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A Partition Assay for Epoxide Hydrases Acting on Insect Juvenile Hormone and an Epoxide-Containing Juvenoid

SUSANNE M. MUMBY AND BRUCE D. HAMMOCK

Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside, California 92521

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An assay for epoxide hydrase activity is described based on the difference in solubility between the epoxide substrate and its corresponding diol. The assay conditions for insect juvenile hormone I and an epoxide-containing juvenoid have been elucidated. In each case, the epoxide partitions into an isooctane phase while the diol partitions largely into an aqueous methanol phase. The assay is faster than chromatographic assays while showing similar or superior precision.

Epoxide hydrases are enzymes which add water to three-membered cyclic ethers (epoxides, oxiranes, or oxides) and convert them to 1,2-diols (glycols). Epoxides are intermediates in the conversion of olefinic and aromatic xenobiotics into hydrophilic materials for excretion. They also occur as natural or man-made xenobiotics, and they may additionally be formed from olefinic compounds normally present in an organism. Some of these epoxides are toxic and/or mutagenic, and it is thus important to understand the role of epoxide hydrases in mammalian systems [for reviews see (1–3)].

Epoxide moieties are also present in all three known insect juvenile hormones and in some juvenile hormone mimics or juvenoids which hold promise as potent and selective insect control agents (4, 5). Epoxide hydrases are considered to be important in the regulation of juvenile hormone titers, and an investigation of their properties is thus needed for an understanding of the endocrine control of insect development (5). As epoxide-containing juvenoids are used for insect control, their metabolism must be studied in resistant and susceptible target and nontarget insects, as well as in man and his domestic animals (6).

The tedious assay methods currently available for the investigations of epoxide hydrases have led investigators to either shun the investigation of these critical enzymes or to use epoxide-containing substrates which can be easily analyzed, and to then generalize the resulting information to other substrates possibly metabolized by totally different enzymes. With mammalian systems an example of this practice is that almost all epoxide hydrase activity has been shown to be membrane bound when styrene oxide, arene oxides, and certain other substrates have been used (1). From such work it has been concluded by many scientists that all epoxide hydrase activity is membrane bound. Other substrates have then been utilized in many studies without doing the necessary subcellular localization of the enzyme activity. Our investigations have shown that with some lipophilic substrates the majority of the epoxide hydrase activity is in the 100,000g soluble fraction of mammalian liver and kidney (6–9). With insect hydrases, investigators in some cases have either used styrene oxide or a cyclodiene oxide as substrates for epoxide hydrase apparently assuming the juvenile hormones to be hydrated by the same enzymes. Other

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experiments have indicated that these model substrates may be hydrated by different enzymes from the ones that hydrate the juvenile hormones and related compounds (10, 11). Thus, rapid analytical methods are needed to monitor the hydration of epoxides in both mammals and insects utilizing other substrates than those previously reported.

MATERIALS AND METHODS

Substrates and products. Juvenile hormone I [JH, methyl(2*E*,6*E*,10*cis*)-10,11-epoxy-3,11-dimethyl-7-ethyl-2,6-tridecadienoate ^{3}H-labeled at C-10, 97% 2*E*, 13.5 Ci/mmol] is available commercially from New England Nuclear Corporation. Its purity was checked by thin-layer chromatography, 250-μm silica gel F254 chromatoplates, EM Laboratories) in hexane:ether (5:1) or by reversed-phase high resolution liquid chromatography (ODS permaphase column, 2 × 250 mm, 5 μm DuPont) eluting with methanol:water (3:1). The radiosynthesis of the juvenoid R-20458 [1-(4′-ethylphenoxy)-3,7-dimethyl-6,7-epoxy-2*E*-octene; (Stauffer Chemical Company)] has been described earlier (12,13) (^{14}C ring, 17 mCi/mmol >95% 2*E*; ^{3}H ring, 600 mCi/mmol, >99% 2*E*). The purity was checked by tlc with multiple developments using benzene:*n*-propanol (20:1) which separates the 2*Z* (high R_f) and 2*E* isomers.

