of JH. The hydrolysis of the ethyl ester of JH by *M. sexta* has been questioned by subsequent workers. These observations were extended to *Samia cynthia* which is similarly capable of hydrolyzing the ethyl ester but not the 2*Z* isomer of JH I. Coudron, T. *et al.* (1981) reported rapid hydrolysis of methyl farnesoate by homogeneous JH esterase from *M. sexta* hemolymph, indicating that the epoxide moiety is not crucial for enzyme binding. Similar conclusions can be drawn from M. Peter's (1981) study showing the C10 enantiomers of both JH I and III are hydrolyzed at equal rates in *M. sexta* hemolymph. The homogeneous *M. sexta* JH esterase appears to have no hydrolytic activity on α -naphthyl acetate, while the *Trichoplusia ni* enzymes show slight activity (Sparks, T. and Hammock, B., 1979a; Yuhas, D. *et al.*, 1982).

The interest in such unnatural substrates stems from several sources. The resistance of the dodecadienoate (2,4-di-unsaturated) juvenoids to hydrolysis probably stems from increased stability from additional conjugation, poorer fit due to decreased rotation of the C3-4 and C4-5 bonds, and steric hindrance due to ethyl and isopropyl esters. This resistance to hydrolysis may increase the biological activity of these compounds. If JH esterases hydrolyzed ethyl, naphthyl, umbelliferyl or other esters, inexpensive, continuous spectrophotometric assays could be devised. With the present state of knowledge, the current tedious assays employing JH itself must be retained. However, additional experiments are needed to determine if the esterases from *Galleria mellonella*, *Pyrrhocoris apterus*, and *T. molitor* hydrolyzing 2*E* and *Z* juvenoid umbelliferyl esters are the same as those hydrolyzing JH (Sláma, K. and Jarolím, V., 1980, 1981). If the JH esterases of *G. mellonella* and *P. apterus* do hydrolyze these substrates, their specificity for the alcohol moiety is much less restrictive than the other esterases studied.

The K_m values for JH esterase activities in the hemolymph of several lepidopterous species are shown in Table 4. Since K_m can be taken as a reasonable estimate of enzyme-substrate affinity with serine esterases and proteases, there is an obvious high affinity for the presumed natural substrates, although there is some variation in the data from different laboratories. Even within the Lepidoptera,

there are huge differences in the JH esterase activity in the hemolymph. It will be interesting to see if this effect is due to quantitative changes in enzyme titer or actual enzyme specific activity. Coudron, T. *et al.* (1981) and Peter, M. (1981) both report that JH I is hydrolyzed slightly faster than JH III with *M. sexta* hemolymph, although JH III apparently binds more tightly than I. Similar conclusions can be made from the data of Sparks, T. *et al.* (1982) showing that TFT is a much more potent inhibitor of JH III than JH I hydrolysis in *M. sexta*. Observations of Sparks, T. and Hammock, B. (1979a) that JH I is hydrolyzed faster than JH III in *T. ni* hemolymph have been confirmed in recent work. However, Wing, K. *et al.* (1982) found that JH III apparently binds more tightly to the esterase than JH I. The rate-limiting reaction for most serine esterases and proteases is deacylation, and other workers have shown that deacylation of acylchymotrypsin depends upon the size of the acyl group. Thus, more rapid deacylation may explain these superficially anomalous observations. Since natural JH titers are far below the K_m of the enzyme, some future work should concentrate on the acylation step of hydrolysis. This step is the rate-limiting step of JH inactivation under physiologically relevant conditions, while the deacylation step is the rate-limiting step of the overall reaction. Thus, the V_m values, and possibly even the K_m values, are kinetically valid at artificially high JH concentrations where deacylation is the rate-limiting step. However, different experimental designs are needed to evaluate the kinetic interactions of JII and JIII esterase at physiological concentrations of the hormone.

Thus, not a great deal is known about substrate specificity of JH esterases. The limited data available certainly suggest enzymes which are highly selective, if not specific, for their natural substrates. Although the receptor sites, carrier molecules and apparently some hydrolytic enzymes all show high specificity for the JH structure, the structural features for recognition seem somewhat different in each case.

2.4 JH esterase purification

Only in the Lepidoptera has much progress been made on purification of JH esterases. Sanburg, L. *et*

al. (1975a) estimated a molecular weight of *Manduca sexta* JH esterase at 67,000 based upon a sedimentation coefficient of 4.98S and a diffusion coefficient of $6.4 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. A similar molecular weight was indicated by gel filtration. Several JH esterase bands of different apparent pI values were found with column isoelectric focusing, but this technique often leads to artifacts. For instance, Wing, K. *et al.* (1982) found JH esterase activity at only a single pI. Coudron, T. *et al.* (1981) purified *M. sexta* JHE to homogeneity (1700 $\times$) by gel filtration, cellulose DE-32 anion exchange, hydroxyapatite, and Sephadex C-50 cation exchange chromatography. The resulting single band on SDS gel electrophoresis had a specific activity of 84 nmol JH hydrolyzed $\text{min}^{-1} \text{ mg}^{-1}$ protein and no activity on α -naphthyl acetate. JH I was hydrolyzed at about twice the rate of JH III. The fact that molecular weight estimates based upon gel filtration, ultracentrifugation and SDS electrophoresis all agree indicates the presence of a single polypeptide chain.

Two approaches have been reported for the purification of JH esterase from *T. ni*. Although polyethylene glycol precipitation will denature some enzymes, it proved very useful with JHE, probably because most of the unwanted proteins are of a much higher or much lower molecular weight than JHE (and also JH carrier protein). A combination of polyethylene glycol precipitation and DEAE chromatography yielded 134-fold purification. Electrophoresis and subsequent isoelectric focusing yielded a single protein band. Although *Trichoplusia ni* JH esterase is an exceptionally stable enzyme in crude hemolymph or even whole body homogenates, the enzyme proved quite unstable in the absence of other protein (Rudnicka, M. and Hammock, B. 1981a,b). Yuhas, D. *et al.* (1982) employed a combination of ammonium sulfate precipitation anion exchange chromatography, gel filtration chromatography and ultracentrifugation to achieve a specific activity of 56 nmol JH hydrolyzed $\text{min}^{-1} \text{ mg}^{-1}$ following a 417-fold purification. The single polypeptide chain was smaller than that from *M. sexta* with an estimated molecular weight of 48,000. The enzyme appeared stable in the sucrose from ultracentrifugation but rapidly lost activity when exposed to charged environments. Unlike *M. sexta* JH esterase, *T. ni* JH esterase seems

to maintain some hydrolytic activity on α -naphthyl acetate (Sparks, T. and Hammock, B., 1979a).

Although initial studies indicated that a variety of esterases were hydrolyzing JH in *Galleria mellonella* hemolymph (Rudnicka, M. *et al.*, 1979), more recent work indicates the involvement of a single enzyme. McCaleb, D. *et al.* (1980) estimate the molecular weight of the *G. mellonella* hemolymph JH esterase to be 60,000, and Wing, K. *et al.* (1982) report a single, discrete pI for hemolymph JH esterase activity. Rudnicka, M. and Kochman, M. (unpublished) report a 2360-fold purification of JH esterase of *G. mellonella* which hydrolyzed JH I at a rate of 920 nmol/min-mg. The analytical conditions used may emphasize the acylation rather than the slower deacylation step.

Some progress has been made on the purification of JH esterases from several insects. When hemolymph from *Blaberus giganteus* was chromatographed on Sephadex G-150, three major peaks of JH esterase activity were found using JH I or III as substrate, while hydrolysis of ethyl or isopropyl esters was minimal (Hammock, B. *et al.*, 1977b). Chromatography of the hemolymph of *Locusta migratoria* on Sepharose 6-B led to a major peak of activity ($> 1.5 \times 10^5$ daltons) and a minor peak (6.7×10^4 daltons) (Peter, M. *et al.*, 1979). Klages, G. and Emmerich, H. (1979b) reported a subunit molecular weight of 35,000 from *Drosophila hydei*, while subsequent work indicates that huge enzyme aggregates are formed (Bisser, A. and Emmerich, H., 1981).

Although a great deal of information can be obtained from physiological studies with JH esterase, homogeneous enzymes are ultimately needed for many studies. The methods described which lead to homogeneous enzymes from *M. sexta* and *T. ni* would be considered adequate for most vertebrate work. However, considering the very limited insect material available, these preparations must be heroic if one hopes to have reasonable quantities of enzyme for subsequent biochemical work. Hopefully, more sensitive and high-yield biochemical techniques for protein purification and characterization will facilitate research in this area.

