consumption and related hormonal events, rather than photoperiod, are primarily responsible for the diurnal pattern in amount and synthesis of SCP.[34] When the functional activity of liver SCP mRNA was measured during the dark–light cycle, no significant changes were found in SCP mRNA levels. Since SCP mRNA is available for translation at all times, the diurnal variation in liver SCP synthesis must involve mechanisms regulating the efficiency of translation of SCP mRNA.[6] These mechanisms are currently under investigation.

Acknowledgments

The author is grateful to Carol Olson for her excellent technical assistance. These studies were supported by the American Cancer Society (BC-378) and the National Heart, Lung, and Blood Institute (HL-28176).

[34] P. A. Stewart, C. D. Olson, and M. E. Dempsey, *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **43**, 1719 (1984).

[16] Epoxide Hydrolases in the Catabolism of Sterols and Isoprenoids

By BRUCE D. HAMMOCK, DAVID E. MOODY, and ALEX SEVANIAN

Background Information

Epoxide hydrolases are enzymes which add water to three-membered cyclic ethers known as epoxides, oxiranes, or oxides of olefinic or aromatic compounds. The products of these reactions are diols (aliphatic systems) or dihydrodiols (aromatic systems). These epoxide ether hydrolases (EC 3.3.2.3) were formerly known as epoxide hydrases or hydratases (EC 4.2.1.63). The hydration leads to a product much more polar than the precursor epoxide. Epoxide hydration, in most cases, represents a detoxification or inactivation reaction, but there are cases where the reaction leads to a more toxic material or is involved in the biosynthesis of a biologically active compound. Numerous reviews are available on these enzymes.[1–4]

[1] F. Oesch, *Xenobiotica* **3**, 305 (1972).
[2] A. Y. H. Lu and G. T. Miwa, *Annu. Rev. Pharmacol. Toxicol.* **20**, 513 (1980).
[3] B. D. Hammock, S. S. Gill, S. M. Mumby, and K. Ota, *in* "Molecular Basis of Environ-

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There is evidence for at least four forms of epoxide hydrolase in vertebrate systems. One of these forms hydrates leukotriene A_4 and will not be further discussed. There is evidence that steroids and isoprenoids are at least incidental if not physiologically significant substrates for the remaining three forms of epoxide hydrolase. Most of the research in this area has been focused on the microsomal form which is responsible for the hydration of arene oxides. It is clear that this form can hydrate some steroids,[5] but since its assay and purification have been reported in this series, it will not be discussed.[6,7] Some of the assay procedures discussed here can be modified for the analysis of the microsomal enzyme. The solubilized form of the microsomal epoxide hydrolase which occurs in preneoplastic and neoplastic lesions in the liver can be assayed by modifications of the techniques used for the analysis of the membrane bound form of the microsomal epoxide hydrolase. It is important that this "preneoplastic antigen" not be confused with the cytosolic epoxide hydrolase discussed below.[8,9]

Methods for the analysis of two other forms will be presented. The predominantly cytosolic form hydrates a variety of epoxides on aliphatic systems with the epoxides of fatty acids being very rapidly hydrolyzed.[10,11] Although epoxides of isoprenoid compounds such as the epoxides of squalene or lanosterol are more slowly metabolized, these compounds have very low K_m values indicating that the enzyme may have a physiological role in the metabolism of such compounds either produced as by-products of steroid biosynthesis or as dietary components.[3] A partition method using the juvenile hormones for the analysis of the cytosolic epoxide hydrolase from vertebrates is first presented. Subsequently simpler methods are presented based on model substrates.

Evidence is good that there is a microsomal epoxide hydrolase which specifically hydrolyzes the α and β epoxides of cholesterol, but is different from the microsomal epoxide hydrolase which metabolizes arene ox-

mental Toxicity" (R. S. Bhatnager, ed.), p. 229. Ann Arbor Sci. Publ., Ann Arbor, Michigan, 1980.

[4] F. P. Guengerich, *Rev. Biochem. Toxicol.* **4,** 5 (1982).

[5] U. Bindel, A. Sparrow, H. Schmassmann, M. Golan, P. Bentley, and F. Oesch, *Eur. J. Biochem.* **97,** 275 (1979).

[6] A. Y. H. Lu and W. Levin, this series, Vol. 52, p. 193.

[7] T. M. Guenthner, P. Bentley, and F. Oesch, this series, Vol. 77, p. 344.