Radioactive JH diol was produced by either enzymatic cleavage of JH using diisopropylfluorophosphate-treated (to inhibit esterase activity, 1×10^{-3} M) mouse liver 100,000 g soluble fraction, or by incubation in sodium acetate buffer (pH 4.0, 0.5 M in double-distilled water containing 25 μg/ml of 2,6-di-*t*-butyl-4-methylphenol and 0.5% ethanol) in a silylated tube under N_2 and in darkness at 40°C. The concentration of JH was always below its critical micelle concentration (cmc) of 1.2×10^{-5} M. The half-life for the chemical conversion is ~17 h and the resulting diol was >97% pure by tlc analysis in hexane:ethyl acetate (3:2) [for kinetic studies on the acid hydrolysis of terpenoid epoxides see (14)] and >99.5% pure when produced enzymatically. The structure was further confirmed by microchemical techniques (15,16). For instance, >93% of the radioactive JH diol reacted with *n*-butylboronic acid to give a product cochromatographing with the cyclic boronate ester of authentic JH diol. The diol of R-20458 was prepared in a similar manner. The unlabeled JH was provided as an isomer mixture by A. J. Manson (Ayerst Laboratories), while syntheses of the unlabeled JH and R-20458 diols were performed as described earlier (7,15,16). All solvents were spectral grade except acetonitrile which was distilled before use.

Analytical procedures. Analytical procedures were similar to those described earlier (17). Siliclad (Clay Adams) or Carbowax (polyethylene glycol, 50,000 MW) were used to treat all glassware contacting aqueous solutions of the substrate to prevent binding to the glass (8,16). The binding phenomenon becomes very important (>90% of JH bound to some types of glass) at low substrate concentrations. The relatively high substrate concentrations used for a recent study of the efficiency of glass treatment may be somewhat misleading (18).

To assess the validity of the partition assay, enzymatic epoxide hydration was monitored by established tlc methods (16,19). The enzyme reaction (100 μl) was halted by the addition of sodium chloride to saturation followed by three extractions with ethyl acetate (3 × 200 μl). The ethyl acetate must be neutral or basic to avoid acid cleavage of the epoxide. Although this cleavage is usually minimal, a trace of pyridine in the extraction solvent eliminates this problem. The ethyl acetate extract was dried over

[1] Abbreviations used: JH, Juvenile hormone I; R-20458, 1-4′-ethylphenoxy)-3,7-dimethyl-6,7-epoxy-2*E*-octene; ODS, octadecyltrimethyloxysilane; cmc, critical micellar concentration; lsc, liquid scintillation counting; OCS, a xylene-based scintillation cocktail; BSA, bovine serum albumin.

Na_2SO_4, concentrated under N_2, and spotted with unlabeled standards on a tlc plate. Hexane:ethyl acetate (1:2) and benzene:*n*-propanol (10:1) were used as developing solvents for JH and R-20458, respectively. In initial studies with a new enzyme source, the plates were scanned or exposed to X-ray plates before and after microchemistry was performed on the products to insure that the corresponding diols were the sole metabolites. Subsequently, the epoxide and diol regions were visualized under ultraviolet light (254 nm), scraped, analyzed by liquid scintillation counting (lsc) in a cocktail consisting of OCS (a xylene-based scintillation cocktail, Amersham Corporation) mixed with Triton X-100 (3:1), and the epoxide:diol ratio was calculated.

The conditions for the partition assays were established by placing radiolabeled pure diol or pure epoxide in 100 μl of sodium phosphate buffer (I = 0.2 M, pH 7.4). Varying levels of water-soluble organic solvents followed by water-insoluble organic solvents (250 μl) were added, the phases mixed on a Vortex, centrifuged (2000g, 3–5°C), and aliquots of each phase removed with a Hamilton syringe and analyzed by lsc. The subsequent counts were corrected for background, quench, and phase volumes. Once a solvent ratio was established, the experiments were repeated varying the substrate levels from the lowest detectable level up to the cmc of the substrate, the buffer system utilized (phosphate, Tris, ammonium chloride, Universal), the pH (5–9.5) and ionic strength of the buffer (0.01–1 M), and the protein concentration using both bovine serum albumin (BSA) and mouse liver microsomal protein. Varying enzyme sources, incubation times, and substrate levels were utilized to give different ratios of the epoxide and diol. Simultaneous tlc assays were employed to assess the validity of the partition assays. The composition of radioactive materials in the isooctane was determined directly by tlc while the remainder of the aqueous methanol was analyzed by tlc after NaCl saturation and extraction as described earlier.