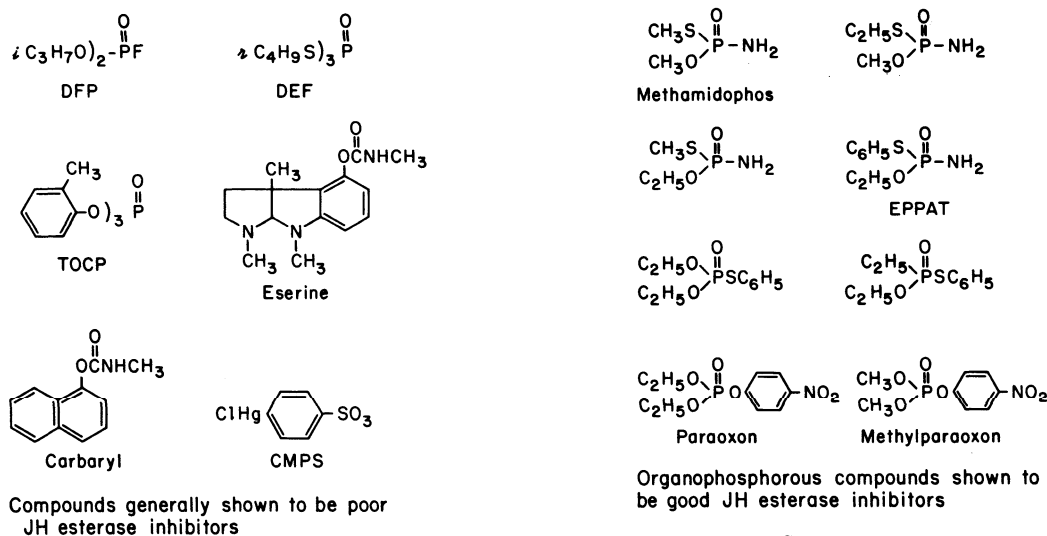
2.5 JH esterase inhibition

The first groups to investigate JH ester hydrolysis

assumed that the reaction was mediated by typical carboxylesterases. In fact, Whitmore, D. *et al.* (1972) reported that the α -naphthyl acetate esterase activity, which probably co-migrated with JH esterase activity when the hemolymph of *Hyalophora gloveri* was electrophoresed, could be inhibited by *O,O*-di-isopropyl phosphorofluoridate (DFP). Kramer, S. *et al.* (1974) first demonstrated that JH esterases from *Manduca sexta* are not typical esterases, by his failure to inhibit them with DFP. Only by using massive concentrations of DFP and extended incubation times can the JH esterase of *M. sexta* be inhibited (Hammock, B. *et al.*, 1975b; Sanburg, L. *et al.*, 1975a). Pratt, G. (1975) found that paraoxon (diethyl-4-nitrophenyl phosphate, $I_{50} = 1.7 \times 10^{-7}$ M) and dichlorvos (O-2,2-dichlorovinyl-O,O-dimethyl phosphate, $I_{50} = 6 \times 10^{-6}$ M) were good inhibitors of the hemolymph JH esterase from *Schistocerca gregaria*, while DEF (*S,S,S*-tributyl phosphorotrithioate) was a notably poor inhibitor. Di-isopropyl paraoxon was used by Hammock, B. (1975) to inhibit JH esterase activity in corpus allatum homogenates from *B. giganteus*. Hammock, B. *et al.* (1977b) screened 42 potential inhibitors on three partially purified JH esterase fractions from *B. giganteus* hemolymph. In some, but not all, cases the JH esterase and α -naphthyl acetate esterases could be differentially inhibited. As shown below, several very potent inhibitors of JH esterase activity were found, including the phosphoramidothiolates and 5-phenylphosphates with I_{50} values ranging from 10^{-7} to 10^{-10} molar. Once again DEF, and all carbamates tested, proved to be very poor inhibitors. Phenylmethylsulfonyl-fluoride (PMSF) was a marginal inhibitor ($I_{50} \cong 10^{-6}$ M).

Peter, M. *et al.* (1979) report a very interesting situation in the hemolymph of *L. migratoria*. Although the JH esterases are in want of further purification, they do seem to hydrolyze α -naphthyl acetate while showing a high specificity for both JH homologs and enantiomers about the epoxide moiety. This high specificity could qualify them as JH specific esterases, although they are susceptible to inhibition with DFP.

The dipterous (and possibly hymenopterous) insects examined appear to be quite different from other insects in regard to their JH esterases. In an extensive study of esterase action on malathion, α -



SCHEME H

SCHEME G

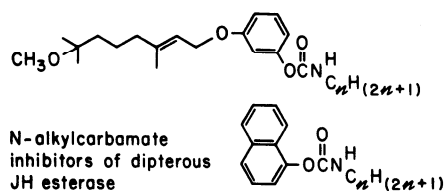
naphthyl acetate, JH, methoprene, and hydroprene in *Culex pipiens pipiens*, Hooper, G. (1976) concluded that JH was hydrolyzed by different enzymes than those hydrolyzing malathion and α -naphthyl acetate. JH was rapidly metabolized by these mosquito homogenates with JH acid as the major metabolite, and the esterase was again surprisingly resistant to tri-orthocresol phosphate (TOCP), *p*-chloromercuriphenyl sulfonic acid (CMPS) and several other classical esterase inhibitors. High concentrations of triphenyl phosphate and paraoxon were needed for significant inhibition of the activity. In *Musca domestica* JH hydrolyzing enzymes were

found both in the cytosolic and microsomal fractions of whole-body homogenates. Yu, S. and Terriere, L. (1978a) successfully used paraoxon to inhibit microsomal esterase activity, while Mumby, S. *et al.* (1979) found that an *N*-ethyl carbamate of a geraniol-based juvenoid was a potent inhibitor of both the microsomal and cytosolic forms of the esterase. However, the *N*-ethyl, propyl and butyl carbamates of 1-naphthol were also excellent inhibitors and are simple to synthesize and purify. The *N*-propyl carbamate appears to be most useful in inhibiting both forms of the enzyme, while the *N*-ethyl was most potent in the microsomal form. Sparks, T. and Hammock, B. (1980) were further

Table 4: Apparent K_m values of hemolymph JH esterases from several species

Species	Substrate	$K_m (\times 10^{-7} \text{ M})$	References
<i>Diatraea saccharalis</i>	JH I	11	Roe, R. <i>et al.</i> (1982)
	JH III	40	Roe, R. <i>et al.</i> (1982)
<i>Galleria mellonella</i>	JH I	18	McCaleb, D. <i>et al.</i> (1980)
	JH III	1.7	Wing, K. <i>et al.</i> (1982)
<i>Heliothis virescens</i>	JH III	3.1	Wing, K. <i>et al.</i> (1982)
<i>Manduca sexta</i>	JH I	8.7 (5.0)*	Peter, M. (1981)
	JH III	14	Vince, R. and Gilbert, L. (1977)
		9.3 (1.4)*	Peter, M. (1981)
<i>Spodoptera exigua</i>	JH III	1.0	Wing, K. <i>et al.</i> (1982)
<i>Trichoplusia ni</i>	JH I	3.2	Wing, K. <i>et al.</i> (1982)
		11	Yuhas, D. <i>et al.</i> (1982)
	JH III	2.0	Hammock, B. <i>et al.</i> (1982)
		1.2	Wing, K. <i>et al.</i> (1982)
		4.0	Yuhas, D. <i>et al.</i> (1983)

*Pre-wandering JH esterase peak (post-wandering).



SCHEME I

able to distinguish the two forms by inhibiting the microsomal form with CMPS and EPPAT (O-ethyl S-phenyl phosphoramidothiolate).

As in all other insects so far examined, the JH esterase activity in the hemolymph of the beetle *Leptinotarsa decemlineata* is resistant to DFP, but it is surprisingly susceptible to inhibition by the 4-*t*-octylphenyl ethoxylate, Triton X-100 (Kramer, S. and de Kort, C., 1976b; Kramer, S. *et al.*, 1977). In *Tenebrio molitor* Triton X-100 also selectively inhibits hemolymph JH esterase, while DFP and paraoxon are poor inhibitors (McCaleb, D. *et al.*, 1980). EPPAT proved to be a useful inhibitor of both microsomal and cytosolic JH esterase from *T. molitor* (Sparks, T. and Hammock, B., 1980). Mane, S. and Rembold, H. (1977) report that high levels of Triton X-100 will also inhibit JH esterase activity in *Apis mellifera*.

Most research has centered on the Lepidoptera. Whitmore, D. *et al.* (1972) used DFP, CMPS, and eserine in their study of JH esterases induced with JH in *Hyalophora gloveri* pupae. DFP caused major, but not total, inhibition of the enzyme stained in the gel with α -naphthyl acetate. This observation raises the possibility that the JH esterase was partially co-electrophoresing with other enzymes. Sixteen potential esterase inhibitors were screened by Ajami, A. (1975) on hemolymph from pupal *M. sexta*. If the hemolymph is free of fat body, JH esterase activity is very low in the pupa. Thus, it is possible that these data refer to inhibition of general esterase activity on JH. PMSF, Abate and Dursban were shown to stabilize radioactive JH in the hemolymph *in vitro*, as well as possibly synergizing hormonal action *in vivo*.

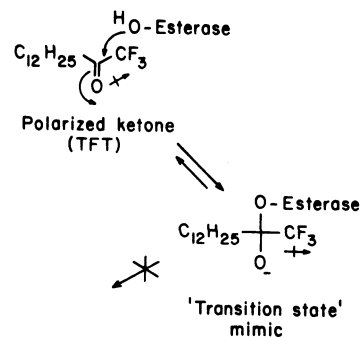
As discussed earlier, the discovery by Kramer, S. *et al.* (1974) that JH esterases from *M. sexta* were surprisingly resistant to inhibition by DFP was a major contribution to the study of JH metabolism. DFP has subsequently been used, and occasionally

misused, in many studies. The information from Mitsui, T. *et al.* (1979) that JH is metabolized by both DFP-susceptible and -resistant esterases in *M. sexta* epidermis is possibly the clearest indication of general vs. specific JH esterase action. Sláma, K. and Jarolím, V. (1980) found that DFP was a potent inhibitor of the hydrolysis of 4-methylumbelliferyl esters of JH III from *Galleria mellonella*, and several other insects. However, the lipase-like enzymes which hydrolyzed oleic acid esters were rather insensitive to this inhibitor. The JH esterases of some insects, although not necessarily the ones studied, will not hydrolyze esters of large alcohols such as methylumbelliferol. However, the resistance of some lipases to DFP inhibition should lead to caution in the use of DFP to distinguish JH esterases. Slade, M. and Wilkinson, C. (1973 and unpublished) took a similar approach and found that some inhibitors of epoxide hydration also inhibited esterase activity in *Spodoptera eridania*. Sparks, T. and Hammock, B. (1979a, 1980 and unpublished) surveyed a large number of esterase inhibitors. Some useful trends emerge from the 30 compounds reported. A variety of "typical" esterase inhibitors including TOCP, DEF, DFP, eserine and several other carbamates, PMSF, CMPS, and EPTA failed to inhibit JH esterase. EPPAT and several phosphoramidothiolates appeared to be good inhibitors, while paraoxon and dichlorvos are more commonly available and useful inhibitors. EPPAT and several paraoxon homologs proved to be excellent inhibitors in *Manduca sexta* (Sparks, T. unpublished). It is somewhat difficult to interpret the data of Brown, J. *et al.* (1977) who studied the propensity of larval α -naphthyl acetate esterases to bind JH and several mimics. Possibly they were observing co-chromatography of the JH carrier with an esterase band on gel electrophoresis, or possibly the acyl enzyme of a conjugated ester is stable enough to isolate.

For *in vivo* studies selective inhibition of JH esterase is important for determining the role of the esterase and for experimental stabilization of JH. At most stages of the insect's life it is important that the inhibitor not be a potent inhibitor of acetylcholine esterase and thus highly toxic. EPPAT and 1,1,1-trifluorotetradecane-2-one (TFT) meet this criterion in *Trichoplusia ni*. When comparing the ratio of inhibition (I_{50}) of hemolymph JH esterase

and α -naphthyl acetate esterase for various inhibitors, it was found that DFP had a ratio of $>25,000$, carbaryl >14 , EPPAT 10 and TFT 0.001, indicating that selective inhibition was certainly possible. The ideal JH esterase inhibitor for physiological studies should have minimal toxicity to insect and experimenter, show high selectivity for JH esterase inhibition over other esterases, penetrate rapidly, have a useful persistence *in vivo*, and allow almost total inhibition in an essentially irreversible manner. EPPAT approaches this ideal for *G. mellonella*, *M. sexta*, *T. ni*, and some other insects in that it irreversibly phosphorylates JH esterase (below). Hopefully better probes can be found. It should be noted that the inhibition patterns and potencies of EPPAT, TFT and related compounds vary with the species examined, and they are by no means specific JH esterase inhibitors.