[8] W. Levin, A. Y. H. Lu, P. E. Thomas, D. Ryan, D. E. Kizer, and M. J. Griffin, *Proc. Natl. Acad. Sci. U.S.A.* **75,** 3240 (1978).

[9] M. J. Griffin and K. I. Noda, *Cancer Res.* **40,** 2768 (1980).

[10] S. S. Gill and B. D. Hammock, *Biochem. Biophys. Res. Commun.* **89,** 965 (1979).

[11] N. Chacos, J. Capdevila, J. R. Falck, S. Manna, C. Martin-Wixtrom, S. S. Gill, B. D. Hammock, and R. W. Estabrook, *Arch. Biochem. Biophys.* **223,** 639 (1983).

ides and substrates such as 16,17-epoxyandrosten-3-one.[5,12,13] Thus, a method for the analysis of this enzyme using cholesterol epoxide is presented.

Epoxide hydration in insects has received much less attention and several reviews cover the available information.[14,15] However, it appears in the few species so far studied that there are several hydrolases predominantly associated with the microsomal fraction which hydrate a number of substrates including cyclodiene insecticides. It is interesting that the most active epoxide hydrolase so far purified has come from an insect.[16] The assays presented here and elsewhere for vertebrate systems certainly need to be applied to insect systems. However, in relation to this volume the activity of primary importance is the one which hydrates the terpenoid insect juvenile hormones as originally reported by Slade and Zibitt.[17] In this regard an assay for epoxide hydrolase activity using the natural juvenile hormones of insects is presented.

Assay with Juvenile Hormone

General Considerations

The partition assay for epoxide hydrolase activity described here is more tedious than some of the partition methods described below. However, use of these substrates led to the discovery of the cytosolic epoxide hydrolase in vertebrates, and they represent commercially available model compounds for the hydration of terpenoid epoxides. Entomologists are fortunate in having these compounds available for studying epoxide hydrolase activity with intrensic substrates. For such studies, the only viable alternatives to this method are a modification of the TLC methods discussed in this volume [32] and with regard to cholesterol epoxide below, or HPLC or GLC methods which are quite laborious.

As discussed in this volume [32] it is important to ensure that epoxide hydration is the only pathway involved in the system being investigated. In the vertebrates studied, most of the carboxyesterase activity on the

[12] T. Watabe, M. Kanai, M. Isobe, and N. Ozawa, *J. Biol. Chem.* **256,** 2900 (1981).

[13] W. Levin, D. P. Michaud, P. E. Thomas, and D. M. Jerina, *Arch. Biochem. Biophys.* **220,** 485 (1983).

[14] B. D. Hammock and G. B. Quistad, *Prog. Pestic. Biochem.* **1,** 1 (1983).

[15] B. D. Hammock, *in* "Comprehensive Insect Physiology, Biochemistry and Pharmacology" (G. A. Kerkut and L. I. Gilbert, eds.), Chapter 12. Pergamon, Oxford, 1984 (in press).

[16] C. Mullin and C. F. Wilkinson, *Insect Biochem.* **10,** 681 (1980).

[17] M. Slade and C. H. Zibitt, *in* "Insect Juvenile Hormones: Chemistry and Action" (J. J. Menn and M. Beroza, eds.), p. 155. Academic Press, New York, 1972.

juvenile hormones is in the microsomes while in the Lepidoptera studied it is in the cytosol. The epoxide hydrolase activity is in the opposite fraction in each case. If esterase activity is a problem, *O,O*-diisopropyl phosphorofluoridate (DFP), or *O,O*-diethyl-*O*-*p*-nitrophenyl phosphate (paraoxon) are suggested as inhibitors. For insects *O*-ethyl-*S*-phenyl-phosphoramidothioate (EPPAT) and 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP) should be added to the list.[18,19] Since glutathione reacts very poorly with trisubstituted, aliphatic epoxides, formation of the conjugates of the juvenile hormones is usually not a problem.

Enzyme Preparation

In vertebrate tissue the enzyme referred to as cytosolic epoxide hydrolase is primarily located in the cytosol fraction, but similar enzyme activity has been described in particulate fractions.[20-22] Therefore, initial studies should consider the ratio of soluble to particulate activity. For common rodents the cytosolic fraction can be prepared by standard protocols. Liver is homogenized in sodium phosphate buffer pH 7.4 with an ionic strength of 0.2 *M*, and the cytosolic fraction is prepared from the postmitochondrial supernatant. While the ionic strength and pH of the buffer are important for reproducible assays, we have found other buffers, such as 0.25 *M* sucrose, 10 m*M* Tris–HCl (pH 7.4), appropriate for tissue preparation. Recently, the purification of cytosolic epoxide hydrolase from rabbit,[23] human,[24] and mouse[25] liver has been described. These references should be consulted for preparation of purified enzyme. Although this discussion applies to murine epoxide hydrolase, it represents a good starting point for optimizing assay conditions in insects.