The assay was routinely performed by adding the radiolabeled substrate in ethanol (1 μl) with a Hamilton repeating dispenser. As earlier reported (17), the SD of this method of addition is ±6%. The reaction was terminated by the addition of methanol (150 μl for R-20458 or 200 μl for JH) using an automatic pipet (Pipetman, Cole Scientific, Calabasas, Calif.). After vortexing, 250 μl of isooctane was added using an automatic dispenser (Repipet, Labindustries, Berkeley, Ca.). The sample was vortexed, centrifuged, and 100-μl (measured with a microcapillary pipet) aliquots of each phase were analyzed by lsc. The percentage of diol present was determined from a standard curve of the percentage of radioactivity in the aqueous phase vs the percentage of diol calculated from the tlc data. Calculations were performed using a hand calculator or a minicomputer (NOVA-3) programmed to accept data on paper tape from the liquid scintillation counters.

Enzyme preparation. The partition assay has been used on epoxide hydrases from the tissue homogenates of several mammals and insects. The comparisons reported here, however, used whole insect homogenates from the white larvae (19,20) of the housefly, *Musca domestica*, and 0- to 6-h-old pupae of the yellow mealworm, *Tenebrio molitor*. Both homogenates were prepared in sodium phosphate buffer (I = 0.2 M, pH 7.4, 1% BSA) at 5 larval eq or 2 pupal eq/ml. Homogenization was performed in a glass Potter–Elvehjem tissue grinder, then the material was centrifuged at 12,000g for 15 min, passed through glass wool to remove fat, centrifuged at 100,000g for 60 min and the resulting "microsomal" pellet resuspended in sufficient buffer to yield 5 larval or 2 pupal eq/ml (unless otherwise noted). Enzyme preparations were performed at 0 to 5°C while incubations were performed at 30°C in 10 × 75-mm treated glass test tubes.

RESULTS

As previously described for another assay (17) halogenated organic solvents were found inappropriate due to poor partition characteristics and severe quench. Hexane and isooctane gave very similar and more desirable results, but the higher boiling point of isooctane allowed centrifugation and analysis at room temperature with minimum evaporation of the organic phase. Isooctane also gave slightly lower quench and less stable emulsions than hexane. Varying levels of methanol (Table 1), dimethylsulfoxide, and acetonitrile were examined as aqueous cosolvents. Acetonitrile and dimethylsulfoxide gave poor partition characteristics and erratic quench and counting efficiency. In 3.5 ml of scintillation solution, isooctane and aqueous methanol (100 μl) led to low background, negligible quench, and relatively stable count rates over time.

As discussed later, the assay conditions can be systematically varied to suit the needs of the experimenter using the partition data presented in Table 1. With our standard conditions, 94.6 ± 0.3% of the JH appeared in the organic phase while 79.1 ± 0.4% of the diol appeared in the aqueous phase. When equal amounts of radioactive JH and JH diol were mixed, assayed by the partition assay, and each phase subsequently analyzed by tlc, JH accounted for 82 and 6.4% of the radioactivity in the organic and aqueous phases, respectively, while JH diol accounted for 18 and 93.6%, respectively. No differences were found between the partition characteristics of chemically and enzymatically produced diol. Similar studies showed that 96.4% of the R-20458 partitioned into the organic phase and 85.0% of the diol occurred in the aqueous phase. The percentage of counts in the aqueous fraction was found to be linearly related to the amount of diol present (assayed by tlc) with both JH and R-20458 using chemical hydrolysis or several different enzyme systems.