Towards this end a variety of polarized ketones were synthesized by Hammock, B. *et al.* (1982). The most active inhibitor of *T. ni* JH esterase in this series was TFT, whose carbon chain length is identical to that of JH I and II. In contrast to irreversible inhibitors such as EPPAT, DFP and paraoxon, the polarized ketones are reversible inhibitors showing pure competitive inhibition kinetics. These polarized ketones are excellent examples of a transition state analog inhibitor. Note how the polarized ketone (below) mimics the tetrahedral intermediate on the reaction coordinate leading to the JH-acyl enzyme (Scheme C, section 1.2). Pauling, L. (1946, 1948) calculated that the affinity of an enzyme for a transition state of a reaction must be much higher than for either the substrate or the product of that reaction. Transition state theory has become a central philosophy in explaining enzyme

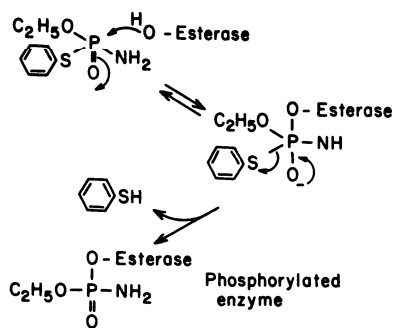


SCHEME K

action, and this theory may be very useful in the design of new enzyme inhibitors. Many inhibitors have been designed to mimic substrates, but even classical insecticides such as paraoxon act because they mimic the transition state (before phosphorylation) rather than the substrate. This effect can be seen above in the diagram showing inhibition of JH esterase by EPPAT.

Unfortunately, TFT has some limitations as a tool for studying JH esterase. In spite of its high selectivity and potency in *T. ni*, it is less active in *M. sexta* and some other insects. The slope of its inhibition curve is shallow, thus obtaining total inhibition of the esterase is difficult with TFT. Also, reversible inhibitors must be much more potent than irreversible inhibitors in order to obtain equivalent *in vivo* effects. However, the class of compounds promises to be very useful as NMR probes for the esterase active site, affinity ligands and as direct inhibitors if the potency can be improved.

Several research groups have used esterase inhibitors to evaluate the similarity of esterases from subcellular fractions, from developmental stages, from different tissues, from different species as well as esterases acting on different substrates. For instance, Mumby, S. *et al.* (1979) used a variety of carbamates to show that the esterase activity of the cytosolic and microsomal fractions of *Musca domestica* could be differentially inhibited. Although purification is ultimately needed to characterize an enzyme, a battery of esterase inhibitors were used in *T. ni* to test the similarity of JH esterase activities (Hammock, B. *et al.*, 1977b; Sparks, T. and Hammock, B. 1979b; Jones, G. *et al.*, 1981; Wing, K. *et al.*, 1981). Of course, similar patterns of inhibition do not prove that two enzymes are identical,



SCHEME J

but different patterns of inhibition serve to distinguish them without intense effort. G. Hooper's (1976) study in mosquitoes is an excellent example of the use of inhibitors to distinguish esterases acting on different substrates, as was the use of DFP in *M. sexta* to distinguish esterases acting on JH and α -naphthyl acetate (Kramer, S. *et al.*, 1974).

Inhibitors traditionally have been used to classify mammalian esterases into four major groups (Aldridge, W., 1953; Holmes, R. and Masters, C., 1967). Some general trends are evident from current knowledge of JH esterases. However, the application of mammalian classification systems should be cautious because insects are exceedingly diverse and all classification schemes used to date are clearly artificial. JH esterase activity does not depend upon the presence of divalent cations, as evidenced by the failure of the enzymes to be inhibited by EDTA. The sulfhydryl reagents tested are also remarkably poor inhibitors of JH esterases with the possible exception of some *M. domestica* esterases. However, the instability of partially purified *T. ni* esterase may indicate the presence of sulfhydryl groups necessary for activity. Although JH esterases are not inhibited by a variety of organophosphates widely used as carboxylesterase inhibitors, they are very susceptible to inhibition by some organophosphates. Eserine is a universally poor inhibitor of enzymes hydrolyzing JH ester, indicating that JH esterase and acetylcholine esterases can be differentially inhibited. Other carbamates tested were similarly poor inhibitors of JH esterase activities in all species tested except for the Diptera where some *N*-alkyl-carbamates proved to be good inhibitors. Thus, JH esterases can best be classified as carboxylesterases (E.C. 3.1.1.1). This classification is certainly realistic since their primary substrate is, in fact, a carboxylic acid ester, and hog liver carboxylesterase does hydrolyze JH. However, this classification must not be over-interpreted since numerous lines of evidence distinguish JH esterases from many other more typical carboxylesterases.

JH esterase inhibitors have found utility in a variety of biochemical and biological studies. For example, Yu, S. and Terriere, L. (1978b) successfully blocked ester hydrolysis of JH in order to study epoxide hydration, and such inhibitors have also been used to investigate JH biosynthesis. Inhibition of JH metabolism may in fact prove crucial in JH

receptor work and in other investigations of JH action (Chang, E. and Emmerich, H., personal communication). In all of these studies it is, of course, necessary to run controls to ensure that the inhibitors used do not influence the biochemical parameter under investigation. To this end it may be useful to test a variety of inhibitors.

A critical physiological application of JH esterase inhibitors is to explore the *in vivo* role of JH ester hydrolysis. For instance, Kramer, S. *et al.* (1977) was able to stabilize radioactive JH *in vivo* with Triton X-100 in *Leptinotarsa decemlineata*, and thus demonstrate a role for the esterase. Sparks, T. and Hammock, B. (1980) found that topical application of EPPAT to pre-wandering *Trichoplusia ni* led to a decrease in hemolymph JH esterase activity, an increase in the *in vivo* stability of radioactive JH and a delay in pupation (discussed further in section 3.3). Inhibition of the second last-instar JH esterase peak blocked pupation (Fig. 4). Although the data on *L. decemlineata*, *T. ni*, and recently *Manduca sexta* (Safranek, L. and Williams, C. personal communication; Sparks, T., unpublished) support the concept that in some insects JH esterase has an *in vivo* role, the inhibitors used are still not as specific as one might hope.

JH esterase inhibitors are exceedingly valuable probes, but for practical insect control their utility is likely to be very limited, in spite of early hopes as illustrated by the work of Solomon, K. and Metcalf, R. (1974) and Ajami, A. (1975). The inhibitors would share the limitations of juvenoids in prolonging the destructive instars of many pests and only acting at specific periods in development. As discussed below (section 3) degradative metabolism may not be crucial in JH regulation in some insects, and in others regulation may involve both biosynthesis and degradation of JH. In *T. ni* JH esterase appears to be in such large excess that the majority of the enzyme must be inhibited before *in vivo* changes are seen. Thus, even potent inhibitors could delay without totally blocking metamorphosis. However, the JH esterase still may be a useful target for biochemical and molecular approaches to insect control. If the enzyme can be caused to appear precociously in the hemolymph, metamorphosis may be accelerated or reproduction inhibited. Antagonistic effects could be obtained by blocking the production or action of the enzyme.

2.6 JH esterase interaction with carrier molecules

Following the early work on JH metabolism, it seemed reasonable that JH would need to have some protection if it were to circulate effectively in those insects which rapidly metabolize the hormone (Slade, M. and Zibitt, C. 1971, 1972; Weirich, G. *et al.*, 1973; see Goodman and Chang, this volume). Although the JHs are water-soluble at physiological concentrations (Kramer, S. *et al.*, 1974), JH will behave as a surfactant. For instance, several independent methods indicate a critical micelle concentration for JH of approximately 10^{-5} M (Kramer, S. *et al.*, 1974, 1976a,b; Mayer, R. and Burke, M. 1976; Hammock, B. *et al.*, 1977b). It is known that such surfactants will accumulate at membrane cytosol interfaces and, in fact, may travel through the cell on membrane surfaces. Akamatsu, Y. *et al.* (1975) thus pointed out that JH carriers may be important in preventing such a surface excess. While the moderate water-solubility of JH is surprising, its lipophilicity is not. The octanol water partition coefficient for JH I of 3.7 certainly explains its tendency to be rapidly absorbed into any lipophilic sink (Hammock, B. *et al.*, 1975b; Nowock, J. *et al.*, 1976; Mumby, S. and Hammock, B. 1979). Thus, for rapid circulation in an aqueous compartment, a JH carrier molecule offers several advantages, as further discussed below. The biochemistry and physiology of JH carriers has been recently reviewed by several authors (Gilbert, L. *et al.*, 1978; Kramer, S. and Law, J. 1980; Hammock, B. and Quistad, G. 1981), most notably in this volume (see Goodman, W. and Chang, E. this vol.). Thus, this section will largely be restricted to the interaction of carrier molecules with JH esterases.

Outside the order Lepidoptera, there are few reports of high-affinity hemolymph carrier proteins. In many of the insects so far examined, high molecular weight lipoproteins, or proteins with hydrophobic sites, may assume the additional role as JH carrier. Although these proteins have a high capacity, the limited number of studies to date indicate that they have low affinity. No data are yet available, but one might also predict that mass transfer will be slower from some of these proteins than the lepidopteran JH carrier molecules. Thus, JH may not reach equilibrium as quickly with the

aqueous environment. It is still questionable if these proteins make a major contribution to JH dynamics due to the low affinity of the proteins for the hormone and the low molarity of JH in circulation. In work with *Leptinotarsa decemlineata* Kramer, S. and de Kort, C. (1978) ultimately demonstrated a minimal role of hemolymph lipoproteins in protecting JH from ester hydrolysis. However, the high levels of lipoproteins required led these workers to question the biological significance of this protection. Sams, G. *et al.* (1978) report that lipoproteins from *Periplaneta americana* hemolymph retard JH metabolism in short-term organ culture. Clearly more work is needed on JH transport in insects lacking high-affinity carriers and on the relative roles of high- and low-affinity carriers in insects with both forms (Trautmann, K. 1972; Emmerich, H. and Hartmann, R. 1973; Emmerich, H. 1976; Kramer, S. *et al.*, 1976a,b; Kramer, S. and de Kort, C. 1976b, 1978; Bassi, S. *et al.*, 1977; Hammock, B. *et al.*, 1977b; de Kort, C. *et al.*, 1977).

