Juvenile Hormone Analogs as *in Vitro* Inhibitors of Rat Liver Microsomal Oxidases

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Three juvenile hormone analogs were found to be *in vitro* inhibitors of rat liver microsomal oxidases. Aniline hydroxylase reactions gave apparent inhibition constants of 8×10^{-5} *M* and 1.4×10^{-4} *M* for (*E*)-4-[(6,7-epoxy-3,7-dimethyl-2-nonenyl)oxy]-1,2-(methylenedioxy)benzene and (*E*)-4-[(6,7-epoxy-3-ethyl-7-methyl-2-nonenyl)oxy]-1,2-(methylenedioxy)benzene, respectively. (*E*)-6,7-Epoxy-1-(*p*-ethylphenoxy)-3,7-dimethyl-2-octene, the least active, had an apparent inhibition constant of 2.5×10^{-4} *M*. Cytochrome P-450 binding for all three compounds was observed to be of the type I category. The results provide evidence that JH analogs react with mammalian tissues and are thus not completely insect specific.

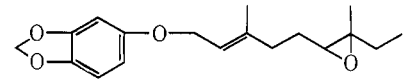
The juvenile hormone (JH), which is one of the hormones that controls maturation in insects (Schneiderman and Gilbert, 1964), was demonstrated as early as 1935 (Wigglesworth, 1935). However, the concept of hormonal control of insects was first suggested by Williams (1956) after he had demonstrated the juvenile hormone activity of extracts from cecropia moths, *Hyatophora cecropia* (L.). The subsequent delineation of the JH structure (IV) from the male cecropia moth (Dahm *et al.*, 1967; Roller *et al.*, 1967) and widespread interest in JH has resulted in the isolation and synthesis of a multitude of compounds possessing JH activity (Bowers, 1968, 1969; Schneiderman *et al.*, 1965; Slama *et al.*, 1968). As a result juvenile hormones and compounds with JH activity are now being considered as likely candidates to succeed conventional insecticides as third-generation insecticides (Williams, 1967).

It was therefore of particular interest to us when Bowers (1968) found that a number of insecticide synergists such as piperonyl butoxide, sesamin, and sesamolin had activity which mimicked that produced by the JH. Most of these same synergists have been shown to improve the efficacy of certain insecticides by inhibiting microsomal oxidases that would normally act in detoxification processes (Anders, 1968; Casida, 1970; Casida *et al.*, 1966; Philleo *et al.*, 1965). Then, later, based on results with *Tenebrio* and *Oncopeltus*, Bowers (1969) found that JH activity increased if terpenoid ethers were synthesized into methylenedioxyphenyl and benzene ring systems. The biological activities of these hybrids were considerably greater than that of methyl *trans,trans*-10,11-epoxyfarsenate and the cecropia hormones.

However, if methylenedioxyphenyl and benzene ring systems are retained in some JH structures, they would be suspected of causing the same effects as their particular synergist analogs on nontarget organisms such as other invertebrates, vertebrates, and man. The present paper therefore presents data on the effects of some JH analogs that are currently of commercial interest on mammalian systems.

MATERIALS AND METHODS

(*E*)-4-[(6,7-Epoxy-3,7-dimethyl-2-nonenyl)oxy]-1,2-(methylenedioxy)benzene (I) and its ethyl homolog (II) were 99% pure. Both compounds were provided by Hoffman-LaRoche, Inc. (*E*)-6,7-Epoxy-1-(*p*-ethylphenoxy)-3,7-dimethyl-2-octene (III), 75% pure (this sample has a slight contamination of less than 5% Z isomer), was supplied by the Stauffer Chemical Company. Glucose 6-phosphate (G6P) and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Sigma. All other materials were of reagent quality or of the highest available purity.