For studies of epoxide hydration in tissues of lepidopteran (*Trichoplusia ni*) tissues were homogenized in sodium phosphate buffer at pH 7.4 with an ionic strength of 0.2 *M* with 0.01% phenylthiourea to inhibit tyrosinases. Activity was either examined in a 20,000 *g* supernatant with esterase inhibitors or in a postmitochondrial pellet. Assay conditions should be optimized for the particular study planned.

[18] T. C. Sparks and B. D. Hammock, *Pestic. Biochem. Physiol.* **14,** 290 (1980).
[19] T. C. Sparks, B. D. Hammock, and L. M. Riddiford, *Insect Biochem.* **13,** 529 (1983).
[20] S. S. Gill and B. D. Hammock, *Biochem. Pharmacol.* **30,** 2111 (1981).
[21] T. M. Guenthner, U. Vogel-Bindel, and F. Oesch, *Arch. Toxicol., Suppl.* **5,** 365 (1982).
[22] F. Waechter, P. Bentley, F. Bieri, W. Staubli, A. Vozkl, and H. D. Fahimi, *FEBS Lett.* **158,** 225 (1983).
[23] F. Waechter, M. Merdes, F. Bieri, W. Staubli, and P. Bentley, *Eur. J. Biochem.* **125,** 457 (1982).
[24] P. Wang, J. Meijer, and F. P. Geungerich, *Biochemistry* **21,** 5769 (1982).
[25] S. S. Gill, *Biochem. Biophys. Res. Commun.* **112,** 763 (1983).

Principle

The enzyme is assayed by the conversion of the epoxides of juvenile hormone I, II, or III to their corresponding diols. The method is described for JH I, but is equally applicable to any of the commercially available compounds. Following the enzyme reaction, the majority of the JH is extracted with a hydrocarbon solvent (isooctane or dodecane is used to reduce volatility) while the majority of the product diol remains in the aqueous phase. The proportion of the diol in the aqueous phase is increased by incorporating methanol.[26] General principles of partition assays are described in more detail elsewhere.[27,28]

Reagents

The juvenile hormones such as JH I [methyl-(2*E*,6*E*,10-*cis*)-10,11-epoxy-3,11-dimethyl-7-ethyl-2,6-tridecadienoate] are available as racemic mixtures with a tritium in the 10 position from New England Nuclear while the unlabeled materials are available from many suppliers including Calbiochem and Sigma. The labeled and unlabeled materials should be mixed to give the desired substrate concentration (see this volume [32]). For routine work a concentration of 1×10^{-4} *M* in absolute ethanol containing approximately 10,000–20,000 cpm per microliter is useful. For developing a standard curve JH diol can be prepared chemically[17] or enzymatically as discussed below. The only variation would be to extract the aqueous methanol several times with pentane to remove all traces of JH. Varying ratios of JH and JH diol can be prepared and subjected to the partition assay for the development of a standard curve. In addition one needs methanol (ethylene or propylene glycols can be substituted), isooctane, or other hydrocarbon solvent, silicon or polyethylene glycol coated tubes, and a scintillation solution capable of handling aqueous solvents.

Assay Procedure

Place 100 μl of enzyme solution in a round bottom 6 × 50 or 10 × 75-mm glass tube treated with a 1% solution of polyethylene glycol (PEG 20,000) to reduce substrate binding to the glass. Equilibrate the tube to 30 or 37.5° (insect or vertebrate) and add substrate to initiate the reaction. The substrate is added with a 50-μl Hamilton syringe in a repeating dispenser designed to give 1 μl yielding a final substrate concentration of 1×10^{-6} *M* (this is just below the critical micellar concentration for JH I). The

[26] B. D. Hammock and T. C. Sparks, *Anal. Biochem.* **82,** 573 (1977).
[27] M. T. Bush, this series, Vol. 77, p. 353.
[28] B. D. Hammock and R. N. Wixtrom, *Adv. Biochem. Pharmacol.* (in press).

reaction is terminated (usually at 0 to 30 min) by the addition of 200 μl of methanol. Isooctane (250 μl) is then added with a repeating dispenser and the mixture vortexed vigorously and then centrifuged. Under these conditions one can expect over 76% of the diol to be in the aqueous phase with less than 5% contamination by the epoxide. Initially one can sample 100 μl of both phases and determine radioactivity by liquid scintillation counting, but for routine work only the aqueous phase needs to be sampled.