R. Hartmann, in 1978, reported an exciting discovery that the hemolymph of the grasshopper *Gomphocerus rufus* contains a glycolipoprotein (molecular weight 220,000) which shows a very high affinity for JH ($K_d = 5 \times 10^{-8}$ M). The protein is apparently produced, at least in part, by the fat body throughout the life of male and female, normal and allatectomized insects. Hartmann, R. (1978) argued that a major role of the carrier was to distribute apparently large amounts of JH from the corpora allata through the insect. Excellent evidence for a physiological role for the protein was provided when the injection of anti-carrier antibodies blocked JH-dependent reproduction in adult females. Peter, M. *et al.* were probably examining a similar protein from the hemolymph of *Locusta migratoria* in their 1979 study. The high-affinity carrier in this case binds JH III more tightly than JH I and the natural enantiomer of JH III more tightly than the unnatural. By first examining the specificity of the hemolymph esterase and then the enantiomeric selectivity of JH metabolism in crude hemolymph, a clear protective role for the carrier was demonstrated. The esterase activity in the hemolymph of *L. migratoria* was much lower than that described in the hemolymph of many Lepidoptera, but it was sufficient for metabolizing unbound hormone. As discussed for *Trichoplusia ni*,

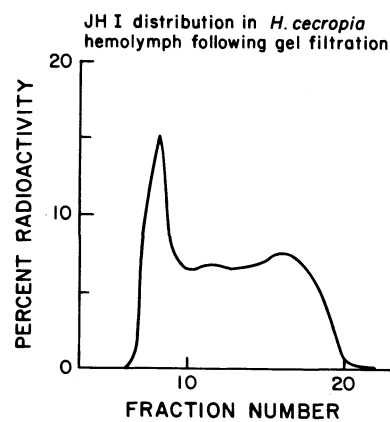
the carrier protein and esterase may function in concert in *L. migratoria* to increase the proportion of circulating JH III should other homologs be produced. Both groups also found high-capacity, low-affinity JH binding proteins of questionable significance in JH circulation.

Carrier proteins in the Lepidoptera have been the subject of a much more intensive investigation. Whitmore, E. and Gilbert, L. (1972, 1974) found that in six lipoproteins from the hemolymph of the saturniids *Hyalophora cecropia*, *Hyalophora gloveri* and *Antheraea polyphemus* will bind JH *in vitro* but that only two will bind JH *in vivo*. The tremendous capacity of these lipoproteins tends to overshadow the presence of a high-affinity carrier similar to that found in other Lepidoptera. The distribution of 10^{-8} M radioactive JH I in undiluted *H. cecropia* pupal hemolymph run on a Sephadex G-100 column is shown below. These data indicate quite clearly the binding of JH to high molecular weight lipoproteins. Rechromatography of the intermediate molecular weight fractions yields a pattern somewhat similar to that seen for *M. sexta* and numerous other Lepidoptera in which a protein of low molecular weight is critical in JH binding (Hammock, B. and Gilbert, L., unpublished).

Certainly in the majority of lepidopterous species examined, the low molecular weight carrier plays the major role in JH distribution in spite of the tremendous capacity of the lipoproteins (Kramer, S. *et al.*, 1974). As shown in Fig. 2, when last-stadium day 4 hemolymph esterase activity is inhibited and the undiluted *Manduca sexta* hemolymph then incubated with varying concentrations of JH I, quantitative binding of JH to very high molecular weight proteins only occurs at unphysiologically high concentrations of JH. In general, such gel permeation columns have been run following equilibration of JII with dilute hemolymph. Such experiments emphasize the equilibrium between the JH carrier and free hormone. The data shown in Fig. 2 emphasize that *in vivo* an important equilibrium is between the JH carrier and lipophilic depots including hemolymph lipoproteins. Even at very low JH concentrations (not shown), a surprisingly large proportion of JH is bound to very high molecular weight proteins. A variety of juvenoids including R 20458, methoprene, hydrophene and Ro 8-4374 bind largely to the

lipoprotein fraction under these conditions, but some binding to the JH specific carrier is observed. Such juvenoid binding to the carrier molecule is deemphasized by competitive binding experiments (Hammock, B. and Gilbert, L., unpublished). Thus, these lipoproteins probably have a less significant role than the JH carrier protein in JH distribution and turnover in the Lepidoptera. However, their role must not be totally discounted with either JH or juvenoids.

The hemolymph JH carrier has been most extensively studied in *M. sexta*. The carrier has an acidic pI, a molecular weight of 28,000, and a single JH binding site with a K_d of 3×10^{-7} M for JH I. The carrier molecule shows a greater affinity for JH I and 0 than for II or III, as well as ability to recognize the appropriate geometrical and optical isomers of the hormone. Its specificity is further indicated by its very low affinity for JH metabolites and for a variety of biologically active juvenoids. The fat body appears to be a major site of synthesis of this protein which is then released into the hemolymph (Kramer, S. *et al.*, 1974, 1976a,b; Akamatsu, Y. *et al.*, 1975; Sanburg, L. *et al.*, 1975b; Nowock, J. *et al.*, 1975, 1976; Nowock, J. and Gilbert, L. 1976; Goodman, W. *et al.*, 1976, 1978a,b; Goodman, W. and Gilbert, L. 1978; Peterson, R. *et al.*, 1977; Law, J. 1978; Gilbert, L. *et al.*, 1978; Schooley, D. *et al.*, 1978). A similar situation has now been observed in a variety of lepidopterous species. Although the concentrations and apparent equilibrium dissociation constants of the JH carriers from different species vary, several workers have calculated that the majority of JH I or II at physiological concentrations will be bound.



SCHEME L

Goodman, W. and Gilbert, L. (1978) reported that the carrier protein undergoes only slight fluctuations correlated with hemolymph protein concentration during the penultimate larval stadium of *M. sexta*. In *T. ni*, as in *M. sexta*, a single protein appears responsible for high-affinity JH binding in the hemolymph. The same number of binding sites occur whether JH I or III is employed as ligand. However, JH I is bound about 10-fold more tightly. JH I binding activity fluctuates only slightly during the last larval stadium of *T. ni*, in contrast to the dramatic changes in JH esterase activity. Since the binding activity is highest in mid-instar, the hypothesis that a disappearance of binding protein leads to a release of free JH into the hemolymph is not supported (Hammock, B. *et al.*, 1977a; Sparks, T. *et al.*, 1979a; Sparks, T. and Hammock, B. 1979b; Wing, K. *et al.*, 1981, 1982). No information is yet available on JH carrier regulation. In spite of the minor fluctuations in titer so far observed, such a study of regulation could be very informative.

The concept that hemolymph JH carriers play a multitude of roles in influencing JH metabolism and distribution has evolved from several laboratories (Table 5) (Kramer, S. *et al.*, 1974, 1976a,b; Ferkovich, S. *et al.*, 1975, 1977; Hammock, B. *et al.*, 1975b; Sanburg, L. *et al.*, 1975a,b; Ferkovich, S. and Rudder, R. 1976; Kramer, S. and Childs, C. 1977; Goodman, W. and Gilbert, L. 1978; Goodman, W. *et al.*, 1976, 1978a; Gilbert, L. *et al.*, 1976, 1978; Sparks, T. and Hammock, B. 1979a; Kramer, S. and Law, J. 1980; Hammock, B. and Quistad, G. 1981; Wing, K. *et al.*, 1981, 1982). The qualitative significance of the protection of JH from general esterases has not been established. However, such a protective role clearly exists. For instance, in *T. ni* JH I is metabolized slightly faster than JH III by the last-instar JH esterase, but in concentrated penultimate larval instar hemolymph JH III (which is weakly bound by the carrier molecule) is hydrolyzed much more rapidly than JH I. The approach of Peter, M. (1981) promises also to yield useful information along this line.

It is likely that the JH carrier plays a major role in effecting rapid distribution of the hormone. The apparent K_d measured for numerous species appears sufficient to account for binding most of the JH present in the hemolymph. Measurements of k_a and k_d (association and dissociation rates) have

Table 5: Possible roles of JH carrier proteins

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| (1) | Protection from general esterases (and possibly other enzymes). |
| (2) | Maintenance of JH in solution rather than in lipophilic depots or at membrane aqueous interfaces to effect rapid and equal distribution of JH throughout the larva. |
| (3) | Extraction of JH from lipophilic depots by mass action. |
| (4) | Interaction with JH esterases to effect rapid degradation. |
| (5) | Vary ratio of JH homologs by preferential binding and protection from metabolism. |
| (6) | Modulation of JH action on target tissues. |
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been less successful but appear very rapid (Goodman, W., personal communication; Peterson, R. and Law, J., unpublished; Peterson, R. 1977; Wing, K. *et al.*, 1982). The k_a is sufficiently high to ensure almost immediate binding when JH is released from the corpora allata. Thus, changes in rates of JH synthesis are rapidly reflected in changes in circulating hormone titers. In contrast to cells and even vesicles which very slowly release lipophilic molecules to an aqueous environment, the k_d of the JH carrier is also very fast. For serine esterases K_m is often proportional to substrate affinity for the enzyme. Under assay conditions where V_{max} is approached, the rate-limiting step in ester hydrolysis is deacylation of the enzyme. However, under physiological conditions with very low JH concentrations, the acylation of JH esterases will be the rate-limiting step in the removal of JH from circulation. The V_{max} of this step is higher, and the K_m lower, than that of the overall reaction as estimated by classical Lineweaver-Burk methods. Many studies only employed substrate concentrations above the K_m . Thus, by mass action JH can be transferred rapidly and irreversibly from hemolymph carrier molecules to JH esterases without the need to envision direct interaction of the esterase and the carrier. Thus, the JH carrier serves to protect and distribute JH in the absence of JH esterases and serves to affect rapid hydrolysis in the presence of these esterases. Without the carrier either maintaining JH in solution or extracting JH from lipophilic depots by mass action, JH catabolism would be much slower. As more is learned of the distribution of JH carriers, it may be possible to correlate their presence with lower JH synthetic rates and with insects or insect stages in which rapid, precise changes in circulating JH titers are critical for development. As with JH esterases, it will be important to somehow block JH binding if

the physiological roles of the carrier molecules are to be established.