The following variables caused no change in the partition characteristics of either JH or R-20458 or their respective diols: substrate concentrations of JH from 1×10^{-9}

TABLE 1

DISTRIBUTION OF R-20458, JUVENILE HORMONE, AND THEIR RESPECTIVE DIOLS BETWEEN ISOOCTANE (250 μl) AND AQUEOUS METHANOL

Methanol in aqueous phase[a] (%)	Percentage in aqueous phase			
	R-20458	R-20458 diol	JH	JH diol[b]
20	0.8	8.5	—	4.8
30	1.3	17.5	—	9.5
35	1.4	20.8	—	13.5
40	1.3	40.0	0.5	20.5
45	1.3	52.0	0.7	30.0
50	2.1	64.0	1.0	41.5
55	2.6	76.5	1.2	54.0
60	3.6	85.0	2.5	67.0
65	6.0	90.0	4.5	76.5
70	10.0	93.5	7.5	85.0
75	18.0	95.0	12.0	91.0
80	32.0	97.0	—	95.5

[a] Varying amounts of methanol were added to 100 μl of I = 0.2 M, pH 7.4 phosphate buffer to yield the appropriate methanol composition.

[b] The same distributions are found for chemically and enzymatically produced JH diol.

to 1×10^{-4} M, and R-20458 from 1×10^{-6} to 1×10^{-4} M, six concentrations of BSA from 0.04 to 10 mg/ml, and up to 10 mg/ml protein from mouse liver homogenate (which contains many components other than protein). High protein levels often require more extensive centrifugation to break emulsions. Potassium chloride concentrations from 0.004 to 0.33 M failed to detectably affect the partition characteristics while 1 M KCl caused a 3% decrease in the amount of diol in the aqueous phase. Many buffer types, pH's, and ionic strengths have been tried in the search for optimum incubation conditions for epoxide hydrases (from mouse, rat, and rabbit liver 100,000 g soluble and "microsomal" fractions, and microsomal epoxide hydrases from insect tissue or whole insect homogenates of *Tenebrio molitor*, *Musca domestica*, and *Trichoplusia ni*). In each case, the partition assay was checked by tlc and no differences have yet been found in the results from the two methods.

The partition assay is compared with the much more tedious tlc assay in several systems below. The hydration of R-20458 by housefly larva microsomes was linearly dependent upon enzyme concentration from 0.5 to 5 larval eq/ml with a linear correlation coefficient of 0.998 by the partition assay and 0.985 by tlc. The hydration showed a V_{max} and apparent K_m of 8.4 nmol/larval eq/h and 7.1 mM with a linear correlation coefficient for the Lineweaver–Burke plot of 0.998 when assayed by the partition assay. The V_{max} and K_m on the same enzyme preparation assayed by the tlc method were 7.5 and 6.6, respectively, with a linear correlation coefficient of only 0.978. Similarly with R-20458 in 0- to 6-h-old pupae of *T. molitor* the hydration of the epoxide was found to be linear with time up to 30 min with a correlation coefficient of 0.976 by partition and 0.961 by tlc. Twenty separate *T. molitor* microsomal incubations were run for 30 min with 5×10^{-6} M R-20458 and half were assayed by partition and half by tlc. The percentage hydration was found to be 45.1 ± 1.8% for partition and 45.7 ± 1.6% for tlc.

DISCUSSION

This partition assay is only valid if the investigator establishes the identity of the product diol and that epoxide hydration is the only route of substrate metabolism occurring. R-20458 is not susceptible to ester hydrolysis. The microsomal and soluble fractions of mouse mammalian liver have low levels of easily inhibited esterases capable of hydrolyzing conjugated esters. Specific JH esterase inhibitors have been developed for several insects which do not inhibit epoxide hydrase activity on the two substrates discussed here (21). Mixed function oxidase activity in microsomal or mitochondrial preparations can be minimized by the exclusion of cofactors, the presence of inhibitors, or anaerobic conditions. Thus, these assays can be applied to a number of only partially purified enzyme systems.

The data presented in this paper provide a close description of the partition characteristics of two epoxides and their diols. Rather than check the delivery rate of solvent dispensers used in the study and use scrupulously pure solvents for each assay, this laboratory has found it quicker to assay several incubations by both tlc and partition, or to assay known diol: epoxide ratios by partition, and use this data to generate a standard curve for subsequent partition assays.

The conditions for the partition assay should not be considered as static. Sensitivity, reproducibility, and analysis time can all be adjusted to the needs of the investigator. For instance analysis of both the aqueous and organic phase leads to much greater reliability of the data, but some investigators may choose to only assay the aqueous phase thus saving time and supplies. For studies of initial enzyme rates,

the percentage of methanol in the aqueous phase could be reduced, or multiple or larger volume isooctane extractions performed to reduce the amount of epoxide contaminating the aqueous phase at the expense of larger amounts of radioactivity and/or time needed per assay. Conversely, larger amounts of methanol in the aqueous phase are acceptable if the enzyme studied will metabolize a high percentage of the substrate in a linear fashion.