Most systems give only a small, consistent level of quenching so that cpm can be used in calculations. One should run the control where methanol, but no isooctane is added to provide the maximum number of counts, determine machine background, and do a zero time experiment to yield the assay background. Spontaneous hydration of these substrates is very small. The percentage of diol formed can be determined from a standard curve by regression or by simultaneous equations.

Assay with *trans*- and *cis*-Stilbene Oxide

General Considerations

A specific and rapid partition assay for cytosolic epoxide hydrolase recently has been devised which uses *trans*-stilbene oxide (TSO) as substrate.[29,30] The geometric isomer, *cis*-stilbene oxide (CSO), can be used to assay the common microsomal epoxide hydrolase. This assay will also be considered at this time. The use of these geometrical isomers to compare the cytosolic and microsomal epoxide hydrolase is attractive since they have similar physical properties. The stilbene oxides can conjugate with glutathione. Tissues which contain significant amounts of glutathione such as rat liver cytosol, should be dialyzed overnight before use. Otherwise, the principles for preparation of the cytosolic fraction are the same as discussed previously. Microsomes can be prepared by conventional methods. The principles of the partition assay are similar as those discussed for juvenile hormone. The differentiation of the cytosolic and microsomal activities depends upon substrate specificity, pH, and subcellular fractions used. Cytosolic epoxide hydrolase is therefore defined as the conversion of TSO to its diol at pH 7.4 using cytosolic fractions. Microsomal epoxide hydrolase is defined as the hydrolysis of CSO to its diol at pH 9.0 using microsomal fractions.

[29] S. S. Gill, K. Ota, and B. D. Hammock, *Anal. Biochem.* **131**, 273 (1983).
[30] D. E. Moody, D. N. Loury, and B. D. Hammock, submitted.

Reagents

The preparation of TSO and CSO and their tritiated counterparts has been previously described in detail and will not be repeated here.[29] The substrates should be dissolved in ethanol at a concentration of 5 m*M* with enough radiolabeled substrate to give 10,000–20,000 cpm per microliter. The tissue to be assayed should be suspended in 76 m*M* sodium phosphate (pH 7.4) for the cytosolic epoxide hydrolase or in 100 m*M* Tris–HCl (pH 9.0) for the microsomal epoxide hydrolase. Dodecane or isooctane (with or without previous addition of up to 25% methanol) is used for the extraction, and scintillation solution appropriate for aqueous solutions is needed.

Assay Procedure for Cytosolic Epoxide Hydrolase

Place 100 μl of enzyme preparation in a round bottomed 10 × 75 or 13 × 100-mm glass tube and preincubate for 1 min at 37°. Initiate the reaction by adding 1 μl of [^{3}H]TSO at 5 m*M* using a 50-μl Hamilton syringe with a repeating dispenser. The reaction is stopped by adding 200 μl of dodecane or isooctane followed by vigorous vortexing. Centrifuge the tubes briefly to ensure phase separation and then withdraw a 50-μl aliquot of the aqueous phase for counting. Blanks are run simultaneously by adding buffer instead of enzyme. Standards are prepared by adding 1 μl of the radiolabeled substrate directly to the scintillation cocktail. Activity is calculated using the assumption that 92% of the diol is left in the aqueous phase. This value may be increased by previous addition of up to 25% methanol in the extracting solvent.

Assay Procedure for Microsomal Epoxide Hydrolase

The procedure for the microsomal epoxide hydrolase is essentially the same as that described above, so only the differences will be pointed out. A similar volume of enzyme is used, but it must be diluted in 100 m*M* Tris–HCl (pH 9.0). After equilibration of the enzyme 1 μl of [^{3}H]CSO at 5 m*M* is added to start the reaction. All the following procedures are identical to those described above.