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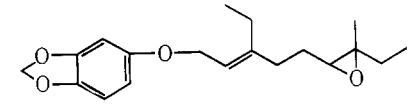

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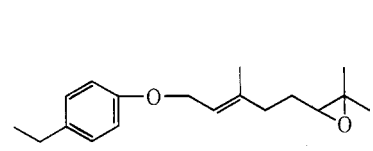

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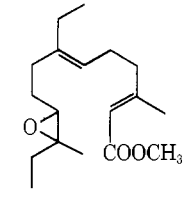

IV

Preparation of Enzyme. Male Sprague-Dawley rats weighing 306 to 322 g were decapitated without anesthesia, and the livers were removed, chilled in cold 0.15 *M* KCl, blotted, weighed, and homogenized in 3 vol of ice cold 0.15 *M* KCl by using a motor-driven Teflon-glass homogenizer. The homogenate was centrifuged at $9000 \times g$ for 20 min at 0–4° in a Sorvall model RC-2B centrifuge. After the floating fat layer was carefully aspirated, the underlying supernatant fraction was decanted and used as the microsomal enzyme source.

Enzyme Assays. Aromatic hydroxylation of aniline by microsomal oxidases was determined by measuring the formation of *p*-aminophenol (PAP) using a modification of the method of Kato and Gillette (1965). The incubation mixtures contained the following: microsomal suspension, 1 ml (equivalent to 250 mg of liver); NADP, 5 μmol; G6P, 25 μmol; $MgSO_4$, 25 μmol; aniline, 0.5 ml; and enough 0.1 *M* phosphate buffer, pH 7.4, to make a final volume of 4.8 ml. The effect of each JH analog on the *in vitro* metabolism of the substrate was determined by adding 0.2 ml of solution of JH analog in spectral grade acetone with agitation to the ice-cold incubation mixture. Acetone (0.2 ml) was added to the control flasks. Four concentrations of aniline were used (0.5, 0.2, 0.1, and 0.05 m*M* final concentration), and two concentrations of each inhibitor were used (0.1 and 1.0 m*M*). The mixtures were incubated for 20 min at 37° under air in a Dubnoff metabolic shaker at 120 oscillations/min. Appropriate recoveries and blanks were used. Apparent Michaelis Menten constants (K_m) and maximum velocities (v_{max}) were cal-

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culated by the computerized method of Wilkinson (1961), and the apparent inhibition constants (K_i) were determined graphically as described in Dixon and Webb (1964). Statistical comparison of the data was made by using the student t test.

Characterization of Difference Spectra. The postmitochondrial fraction was centrifuged at 105,000 × *g* for 1 hr at 0–4° in a Beckman model L2-65 ultracentrifuge. The resulting microsomal pellet was resuspended in 0.15 *M* KCl, recentrifuged at 105,000 × *g* for 1 hr, and stored as the pellet at −15° overnight. Storage of microsomes in this fashion was determined to have little or no affect on cytochrome P-450 (Wade *et al.*, 1972). Thawed, washed microsomes were resuspended in 0.15 *M* KCl, and the protein content was determined by the method of Gornall *et al.* (1949). The microsomes were then diluted to 2 mg of protein per ml with 0.3 *M* phosphate buffer, pH 7.4. A modification of the method of Remmer *et al.* (1966) was used to add 0.5 to 2.5 μmol of each JH analog in acetone (usually 3–7 μl) to 3.0 ml of microsomes in the sample cuvette; an equal volume of acetone was added to the microsomes in the reference cuvette. The difference spectrum was recorded on oxidized microsomes between 340 and 490 nm by using an Aminco Chance spectrophotometer in the split beam mode.

RESULTS

Inhibition Kinetics. Compounds I, II, and III inhibited the metabolism of aniline at concentrations of 0.1 and 1.1 m*M*. Table I shows the kinetic data describing this inhibition. Since both the apparent v_{max} and K_m were usually altered by the inhibitors, the observed inhibition is a mixture of competitive and noncompetitive effects. In the case of compound II, low inhibitor concentration resulted in noncompetitive inhibition. Graphic estimation of the apparent inhibition constant indicates that compound I is the most potent inhibitor and III is the least. The high K_m obtained for aniline hydroxylation probably results because of its nonspecific binding to the soluble proteins of the 9000 × *g* supernatant which were included to ensure solubilization of the inhibitors in the incubation mixtures.