3 REGULATION OF JH DEGRADATION

Most models for endocrine regulation in the vertebrates are based upon the assumption that degradation and elimination of hormones remain rather constant and that circulating hormone titer is determined largely by biosynthesis. Similar arguments have been made for JH regulation in insects. However, evidence is building in at least three orders that changes in the rates of JH degradation, as well as changes in biosynthetic rates, are crucial in regulating circulating as well as tissue hormone titers. In some Lepidoptera, for instance, the insect seems to turn down but not turn off JH biosynthesis. Thus, an increase in JH metabolism is necessary for reducing JH titers to a level which allows prothoracicotropic hormone (PTTH) release and the initiation of metamorphosis (see Steel and Davey, vol. 8). Thus, the factor(s) which causes the production of JH esterases may initiate the cascade of endocrine events leading to pupation. For proper maturation and reproduction in several insects it also appears that JH metabolism must be precisely turned off. Thus, it is likely that regulation of JH degradation will be an important area of investigation in the future.

3.1 Regulation in the Orthoptera

Retnakaran, A. and Joly, P. (1976) found that cautery of the A and B neurosecretory cells of *Locusta migratoria* led to a reduction in hemolymph JH esterase activity. Further work on regulation of JH metabolism in the Orthoptera was not pursued until very recently. Sparks, T. and Hammock, B. (unpublished) found increases in JH esterase activity during the last stadium of *Blaberus giganteus*. This work was halted due to difficulties encountered in timing experimental animals. Using well-timed animals, Szibbo, C. *et al.* (1982) found that the hydrolysis of JH by hemolymph enzymes increased dramatically during the last half of the last stadium of *Diploptera punctata*. This increase in JH esterase activity correlated well with a reduction in *in vitro* JH biosynthesis. JH esterase activity was high at

adult emergence and then declined in mated females during the time that JH appears to stimulate vitellogenesis. A subsequent sharp rise in JH esterase activity was then observed and maintained during pregnancy. The low titers of JH esterase observed in allatectomized females were restored by juvenoid application. Whether this induction occurs directly or through another hormone is not yet known (Rotin, D. *et al.*, 1982). Thus, the hemolymph JH esterase activities correlate well with suspected declines in JH titers, as well as with declines in JH biosynthesis in *D. punctata*.

3.2 Regulation in the Coleoptera

The regulation of JH degradation in larval Coleoptera has not been well studied, possibly because the insects are hard to stage. However, as discussed earlier, high JH esterase and epoxide hydrolase activities occur in the late larvae and pupae of *Leptinotarsa decemlineata* and *Tenebrio molitor* (Kramer, S. and de Kort, C. 1976a; Sparks, T. and Hammock, B. 1980) which suggests that JH catabolism in these larvae may play an analogous role to that observed in the Lepidoptera.

Regulation of JH metabolism has been studied in more detail in adult *L. decemlineata*. Although polar conjugates were the major *in vivo* metabolites, tissue levels of JH esterase and epoxide hydrolase were very low, allowing the workers to concentrate on the hemolymph enzyme. Hemolymph JH esterase activity increases in both long and short photophase adults, but the levels rise much higher in the short-day animals which will enter diapause soon after adult emergence. The increases in JH esterase activity correlate with drops in *in vivo* JH titer in both long- and short-day animals. Since low JH titers are needed for short-day *L. decemlineata* to enter diapause, the appearance of very high hemolymph JH esterase activity suggests a biological role. However, JH titers are dramatically reduced before peak esterase activity is detected. This information led de Kort, C. and Granger, N. (1981) to conclude that JH esterase is not a primary factor in reducing JH titer. However, studies of the rates of *in vivo* biosynthesis indicate that a decrease in biosynthesis rather than a cessation of biosynthesis correlates with decreases in *in vivo* JH titers. Thus, the data of Kramer, S. (1978b) suggest that a combination of

degradation and decreased synthesis account for the observed reduction in JH titer. Possibly the use of selective esterase inhibitors or pharmacokinetic calculations will allow the role of hemolymph esterases to be accurately assessed (de Wilde, J. *et al.*, 1968; Kramer, S. and de Kort, C. 1976a,b, 1978; Kramer, S. *et al.*, 1977; Kramer, S. 1978a,b).

Kramer, S. (1978a) further reports that JH treatment rapidly increases JH esterase activity in diapausing beetles. Several experiments indicate that this effect is not direct. For instance, when short-day beetles were transferred to a long-day photoperiod, the expected rise in JH esterase did not occur, and its rise could also be blocked by ligation even with JH application. The rise in hemolymph JH esterase in short-day beetles also could be blocked by JH application shortly after emergence, possibly suggesting that the hemolymph JH titers had to be reduced before the beetle could reach the stage necessary for the high JH esterase titers needed for complete clearance of JH from the hemolymph. Such a cascade effect where one endocrine change signals another could be an effective mechanism for timing such events. Based on his studies Kramer, S. (1978a) concluded that a complex of neuroendocrine events control hemolymph JH esterase activity in the adult. A model in which JH esterase activity is induced by a head factor (other than JH) in response to a circulating titer of JH seems to fit the data.

3.3 Regulation in the Lepidoptera

The regulation of JH metabolism is best understood in the Lepidoptera, and this regulation deals largely with fat body and hemolymph JH esterases. As indicated earlier in *Manduca sexta* and *Trichoplusia ni*, epoxide hydration and conjugation seem unlikely to be rate-limiting steps of JH degradation. The midgut JH esterase activity and the fat body and midgut epoxide hydrolase activities are approximately an order of magnitude lower than the peak fat body and hemolymph JH esterase activities. As discussed earlier, the changes in hemolymph JH binding activities are not dramatic. Models of JH dynamics may ultimately show these changes to be significant, but the crude titers in *T. ni* suggest the JH carrier has as much a role in clearance as in preservation of the hormone. Al-

though many tissues are capable of rapid JH metabolism, the high specific activity of the JH esterases in hemolymph and fat body (and to a much lesser extent epidermis) undoubtedly account for the majority of JH hydrolysis. This is not to say that metabolism in a target tissue is unimportant in limiting JH action, but rather that this metabolism does little to alter total *in vivo* JH titers. The high rates of JH degradation in epidermis and imaginal disks in fact support the concept that metabolism may have a crucial microscopic role in the tissue concerned. At peak levels of JH hydrolytic activity a single enzyme appears responsible for the vast majority of JH hydrolysis. Using a number of biochemical techniques, no differences have been found among the JH esterases collected at several times during development or from the major tissues of *M. sexta* and *T. ni*. Only the JH esterase activity in the midgut appears to be due to different enzymes (Sparks, T. and Hammock, B. 1979a,b; Hammock, B. *et al.*, 1981; Wing, K. *et al.*, 1981).

In most of the lepidopterous larvae examined, two major bursts of hemolymph JH esterase activity are observed in the last larval instar (Fig. 3). One burst occurs early in the instar (e) supposedly correlated with a decline in circulating JH titers (b). Near this time the insect reaches a critical weight which can be used as an indication of commitment to pupation. When the JH titer reaches a sufficiently low level, PTTH is released which initiates a cascade of events leading to pupation. These subjects are discussed in greater detail elsewhere (Steel and Davey, vol. 8). This burst is referred to as the pre-wandering JH esterase peak. The second (prepupation) burst occurs late in the last stadium (f), presumably in response to a prepupal pulse of JH (c). In various organisms the relative activities of these two peaks of JH esterase activity vary from *Pectinophora gossypiella* and *Ephestia elutella* where the prepupal activity is larger than the pre-wandering activity, to *Spodoptera exigua* and *Trichoplusia ni* where peaks e and f are of similar specific activity, to *Manduca sexta* and *Pieris rapae* where the second peak (f) is relatively minor. In a few insects a second peak has not been detected (Weirich, G. *et al.*, 1973; Weirich, G. and Wren, J. 1976; Vince, R. and Gilbert, L. 1977; Reddy, G. *et al.*, 1979; Sparks, T. *et al.*, 1979a; Hammock, B. *et al.*, 1981; Jones, D. *et al.*, 1982; Ozyhar, A. *et al.*, 1982).

The fat body seems to be the major source of hemolymph JH esterase activity in both the pre-wandering (e vs. g) and the prepupation peak (f vs. h). Although the work of Whitmore, D. *et al.* (1972, 1974) has been criticized because α -naphthyl acetate was used to follow JH esterase activity; it is possible that the JH esterases of the saturniids studied hydrolyze both substrates. It is more likely that the JH esterase activity studied co-chromatographed with other esterases since DFP inhibition was incomplete. Regardless of these criticisms, the conclusion of Whitmore, D. *et al.* (1974) that JH esterase arises largely from the fat body has been supported by subsequent experiments. Hammock, B. *et al.* (1975b) noted that *M. sexta* fat bodies released JH esterase activity in culture, and Nowock, J. and Gilbert, L. (1976) found that the JH esterase activity in fat body homogenates or released by fat body *in vitro* correlated with hemolymph JH titers in pre-wandering *M. sexta* and similar results were obtained in *Galleria mellonella* by Reddy, G. *et al.* (1979). Wing, K., *et al.* (1981) monitored fat body midgut and hemolymph JH esterase activity during *T. ni* development. The activity in the fat body (g) correlated well with the pre-wandering burst of hemolymph JH esterase, and the rise in fat body JH esterase also correlated with the rise in hemolymph JH esterase leading to the post-wandering peak (h). However, JH esterase activity in the fat body remained high into the pupal stage, indicating that synthesis and release are under separate control if JH esterase is in fact produced by the fat body (i). Subsequent studies with *T. ni* indicate that release rates from fat body, and to a lesser extent the epidermis *in vitro*, correlate well with *G. mellonella* and *T. ni* hemolymph JH esterase levels (Reddy, G. *et al.*, 1979; Wing, K., 1981). Thus, the definitive experiments to demonstrate unequivocally the fat body as the source of hemolymph JH esterase have yet to be run, but all evidence to date points to the fat body as the major source of JH esterase.