The partition assays reported here have been found to be much faster and less expensive than the currently used tlc assays. When carefully performed, they also give greater reliability and higher recoveries of radioactivity than tlc assays. The partition assays have been used for over 2 years in this laboratory to examine epoxide hydration in a number of biochemical systems. Their reliability under many conditions indicates that they can be readily adapted to studies in other laboratories. The speed and reproducibility of the assay should enhance studies on the epoxide hydration of JH and epoxide-containing juvenoids. This and a previous study (22) have shown that there are large differences in aqueous solubility between the epoxides and diols of even very lipophilic molecules. Thus, this partition assay should be easily adaptable to the investigation of the metabolism of numerous epoxides, including arene oxides, by a variety of enzyme systems. The isooctane:aqueous methanol system allows the rapid analysis of both phases and a wide range of aqueous layer polarities to be used.

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REFERENCES

1. Oesch, F. (1973) *Xenobiotica* **3,** 305.
2. Sims, P., and Grover, P. L. (1974) *Advan. Cancer Res.* **20,** 165.
3. Jerina, D. M., and Daly, J. W. (1974) *Science* **185,** 573.
4. Gilbert, L. I., and King, D. S. (1973) *in* The Physiology of Insecta (Rockstein, M., ed.), p. 249, Academic Press, New York.
5. Hammock, B. D., and Quistad, G. B. (1976) *in* The Juvenile Hormones (Gilbert, L. I., ed.), p. 374, Plenum, New York.
6. Gill, S. S., Hammock, B. D., Yamamoto, I., and Casida, J. E. (1972) *in* Insect Juvenile Hormones: Chemistry and Action (Menn, J. J., and Beroza, M., eds.), p. 177, Academic Press, New York.
7. Gill, S. S., Hammock, B. D., and Casida, J. E. (1974) *J. Agr. Food Chem.* **22,** 386.
8. Hammock, B. D., Gill, S. S., Stamoudis, V., and Gilbert, L. I. (1976) *Comp. Biochem. Physiol.* **53B,** 263.
9. Gill, S. S., and Hammock, B. D. *Biochem. Pharmacol.* in preparation.
10. Brooks, G. T. (1973) *Nature (London)* **245,** 382.
11. Slade, M., Brooks, G. T., Hetnarski, H. K., and Wilkinson, C. F. (1975) *Pest. Biochem. Physiol.* **5,** 35.
12. Kalbfeld, J., Hoffman, L. J., Chan, J. H., and Hermann, D. A. (1973) *J. Label. Compounds* **9,** 615.
13. Kamimura, H., Hammock, B. D., Yamamoto, I., and Casida, J. E. (1972) *J. Agr. Food Chem.* **20,** 439.
14. Mumby, S. M., and Hammock, B. D. *J. Agr. Food Chem.* submitted (1978).
15. Hammock, B. D., Gill, S. S., and Casida, J. E. (1974) *J. Agr. Food Chem.* **22,** 379.
16. Hammock, B., Nowock, J., Goodman, W., Stamoudis, V., and Gilbert, L. I. (1975) *Mol. and Cell. Endocrinol.* **3,** 167.
17. Hammock, B. D., and Sparks, T. C. (1977) *Anal. Biochem.* **82,** 573.
18. Giese, Ch., Spindler, K. D., and Emmerich, H. (1977) *Z. Naturforsch.* **32c,** 158.
19. Hammock, B. D., Gill, S. S., and Casida, J. E. (1974) *Pestic. Biochem. Physiol.* **4,** 393.
20. Hammock, B. D., Mumby, S. M., and Lee, P. W. (1977) *Pestic. Biochem. Physiol.* **7,** 261.
21. Hammock, B. D., Sparks, T. C., and Mumby, S. M. (1977) *Pestic. Biochem. Physiol.* **7,** 517.
22. Schmassmann, H. U., Glatt, H. R., and Oesch, F. (1976) *Anal. Biochem.* **74,** 94.