Spectral Shift Studies. Remmer *et al.* (1966) showed that drugs and other foreign compounds that bind with cytochrome P-450, the terminal oxidase of microsomes, produce difference spectra of two general types, type I and type II. Type I compounds are compounds that interact with cytochrome P-450 to give a difference spectrum with a λ_{max} in the range of 385–392 nm and a λ_{min} in the range of 418–427 nm; type II compounds, such as pyridine or aniline, produce difference spectra with λ_{max} of 425–435 nm and λ_{min} of 390–405 nm. Recently, a third type of spectra (type III) has been suggested to describe a NADPH-dependent interaction between piperonyl butoxide and cytochrome P-450 (Philpot and Hodgson, 1971, 1971/72). There is no conclusive evidence as to exactly how the binding of various molecules to cytochrome P-450 gives these two general types of spectra; Mannering (1971) gives an excellent review on the spectral types and on ideas concerning the binding mechanisms which may be responsible for these spectra. A current theory with much support is that type I compounds bind to the protein portion of cytochrome P-450, whereas type II compounds bind directly to the heme grouping.

In our test, when the JH analogs were added to washed buffered microsomes, typical type I spectra were obtained. Compound I produced a spectrum with λ_{max} of 390 nm and λ_{min} of 427 nm; compound II produced λ_{max} 392 nm and λ_{min} 425 nm; and compound III gave λ_{max} of 390 nm and λ_{min} of 424 nm. Thus, these inhibitors produced difference spectra similar to the type I drug metabolism inhibitor SKF 525A (Remmer *et al.*, 1966).

DISCUSSION

Although the compounds discussed here differ somewhat in structure from the original JH (IV) identified by Roller *et al.* (1967) and Damn *et al.* (1967), they do elicit a response not unlike that observed for JH (Bowers, 1969; Pallos *et al.*, 1971). In studies in which both the cecropia moth hormone (IV) and these JHA's were applied to several species of insects, the latter compounds were found to have greater activities for most of the insects studied (Bowers, 1969; Pallos *et al.*, 1971).

Thus, these third-generation pesticides do, in fact, interact with animal systems *in vitro*. Analogs I, II, and III inhibit aniline hydroxylase from rat liver microsomes as sesamex, tropital, and piperonyl butoxide do (Anders, 1968). However, the concentrations of I, II, and III needed to produce inhibition (10^{-4} *M* to 10^{-3} *M*) were slightly higher than the concentrations of sesamex needed to produce inhibition in microsomal systems of the housefly, *Musca domestica* L. (Philleo *et al.*, 1965) and several orders of magnitude greater than the concentrations of IV needed for induction of housefly microsomal oxidases (Yu and Terriere, 1971). Moreover, these inhibitory concentrations are substantially higher than those necessary to produce JH effects in insects; *e.g.*, larval adult intermediates (Bowers, 1969; Pallos *et al.*, 1971).

Difference spectra of JHA's I, II, and III with cytochrome P-450 indicate that type I binding takes place when oxidized microsomes are used. Piperonyl butoxide gives the same results under identical conditions but it will also give a type III spectra when NADPH is added to the solution. It is not known whether or not JH's or JHA's will display a NADPH-dependent type III spectra (Philpot and Hodgson, 1971, 1971/72).

Wagstaff and Short (1971) found that piperonyl butoxide (PB) analogs effect induction of microsomal enzymes in rat livers, and these investigators are of the opinion that induction as well as inhibition may come about as a result of PB and PB analogs acting as alternate substrates. Because the results shown in Table I indicate that a mixture of noncompetitive and competitive effects are involved, we feel that this suggests the JH analogs used here are acting as alternate substrates and will bring about induction of microsomal enzymes in a manner similar to that of PB and PB analogs. Indeed, current work shows definite weight and microsomal protein increases in livers that have been taken from guinea pigs fed JHA containing benzene and methylenedioxyphenyl ring systems (Smalley, 1972). In addition, the induction of microsomal oxidases by JH (IV) has been shown to occur in houseflies (Yu and Terriere, 1971). Such induction of microsomal systems is important when one considers that induction can occur at lower concentrations than those needed to produce inhibition [induction of microsomal enzymes by PB analogs in rat liver occurs at thresholds only 100 times higher than those of DDT (Wagstaff and Short, 1971) and