Although the two peaks of hemolymph JH esterase activity are due to the same enzyme which seems to be produced by the fat body at two separate times during development, the regulation of each peak appears to be quite distinct. A head factor other than juvenile hormone appears necessary for the pre-wandering peak (e) (Fig. 4a). The pre-wandering level of JH esterase drops dramatic-

ally following head ligation in *G. mellonella*, *M. sexta* or *T. ni*. Reimplantation of the brain or subesophageal ganglion (or extracts thereof) will partially restore the JH esterase activity while JH and juvenoids have little effect in isolated abdomens of pre-wandering larvae. JH seems to speed the elimination of JH esterase from the hemolymph of debrained *G. mellonella* larvae. Application of JH to very early last-instar *G. mellonella* decreases hemolymph JH esterase activity. Since appearance of the first JH esterase peak (e) can be blocked by prior treatment with JH or juvenoids, the esterase is probably not released until the insect determines that it is in the last larval instar. In normal and black mutant pre-wandering *M. sexta* and *T. ni* larvae, application of JH causes a slight increase in JH esterase activity. Allatectomy at this stage of development leads to a decrease, but not an elimination, of JH esterase activity. However, these minor effects of JH are either mediated through the head or JH acts in concert with a head factor. Possibly at this stage JH acts in a positive feedback role through the head to fine tune its own metabolism (Fig. 4) (Reddy, G. *et al.*, 1979; Sparks, T. and Hammock, B. 1979a,b; Hammock, B. *et al.*, 1981; Jones, G. *et al.*, 1981; Wing, K. *et al.*, 1981; Hammock, B. and Riddiford, L., unpublished; Jones, G. unpublished).

Jones, G. *et al.* (1981) monitored this head factor in *T. ni* by implanting brains and/or subesophageal ganglia or injecting their homogenates into isolated abdomens and then monitoring hemolymph JH esterase activity. Two peaks of apparent JH esterase inducing factor activity were observed. One peak (j) correlates with a very minor burst of JH esterase activity that occurs at the larval/pupal molt (d); the second peak (k) correlates with the major pre-wandering burst of JH activity (e). Homogenates from still later times actually reduce the JH esterase activity in isolated abdomens (l), while the brain, subesophageal ganglion or homogenates thereof from late last-instar larvae (m) have no effect in inducing the prepupal peak of JH esterase activity (f). Thus, the JH esterase-inducing factor from the head is present during the major pre-wandering JH esterase peak (e), but it also occurs where there is a very minor JH esterase peak (d) and fails to occur where there is a post-wandering peak (f).

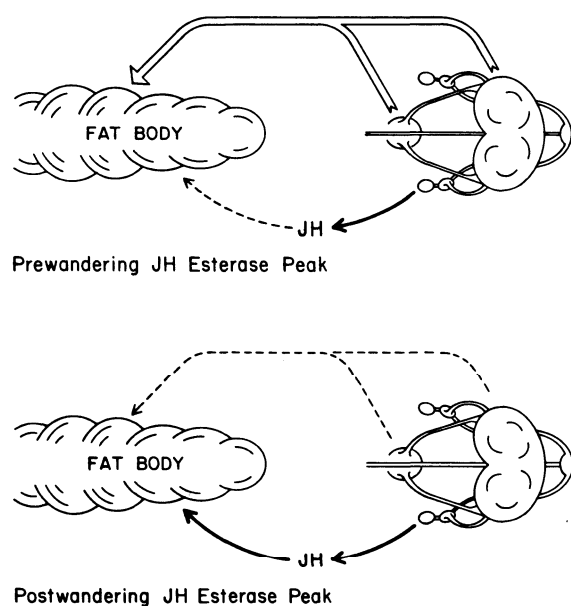


FIG. 4. Possible mechanism of regulation of the two major JH esterase peaks in *Manduca sexta* and *Trichoplusia ni*. Production of JH esterase leading to the pre-wandering peak appears to be stimulated by a water-soluble factor from the brain and/or subesophageal ganglion ($\longrightarrow$). JH will inhibit JH esterase production if applied early and slightly stimulate it if applied later, but it has no effect on isolated abdomens ($\dashrightarrow$). Production of the JH esterase comprising the second peak is directly stimulated by JH itself, even in isolated abdomens ($\longrightarrow$). The process which controls this change in tissue response to JH is as yet not understood. Thus, in some insects JH titers may be controlled at times by a decrease in biosynthesis coupled with an increase in degradation.

This apparent anomaly was explained by monitoring the sensitivity of the abdomen to inducing factor. The sensitivity of isolated abdomens is low in the penultimate and early last instar (n). Thus, the first burst of inducing factor (j) may act on an unresponsive abdomen to produce a minor amount of JH esterase (d) at the larval-larval molt (a). Possibly this event prepares the larva to respond with a major peak of hemolymph JH esterase (e) activity when the second burst of inducing factor occurs in the pre-wandering stage (k). During the mid instar, the fat body again becomes unresponsive (p). By the time of the second major peak of hemolymph JH esterase, the fat body is again competent to respond (q).

A great deal remains to be done with the inducing

factor. It is not known if it is one or several factors, its chemical nature is unknown, and its (or their) apparent presence in both the brain and the subesophageal ganglion (but not other nervous tissue) is disconcerting. This factor may have a number of roles in inducing adult development, and its role in changing the response of the fat body to JH is also unclear (Hammock, B. *et al.*, 1981; Jones, G. *et al.*, 1981).

Ozyhar, A. *et al.* (1981) state that they were unable to cause an increase in hemolymph JH esterase levels in *Galleria mellonella* isolated abdomens by brain implantation, although they did find increases in other esterases apparently involved in lipid metabolism. They were able to obtain increased JH esterase activity by dipping the larvae in 20-hydroxyecdysone. Similar results were obtained by Sparks, T. and Hammock, B. (1979b) who concluded that the esterase induction observed was due, in part, to fat body lysis. However, much lower ecdysteroid levels were active in *G. mellonella*. The role of ecdysone, either alone or in concert with other endocrine factors in JH esterase induction, needs re-examination.

The pre-wandering JH esterase activity is sensitive to the external environment of the insect. For instance McCaleb, D. and Kumaran, A. (1978, 1980) demonstrated in *G. mellonella* that chilling and injury affected JH esterase activity. Reddy, G. *et al.* (1979) demonstrated in the same insect that starvation reduced hemolymph JH esterase activity, and this work was then repeated in *Trichoplusia ni* and *Manduca sexta* (Fig. 5) (Sparks, T. and Hammock, B., 1979b; Hammock, B. *et al.*, 1981; Cymborowski, B., *et al.*, 1982; Sparks, T., Hammock, B. and Riddiford, L., unpublished). Further studies in *T. ni* indicate that the JH esterase titers are not influenced by the mere bulk of the food consumed, but rather the quality of the food. Protein appears to be the single most important nutritional factor in influencing JH catabolism in that the hemolymph JH esterase levels were directly proportional to the amount of casein added to a minimal diet. A variety of plant natural products which lengthened the feeding stage of the insect also decreased the JH esterase titer, indicating a relationship between plant antibiosis and insect endocrinology (Levinson, and Hammock, B., unpublished; Hammock, B. *et al.*, 1981).

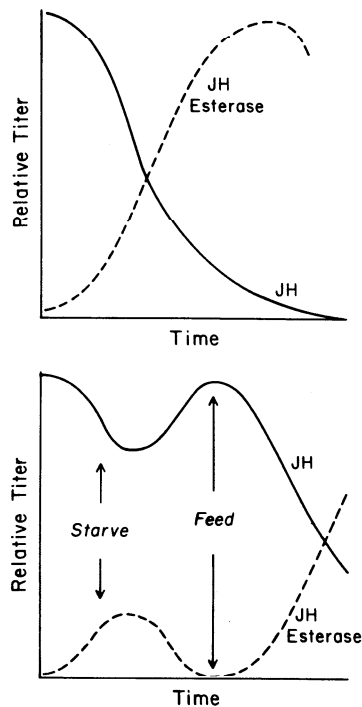


FIG. 5. Schematic representation of the effect of starvation and refeeding on hemolymph JH and JH esterase titers in the early last-larval instar of *Manduca sexta* and *Trichoplusia ni*. In normal larvae (top) JH titers drop as the insect approaches the wandering stage while JH esterase titers increase. If the larvae are starved or placed on an inadequate food supply, JH titers soon increase while JH esterase titers drop. This treatment may result in supernumerary molting or a lengthened prewandering stage. Subsequent refeeding may lead to normal development. Drawn from Cymborowski, B. *et al.*, 1982; Hammock, B. and Quistad, G. 1981; and Hammock, B. *et al.*, 1981.

The above information seems to explain the pre-wandering, but not the post-wandering, JH esterase peak. The clue to its regulation was found by Sparks, T. and Hammock, B. (1979b) who demonstrated that JH and some JH mimics could induce the appearance of JH esterases in post-wandering isolated abdomens of *T. ni* (Fig. 4b). In a series of subsequent studies this induction was shown to depend upon juvenoids or JH homologs having an ethyl branch at the epoxide carbon. The induction phenomenon occurred at JH doses which yielded physiologically reasonable levels of circulating JH. Of crucial significance were classical corpora allata extirpation-replacement experiments. Larvae allatectomized before the prepupation burst of JH occurred produced little JH esterase in both *M.*

sexta and *T. ni*, while allatectomy later in life had a smaller effect on reducing JH esterase titers. The allatectomized larvae retained their capacity to respond to topical JH application by producing JH esterase. Corpus allatum reimplantation or JH treatment led to normal JH esterase levels. The ability of JH mimics to induce JH esterase could be blocked by ETB (as could be predicted from its hormone antagonist role; see Bowers, vol. 8) as well as by the biosynthesis inhibitors *o*-bromophenoxy-methylimidazole and fluoromevalonate. Induction of JH esterase activity in prepupal fat body slightly preceded, then paralleled hemolymph induction *in vivo* and the post-wandering (but not the pre-wandering) fat body could be induced *in vitro*. The ability of the prepupation burst of JH (c) to induce its own hydrolysis has now been demonstrated in several moth and butterfly species (Sparks, T. and Hammock, B. 1979b; Sparks, T. *et al.*, 1979b; McCaleb, D. and Kumaran, A. 1980; Hammock, B. *et al.*, 1981; Hammock, B. and Quistad, G. 1981; Jones, G. *et al.*, 1981; Jones, D. *et al.*, 1982; Kumaran, A. *et al.*, 1981; Wing, K. *et al.*, 1981; Wing, K. 1981; Sparks, T., Hammock, B. and Riddiford, L., unpublished).