Assay with Cholesterol Epoxide

General Considerations

It is now known that a distinct enzyme(s) exists in the microsomes of vertebrates which specifically hydrates Δ^5-steroid epoxides. This enzyme

has been shown to be distinct from the more commonly studied microsomal epoxide hydrolase by studies on its substrate specificity, pH optima, inhibition, induction, and immunochemical properties.[12,13,31,32] While studies comparing this enzyme with cytosolic epoxide hydrolase are few, little or no activity for hydrolysis of the Δ^5-steroids in the cytosol has been found suggesting that these two enzymes are also distinct.[32] Assays for cholesterol epoxide hydrolase have been described using isotope dilution as detected by GC-MS,[33,34] HPLC, or GC analysis of column separated extracts,[35] capillary GC,[34] and TLC separation of radiolabeled substrate and product.[13,32] The GC-MS assay is quite precise and sensitive and can be useful for assays when the recovery of activity is low or when radiolabeled substrates are not available. The most convenient and still relatively sensitive assay is the TLC method, and such an assay will be discussed below.

Enzyme Preparation

Cholesterol epoxide hydrolase is commonly assayed using microsomal fractions. Sodium cholate detergent extracts have also been found appropriate, and while not studied, postmitochondrial supernates, tissue homogenates, and cell suspensions may also be appropriate. Microsomes can be prepared by any conventional means, but before assay they should be suspended in 100 m*M* potassium phosphate (pH 7.4), as both the buffer used and its pH have been shown to effect activity.[13,32] At this time a method for purifying the cholesterol epoxide hydrolase has not been described.

Principle

The enzyme is assayed by the conversion of cholesterol epoxide (other 5,6-epoxysteroids may also be used) to the corresponding cholesterol triol (5,6-glycol) using radiolabeled substrate. The products formed then are separated from the substrate by TLC, both recovered by scraping, and the radiolactivity quantitated. This method was first described by Jerina *et al.*[36] for the assay of a number of epoxides, and subsequently has been modified specifically for the cholesterol epoxide hydrolase.

[31] L. Aringer and P. Eneroth, *J. Lipid Res.* **15,** 389 (1974).
[32] A. Sevanian, R. A. Stein, and J. F. Mead, *Biochim. Biophys. Acta* **614,** 489 (1980).
[33] N. L. Petrakis, L. D. Gruenke, and J. C. Craig, *Cancer Res.* **41,** 2563 (1981).
[34] J. Gumulka, J. S. Pyrek, and L. L. Smith, *Lipids* **17,** 197 (1982).
[35] L.-S. Tsai and C. A. Hudson, *J. Am. Oil Chem. Soc.* **58,** 931 (1981).
[36] D. M. Jerina, P. M. Dansette, A. Y. H. Lu, and W. Levin, *Mol. Pharmacol.* **13,** 342 (1977).

Reagents

The synthesis of unlabeled or radiolabeled cholesterol epoxide and cholesterol triol from cholesterol can be achieved by a few different methods which will not be described further.[12,13,31,32] It is important that the relative amounts of the α- and β-epoxide are known for their rates of spontaneous and enzymatic hydrolysis are different. Therefore, pure isomers are the preferred substrates. The delivery solvent for the cholesterol epoxide is also critical and maximal activity can be achieved by using acetonitrile leading to a final concentration of 6.25%. Siliconized test tubes are recommended to prevent adherance of the substrate. TLC separation has most commonly been carried out using Whatman LK5DF silica gel plates.

Assay Procedure

Add 150 μl of microsomes (200–500 μg/ml) in 100 m*M* potassium phosphate (pH 7.4). Preincubate microsomes for 1 min at 37° and then add cholesterol epoxide (final concentration 20–100 μM) dissolved in enough acetonitrile to give a final concentration of 6.25%. After 2–20 min incubation at 37° the reaction is terminated by addition of 50 μl of tetrahydrofuran containing cold carrier substrate and product. Use of unlabeled standards is particularly important if low amounts of product are expected. Aliquots (up to 70 μl) are then quickly transferred to the preabsorbant zone of the silica gel plate (Whatman LK5DF). The plate is then developed in benzene–ethyl acetate (3 : 2). Spots can then be visualized with iodine vapor and are then scraped for quantitation by scintillation counting.

[17] Biosynthesis and Interconversion of Sterols in Plants and Marine Invertebrates

By L. J. GOAD

The broad outline of the sterol biosynthetic pathway leading to plant sterols is now understood[1] although some facets remain to be clarified. However, many different sterols, varying in the structure of the side chain, the position of double bonds and the presence of methyl groups at

[1] L. J. Goad, *in* "Lipids and Lipid Polymers in Higher Plants" (M. Tevini and H. K. Lichtenthaler, eds.), p. 46. Springer-Verlag, Berlin and New York, 1977.

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