Table I. Apparent K_m's, v_{max}'s, and K_i's in the Presence of Compounds I, II, and III

Inhibitor, m*M*		K_m,[a,b] m*M*	v_{max},[a,b] nmol PAP formed/min/g liver	K_i, m*M*
I	1.0	3.022 ± 0.77[c]	59.76 ± 13.76[d]	0.08
	0.1	1.211 ± 0.081[d]	129.44 ± 6.30[d]	
II	1.0	1.636 ± 0.200[d]	52.12 ± 5.18[d]	0.14
	0.1	0.688 ± 0.056	95.72 ± 5.09[d]	
III	1.0	0.903 ± 0.122	67.21 ± 6.50[d]	0.25
	0.1	0.333 ± 0.041[d]	94.28 ± 6.21[d]	
Control		0.624 ± 0.047	212.74 ± 10.33	

[a] Values are mean ± S.E. [b] Values obtained from a 4-point double reciprocal plot. [c] Value different from control ($p < 0.05$). [d] Value different from control ($p < 0.01$).

physiological aberrations due to endocrine hormone turnover by induced enzymes may result (Street *et al.*, 1969)].

Therefore, the data presented herein indicate that benzene and methylenedioxyphenyl-containing JHA's do resemble insecticide synergists in their reactions with microsomal systems. They do not appear to have any acute toxicity where mammals are concerned (Smalley, 1972), but one should be wary of long-term effects such as induction.

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A Screen for Pesticide Toxicity to Protein and RNA Synthesis in HeLa Cells

Brian C. Myhr

Suspension cultures of HeLa cells were used in a screen for toxicity based upon an initial biochemical response rather than the usual measurements of growth inhibition. Cells were exposed for 30 min to 350 μg/ml dosages of 30 different pesticides, and the effects on [^{3}H]uridine and ^{14}C-labeled amino acids incorporation into RNA and protein were determined. Dose–response curves for DDT, aldrin, carbaryl, and parathion yielded ID_{50} values that showed this method was as sensitive as 48-hr growth measurements. One-half of the compounds studied had no effect on one or both incorporation activities. Eight pesticides selectively inhibited amino acid incorporation, while only one, chlorpropham, was a selective inhibitor of uridine incorporation. Propham, however, inhibited both processes. The actions of the organophosphorus compounds were as varied as the side chains, but all of the chlorinated hydrocarbons except dieldrin strongly inhibited amino acid incorporation. Uncouplers of oxidative phosphorylation were highly inhibitory.

Heightened concern over the potential health hazards of environmental agricultural chemicals has prompted several recent studies of the toxic effects of a number of insecticides on mammalian cell cultures (*i.e.*, Gabliks and Friedman, 1969; Litterst *et al.*, 1969; North and Menzer, 1970) and on chick embryo cells (Wilson and Stinnett, 1969; Wilson *et al.*, 1968). This use of cell cultures represents an attempt to avoid the expense, long time periods, and the complexity of interactions that apply to the usual animal toxicity studies. Such studies have emphasized measurements of the inhibition of cell growth or qualitative changes in cell morphology caused by exposures to several concentrations of different chemicals over time periods usually ranging from 24 to 72 hr. In this way, relative ratings of cytotoxicity or growth inhibition have been obtained. The interpretation of these ratings has been hampered, however, by the lack of knowledge of the many possible changes in both cellular physiology and the nature of the chemical challenge over the long exposure periods.

The purpose of this paper is to present the results of a screen for chemical toxicity based upon initial cellular responses instead of growth inhibition. Such a screening procedure attempts to indicate the relative sensitivity of a prechosen cellular activity to various added chemicals. Thus, the effects of 30 min of exposure to pesticides on precursor incorporation rates into protein and RNA in HeLa cells are described herein. Organophosphorus pesticides are known to inhibit various esterases in cell cultures (DuBois *et al.*, 1968; North and Menzer, 1970), liver glutamate dehydrogenase (Freedland and McFarland, 1965), and trypsin (Ooms and VanDijk, 1966), while some organochlorine compounds inhibit lipase and hexokinase (Sadar and Guilbault, 1971), yet the effects on protein and nucleic acid synthetic rates in animal cells have been

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