Whitmore, D. *et al.* (1972, 1974) and Reddy, G. *et al.* (1979) found that JH could induce JH esterase in saturniid and *G. mellonella* pupae. At this time fat body JH esterase levels are very high, but in saturniids the fact that transcription and translation appear necessary for release from the fat body supports the hypothesis that JH leads to synthesis of JH esterase. In *T. ni* pupae Wing, K. *et al.* (1981) demonstrated that more JH esterase is detectable in the pupa following JH treatment than can be accounted for by that previously present in the fat body. These workers also demonstrated that fat body lysis does not account for the apparent induction. Whether JH treatment and JH esterase-inducing factors lead to *de novo* synthesis, or simply activation of the enzyme, awaits further study. The roles of both the pre-wandering and the prepupal bursts of JH esterase are explained below, and possibly the JH esterase activity in the fat body remains high (i) to keep JH titers low during the early pupal stage. However, why hemolymph JH esterase levels fall dramatically in the early pupal stage, yet remain responsive to JH treatment, is unknown. The mechanism by which the fat body's production of

JH esterase shifts from being unresponsive to JH in the pre-wandering stage (e) to being responsive to JH in the post-wandering stage (f) similarly is unknown. This shift in sensitivity occurs simultaneously with wandering in *T. ni*. However, both normal and allatectomized *M. sexta* are not sensitive to JH induction of JH esterases until shortly before pupation.

Before one can invoke a biological rationale for the mechanisms by which insects regulate JH esterase, the role for these enzymes in insect development must be established. Nijhout, H. (1975) was justified in questioning the role of JH metabolism in hormone regulation. Simply correlating the appearance of an esterase which metabolizes JH with the theoretical disappearance of JH does not prove its role in regulation. The so-called JH esterase could be, for instance, an amidase which just happens to degrade JH, or the enzyme could be serving as a scavenger which is only important in case of a failure of corpora allata to halt biosynthesis. In many insects, as apparently with most vertebrate hormones, degradation may play a secondary role to biosynthesis and release in endocrine regulation.

In the Lepidoptera the properties of the JH esterases (discussed in section 2) provide strong circumstantial evidence for a role in JH regulation (Fig. 3). The best direct evidence for the role of JH esterase in JH titer regulation came from the application of the JH esterase inhibitor EPPAT to *T. ni* larvae. When JH esterase was inhibited in pre-wandering larvae (e), the feeding stage was extended and thus pupation was delayed. This effect could be duplicated by the application of JH or juvenoids. EPPAT was shown to inhibit JH esterases and thus slow JH metabolism *in vivo*. One can thus envision the JH titer not being sufficiently reduced to allow PTTH release and thus blocking the cascade of events leading to pupation. EPPAT and paraoxon could similarly inhibit the prepupation burst (f) of JH esterase activity in *T. ni*. Application of these compounds led to leakage of molting fluid, an inability to shed the larval cuticle, and some traits suggesting a larval-pupal intermediate. These results are difficult to rationalize with our current understanding of the pupation process. Like other lepidopterous larvae, *T. ni* were shown by ligation studies to be committed to pupation long before the application of the JH esterase inhibitors. However,

application of JH and juvenoids to the prepupa gave similar symptoms to those following inhibitor application, while treatment with the potent acetylcholine and α -naphthyl acetate esterase inhibitor DFP failed to block pupation. These studies with EPPAT do not prove unequivocally that JH esterase is crucial for the regulation of JH titer. However, they provide strong evidence that JH esterase is important in JH regulation and that the release of JH esterase may be the critical factor in initiating the decline in JH titer allowing PTTH release and ultimately pupation (Sparks, T. and Hammock, B. 1980).

With this role in mind it becomes easier to hypothesize reasons for regulatory mechanisms involved in JH degradation. Since high JH titers are needed to maintain normal development in the larval stage, it would be very uneconomical for JH to directly induce its own degradation until the insect is well into its last larval instar. In addition, there are definite advantages to having the esterase under neurosecretory control. As discussed earlier, JH esterase titers drop very quickly when *Galleria mellonella*, *Manduca sexta* or *Trichoplusia ni* are either starved or placed on an inadequate diet, while Cymborowski, B. *et al.* (1982) found that JH titers increase in starved insects (Fig. 5). Since a circulating titer of JH blocks PTTH release, the insect can control the initiation of pupation by regulating JH esterase and JH biosynthesis. This ability is likely to be critical to the insect's survival. Due to high mortality in the larval stage, there is a selective advantage for insects to reach the reproductive stage quickly. However, the insect must have sufficient dietary reserves to survive the pupal stage and efficiently reproduce. Thus, the ability to delay pupation by increasing the length of the feeding stage or inducing a supernumerary molt in response to inadequate food supply offers a distinct advantage in some insects such as *M. sexta* and *T. ni*. Neuroendocrine regulation of JH degradation, as well as biosynthesis, provides a mechanism to accomplish this delay in response to dietary and other factors. Such regulation in the future may be shown to be involved in a variety of developmental and behavioral responses of insects to environmental cues. However, a diversity of insect responses can be anticipated. For instance, when early-instar *G. mellonella* larvae are exposed to an inadequate diet, they may undergo precocious pupation.

In contrast to the regulation of events discussed above, the release of PTTH initiates a cascade of events leading inexorably to pupation. Once PTTH is released, the timing of the numerous events involved in the pupation sequence becomes too complex for any one of the events to be tied to environmental cues. Such is the case with JH esterase. No longer is the esterase titer controlled by neuroendocrine factors which can respond to environmental cues. Rather, in the post-wandering larvae the JH esterase is induced by the presence of JH itself.

Numerous workers have noted the presence of elevated JH titers in lepidopterous prepupae (c) (see Jones, D. *et al.*, 1982, for review), but what this JH does has remained a mystery. Kiguchi, K. and Riddiford, L. (1978) report that when *M. sexta* are allatectomized so that this prepupal burst of JH is eliminated, adultoid structures are observed in the pupa. Thus in *M. sexta* the JH may serve to synchronize developmental processes by retarding the rate of maturation of tissues which are especially sensitive to ecdysone in the absence of JH. Allatectomy in *T. ni* actually stops development at a specific prepupal marker. These prepupae can be induced to pupate by the application of JH. Therefore, the prepupal pulse of JH serves to synergize the effects or stimulate the production of ecdysone in facilitating pupation of *T. ni* (Jones, G. and Hammock, B., unpublished). Since in both *M. sexta* and in *T. ni* this prepupal pulse of JH (c) is critical for normal development, it is essential that the first peak of JH esterase activity is eliminated after JH reaches low enough levels for PTTH release. Thus, the appearance of a factor which reduces JH esterase levels (I) may be of physiological significance (Jones, G. *et al.*, 1981). Although complete elucidation of the role of the prepupal pulse of JH will require additional research, the disastrous effects of later application of JH to the late prepupa and early pupa have been long known. Thus, it is essential for normal development that the hemolymph titer of JH be dramatically reduced before pupation. The Lepidoptera seem to have a very effective means of carrying this reduction out if JH in fact induces its own catabolism at this developmental stage. The model for JH esterase regulation shown in Fig. 4 is consistent with most of the data currently available.

Little is known about the regulation of JH metabolism in adult Lepidoptera. The possibility that JH acid may be remethylated by extra-allatal tissues raises the possibility that epoxide hydrolases may be important in the irreversible inactivation of the hormone (Weirich, G. and Culver, M. 1979). Lessman, C. and Herman, W. (1981) raise the possibility that JH metabolism may increase during the migration of *Danus plexippus* and thus prevent reproduction during migration. As more developmental and behavioral events are found to be controlled by JH, it seems reasonable to examine JH degradation as well as biosynthesis in endocrine regulation. In fact, it may be prudent to re-examine the rates of degradation of some vertebrate hormones.

4 RESISTANCE AND JH METABOLISM

In his 1967 article on third-generation pesticides, C. Williams enumerated the many potential advantages which could be obtained by designing pesticides based upon an insect's own hormones. Many of these potential attributes have proven to be true. However, the statement that "insect hormones promise to provide insecticides that are ... proof against the evolution of resistance" was immediately questioned by Ellis, P. (1968) and Schneiderman, H. (1971, 1972). Shortly thereafter Dyte, C. (1972), and Cerf, D. and Georgiou, G. (1972) showed that insect strains resistant to pesticides could also be resistant to JHs and to their mimics or juvenoids. Vinson, S. and Plapp, F. (1974) examined the potential of insects to become resistant to juvenoids, and in a review of insect resistance to juvenoids and benzoylphenyl ureas, Sparks, T. and Hammock, B. (1982) cataloged cross-resistance to juvenoids in 13 insect species, and induced resistance in still other species. Such resistance is not surprising since any exogenous molecule, even if it is the natural hormone, could be expected to be treated as a xenobiotic or foreign compound. Exogenously applied hormones are thus likely to be subjected to the same penetration and distribution barriers and inactivation processes associated with other forms of pesticide resistance. Also, insects have the ability to regulate their own hormones, and thus have some ability to adjust their physiology towards normal

development even in the face of exogenous hormone application. Based upon the structures of most of the juvenoids under serious consideration for development, insects previously selected for high mixed-function oxidase levels could be expected to rapidly become resistant when subsequently pressured with juvenoids (Hammock, B. 1981; Sparks, T. and Hammock, B. 1982).

However, Williams, C. (1967) predictions regarding resistance have some validity. In the cases of resistance and cross-resistance to juvenoids so far studied, the resistance seems to result largely from reduced penetration and increased metabolism. There is no indication of resistance at the sites of juvenoid action. As illustrated by recent problems with the pyrethroid insecticides, such resistance mechanisms have the potential to devastate insect control programs. Since potent juvenoids probably act by being imperfect hormone mimics (mimicking JH at some but not all sites and possibly acting as antihormones at still others), a site of action resistance is likely to develop if insect populations are intensely pressured.

There is, however, cause for optimism about juvenoids. It is unlikely that their use will be heavy enough to induce direct resistance, and cross-resistance problems due to metabolism can be overcome by using different structures. If an insect population can become resistant to a new class of compounds using the same mechanisms of increased metabolism and decreased penetration that were effective for other classes of insecticides, little additional pressure is put on a population. However, if the insect population must overcome a new mechanism of action in order to become resistant, additional pressure is put on that population. Thus, the juvenoids, benzoylphenyl ureas and possibly new insect growth regulators which hit still other novel sites may be very valuable, although possibly limited, control agents for many years.

Hammock, B. *et al.* (1977a) and Yu, S. and Terriere, L. (1978a) demonstrated that *Musca domestica* strains which were either resistant or cross-resistant to juvenoids due to oxidative metabolism also metabolized JH at a greatly increased rate. Neither the esterases nor the epoxide hydrolases which normally are involved in JH metabolism appeared induced, further indicating that these **enzymes are involved in hormone regulation**. The

fact that JH was rapidly metabolized by oxidases bodes both ill and well for insect control. First, it probably indicates that insects have mechanisms to regulate hormone titer in spite of perturbing forces. Second, it may indicate that high oxidase levels due to selection or induction may be biologically expensive, since high levels of resistance commonly decline when selection pressure is removed. Edwards, J. and Rowlands, D. (1978) found that JH was metabolized somewhat faster in a resistant strain of *Sitophilus granarius* by hydrolytic pathways.

In addition to the influence of insecticide resistance upon JH titer, any insect hormone or hormone mimic has profound changes on the biology of the insect. As the insect's biology changes under the influence of a hormone, it is likely that its response to the environment, biological control agents, behavioral control agents as well as insecticides also will change. This possibility has recently been illustrated by the work of Gayen, A. and Knowles, C. (1982) who demonstrated alterations in chlor-dimeform metabolism were correlated with JH-mediated physiological changes in *Diatraea grandiosella*.

5 CONCLUSION: THE PRACTICAL EXPLOITATION OF INSECT REGULATORY BIOLOGY

5.1 Research in insect neuroendocrinology — why bother?

The investigation of the biosphere, and especially its six-legged inhabitants, has provided entertainment for generations of scientists. Considering the diversity and complexity of insects, there should be ample entertainment for many generations to come. The reasons for such investigations into insect biochemistry and physiology, as with any basic science, are clear to any who have studied the evolution of science or technology. The greatest applications for the research are likely to be the ones that are unforeseen. The elucidation of the concept of neuroendocrinology by a handful of entomologists is one of many examples (Scharrer, B. 1976, 1978).

Yet, we are at a time when all science is being questioned by numerous governments around the

world. The concept that all aspects (good and ill) of life in all advanced societies rests upon the basic research of previous generations appears too complex or too radical for most politicians to grasp. The general public has only the vaguest appreciation that cheap food, television, and even canned beer are available only thanks to basic science. The long-term fate of research rests upon a worldwide need for education. In the short term each discipline must also tell society not what we will do for man in the long term, but how basic research will benefit him immediately. This goal has been difficult for insect endocrinologists, especially neuroendocrinologists. Since vertebrate researchers have embraced the concept of neuroendocrinology, the "practical" justification for insect research has been tenuous. Rapid strides in a variety of biological fields have put us on the threshold of a new concept of insect control. The basic investigation of insect regulatory systems is the cornerstone of this concept which could revolutionize the procedures which are used to control insect pests in the future.

5.2 Classical exploitation of discoveries in neuroendocrinology

It is still widely held that peptide hormones and neurotransmitters cannot be exploited in terms of practical insect control. In fact, there are numerous rather conventional ways in which these materials could be exploited. The active portion of small peptides could be mimicked by a classical chemical, as illustrated by the opiate drugs. Alternatively, such peptides could be derivatized to alter their stability or lipophilicity. Many chemical procedures applicable to such derivatization have been known for years and used in peptide synthesis and analysis. Recently, such techniques have been used to profoundly alter the biological and chemical properties of carbamate insecticides, herbicides and other chemicals (Fukuto, T. and Fahmy, M. 1981). Alternatively, one could disrupt processing enzymes involved in the activation of a prohormone or enzyme. In mammalian systems this approach has been successfully exploited with the development of the drug Captopril which blocks the conversion of angiotensin I to II. Possibly the benzoyl-phenyl ureas such as Dimilin[®] act as "transition state" analog inhibitors of proteases which act as

processing enzymes. Many ramifications of the above approach could be employed, yet these are not radically different from some current approaches in pesticide chemistry. It will soon be possible to employ fundamentally new approaches to exploit insect peptide chemistry for insect control.

5.3 Third-generation concept of pesticide development

Sparks, T. and Hammock, B. (1982) argued that one should define first-, second- and third-generation pesticides in terms of the approach used to develop the compound rather than the final product. This concept only slightly alters the arguments put forward by Ordish, G. (1967) and Williams, C. (1967), yet makes the concept more useful in describing present and future trends in pesticide chemistry. Thus, the first generation of pesticides are those which arose from decades or centuries of a trial-and-error approach to insect control. The arsenicals, oils and botanicals clearly fit into this category. DDT, carbamates and organophosphates characterize the second-generation approach in which effective structures were elucidated using a vigorous synthesis-screening program (Ordish, G. 1967). Those pesticides based upon insect hormones and pheromones appear to have been classified as third-generation compounds by Williams, C. (1967). Extension of this procedure could lead to fourth-, fifth-, and sixth-generation pesticides every time a new lead structure is elucidated (Bowers, W. 1976). This problem is avoided if we consider the approach rather than the end-point to classify pesticides. Thus, the second-generation approach to pesticide development involves the process of screening and bioactivity-directed synthesis. A third-generation approach involves an appreciation of the physiology and ecology of target and non-target species which directs a synthetic program to produce environmentally acceptable chemicals.

In most cases modern pesticides will be developed using a combination of second- and third-generation approaches. J. Siddall's (1976) review of the development of some juvenoids illustrates this concept. The natural juvenile hormones clearly represented the lead structure, but much of the structure optimization resulted from a

second-generation approach. The biodegradable DDT mimics and the organophosphates and carbamates which have been derivatized to increase safety and selectivity are excellent examples of the third-generation approach. The parent compounds obviously resulted from a second generation approach, but the subsequent modifications were based upon knowledge of target and non-target biology, as well as environmental chemistry.

Other insect growth regulators such as the benzoylphenyl ureas and anti-juvenile hormones were discovered by a second-generation approach using innovative screening methods. Even the microbial insecticides such as *Bacillus thuringiensis* are classic examples of the second-generation approach. Thus, both second- and third-generation approaches alone and in combination are very useful in pesticide development, and will likely continue to lead to valuable compounds (Sparks, T. and Hammock, B. 1982). Yet, we are starting an era of a radically new concept in pesticide development in which the biologist will play a central role.

5.4 The evolving role of the biologist

The role of the biologist has changed as new approaches to pesticide development have evolved. In the classical second-generation approach the biologist's role is largely that of a technician, screening compounds which emerge from a chemistry program or isolates of micro-organisms. There is certainly room for innovation in terms of improved bioassays, suggestions of possible natural extracts to screen, and other ways. However critical the role of the biologist to the success of the development program, his role is clearly limited. A more exciting role exists in the third-generation approach. The biologist may provide lead structures, as in the case of JHs, he may elucidate differences in mode of action between target and non-target organisms, and he may elucidate a mechanism of action for a new class of compounds. However innovative his approach, the biologist soon finds himself again involved in a screening program. This screen may be at a physiological or biochemical level, yet it is nevertheless a screen. At best the biologist can hope to provide leads which can be exploited chemically. Regardless of these leads, an empirical approach is invariably demanded, requiring luck, as well as a

tremendous investment in exploratory chemistry. As a new or fourth-generation approach to pesticide development is initiated, the biologist faces the exciting and demanding new role of directing pesticide development at each step from the observation of natural history through commercial exploitation. This route may be exceedingly challenging, yet the goal is clear and the approach is no longer empirical.

5.5 The fourth-generation approach to pesticide development

The way in which one can exploit basic entomological research can be illustrated by the hypothetical fate of a cotton bollworm that feeds on a very special, and only slightly futuristic, cotton plant in California's Imperial Valley. The insect's finely tuned physiology is allowing it to survive the intense, desiccating conditions of its environment until it consumes plant material containing its own diuretic hormone. If the exogenous diuretic hormone reaches the insect's hemolymph in effective levels, the life expectancy of such an organism is very short.

At a time when plant genetic engineers are seriously considering changing the entire biology of crops to enhance salt tolerance, fix nitrogen, or even alter the photosynthetic process, the insertion and expression of a single gene will be technically foreseeable in the near future.

The research on vectors, expression, and regulation in plants is progressing rapidly, and the technology to carry out the above manipulations is likely to be available in a few years, but there are also courses of action which can be taken immediately. The technology to synthesize small genes and to insert and express synthetic or natural genes in yeast or bacteria now exists. The micro-organism acts not only as the factory to produce the critical peptide; it also serves as an encapsulation agent to help protect and distribute the biologically active ingredient. The potential of *Bacillus thuringiensis* and *Bacillus israeliensis* as major insecticides illustrates the possible utility of this approach. Although the genetics are less well understood, commercial organisms of both plants and insects also offer possibilities for such manipulation. If we had the messages for critical regulatory peptides from

pest insects, they could be exploited now for insect control. Considering the relative support for plant molecular biology and insect regulatory biology, it is likely that we will be able to manipulate the plant genome long before we know how to manipulate it to affect insect control. Numerous gaps in our knowledge exist, and many problems can be foreseen with this approach, but knowledge can be expanded and the problems will be solved. For example, although there is evidence that some insects absorb even large proteins through the gut wall intact, our knowledge of absorption and digestion in insects is woefully lacking. As we exploit such new concepts in insect control, new problems — as well as new ramifications of old problems — will arise. How does one effect patent protection; how does one prevent the escape of a gene from a crop cucurbit to a closely related weed; will new forms of resistance develop; will such research ultimately be used to justify further reduction in the natural plant gene pool?

Almost every chapter in this volume describes insect systems which can be exploited as described here to affect practical insect control. This chapter alone describes a variety of leads. The time to move on this fourth-generation approach to pest control is now. In a social context, such a move is critical. Metcalf, R. (1980) has charged that our war on insect vectors of human and animal disease and pests of crop plants is faltering. Even new leads from natural products chemistry will provide at best stop-gap measures. Here we simply summon the "experience" of plants in screening xenobiotics against insect pests. The chances of a plant successfully breaking the sequence code of even a short regulatory peptide by random evolutionary pressure are frustratingly small. Yet, the production of certain insect neurohormones by a crop plant should provide it with almost absolute protection against phytophagous insects. When entomologists instruct the plant as to the nature of these critical codes, science will have enhanced greatly the powers of man's already powerful tool in the war on insects — the plant itself. With a fourth-generation approach to pesticide development, we have the ability to take basic knowledge directly to commercial application using the tools of genetic engineering. This new horizon in pesticide chemistry certainly supports the concept that the most applied

research that our society can support is, in fact, basic research.

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