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## Gas Chromatographic Assay of Epoxide Hydrase Activity with 3-Methylcholanthrene-11,12-Oxide

Abstract. *Epoxide hydrase has been measured in rat tissue with 3-methylcholanthrene-11,12-oxide as substrate; diol formation was assayed by gas chromatographic separation of the trimethylsilylated derivative of* trans-*11,12-dihydro-11,-12-dihydroxy-3-methylcholanthrene from the corresponding derivative of the 11 (or 12)-hydroxy-3-methylcholanthrene on 3 percent OV-17, which is formed from the 11,12-oxide during the derivatization. The polycyclic hydrocarbons were extracted initially from the incubation mixture with ethyl acetate. The assay is simple, inexpensive, and sensitive.*

Chemical carcinogens of the polycyclic hydrocarbon type are thought to exert their effect after modification to metabolically active derivatives (*1*). In this regard, Boyland proposed that epoxides may be formed from aromatic ring structures as reactive intermediates in the transition to diols, phenols, and conjugates of glutathione (*2*). Since that time, epoxides have been reported as intermediates in the metabolism of polycyclic hydrocarbons (*3*). The K-region oxides are highly reactive substances and produce malignant transformations of cells in vitro (*4*).

Not only is the rate of synthesis of the K-region oxides of the polycyclic hydrocarbons important, but consideration must also be given to their rate of metabolism. Thus, it has been shown that these reactive substances may be converted to *trans*-dihydrodiol derivatives by a microsomal epoxide hydrase (*5*). The hydrase has been partially purified from the microsomes of guinea pig liver and may actually represent a heterogeneous class of enzymes (*6*). Consequently, the intracellular quantity of the reactive epoxide derivative will be a function of the levels of epoxide synthetase and hydrase enzymes.

Although the hydration of styrene-oxide has been employed as the basis for the hydrase assay in previous work (*7*), this reaction might not reflect an accurate assay of the formation of *trans*-dihydrodiol derivatives of polycyclic hydrocarbons. Accordingly, we have developed a relatively simple and sensitive gas chromatographic method in which the formation of the *trans*-11,12-dihydrodiol of 3-methylcholanthrene (3MC) is measured as a trimethylsilyl derivative. We have reported the gas chromatographic characteristics and the mass spectral patterns of the trimethylsilylated derivatives of the 11,-12-oxide and of the *cis*- and *trans*-11,12-dihydrodiols (*8*). We now report the details of the assay system.

Gas chromatography was conducted on an instrument (Nuclear-Chicago model 5000) containing a U-tube glass column (4 mm by 1.8 m) which was packed with Gas Chrom Q (100 to 120 mesh) coated with 3 percent OV-17 (Applied Science). The injection temperature was 335°C; the detector temperature was 255°C; the carrier gas was $N_2$, flowing at 60 ml/min. The chromatogram was programmed from 220° to 280°C at 3° per minute. The 11,12-oxide and *trans*-dihydrodiol derivatives were prepared by the procedure of Sims (*9*). Microsomes were prepared from livers of adult male Charles River rats after homogenization of the tissue in 0.25*M* sucrose (1 g of tissue per 5 ml of sucrose). The homogenate was centrifuged at 9,000*g* for 15 minutes at 0°C, and the supernatant was recentrifuged at 100,000*g* for 1 hour at 0°C. The

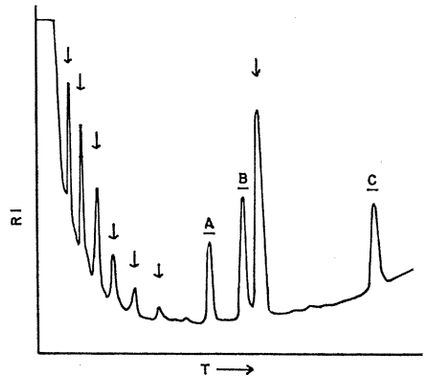


Fig. 1. Gas chromatogram of participants in the epoxide hydrase assay. The chromatogram was programmed from 220° to 280°C at 3° per minute. *A*, Silylated *trans*-diol; *B*, $C_{32}$ standard [dotriacontane (Applied Science)]; *C*, silylated phenol (from the oxide). Rat liver microsomes (2.3 mg of protein) were first incubated in 0.1*M* sodium phosphate buffer, *p*H 8, in a total volume of 0.4 ml for 2 minutes at 37°C. The 11,12-oxide (50 μg in 0.05 ml of dimethyl sulfoxide) was then added, and the incubation mixture was shaken at 37°C for an additional 10 minutes. This amount of dimethyl sulfoxide had no effect on the enzyme activity. Boiled microsomes served as controls in this and all further experiments. After the incubation, the tubes were plunged into an ice bath to halt the reaction, and 2.0 ml of ethyl acetate was added. The polycyclic hydrocarbons were extracted with shaking for 10 minutes and the suspension was then centrifuged at 600*g* for 5 minutes. The organic solvent layer (top) was withdrawn, and the aqueous layer was reextracted with an additional 1.0 ml of ethyl acetate. The ethyl acetate extracts were combined, a 2.5-ml portion was removed, and the organic solvent was evaporated under nitrogen. The residue was washed with approximately 1 ml of anhydrous ethyl ether and the latter was also evaporated off under nitrogen. The remaining residue was trimethylsilylated under the following conditions: To each tube was added 20 μl of Tri-Syl (Pierce) and, after the resultant stoppered mixture was heated at 60°C for 30 minutes, 10 μl of *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (Pierce) was introduced. The solution was brought to a final volume of 70 μl by the addition of acetonitrile, and the derivatives were stored at 5°C for no longer than 12 hours. At this juncture thin-layer chromatography of the silylated derivative in a benzene-ethanol system (19 : 1) showed no underivatized diol present; then 5-μl portions were injected into the gas chromatograph for analysis. The ordinate is given as relative intensity (*RI*) and abscissa as increasing temperature (*T*). The derivatives are stable under these conditions.

top of the resultant pellet was carefully washed with 1.15 percent KCl, and the pellet was suspended in 1.15 percent KCl. Protein concentration was determined by the method of Lowry *et al.* (*10*).

The gas chromatographic analysis is presented in Fig. 1. We observed that the presence of microsomal protein did not significantly influence the quantitative aspects of the extraction of *trans*-dihydrodiol. More than 90 percent of diol was recovered in the presence of 2.5 to 10 mg of microsomal protein over a range of diol concentrations of 20 to 100 $\mu$g per 0.4 ml of assay system. Peaks showing low retention times (indicated by arrows) were also present in control extracts obtained from microsomes alone.

The large peak following the $C_{32}$ standard (*B*) was identified by mass spectrometry (*11*) as the trimethylsilyl ether of cholesterol. This compound has the same retention time as the silylated derivative of *cis*-11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene (*cis*-diol). However, under our assay conditions, no *cis*-diol could be detected from the mass spectral fragmentation pattern associated with this peak, indicating that the microsomal reaction is stereospecific.

The major enzyme activity is associated with the microsomal fraction, although the fraction sedimenting at 600 to 20,000*g* had a specific activity which was approximately 30 percent that of the 100,000*g* pellet (4.0 as compared to 11.2 nmole of diol formed per milligram of protein per 10 minutes). A good deal of the former activity may be associated with microsomal contamination. No activity could be found in the 100,000*g* supernatant.

The reaction appeared linear for up to 10 minutes and, consequently, the latter time was chosen as a part of the assay conditions. A study of velocity of the reaction plotted against substrate concentration indicated that 0.44 m*M* oxide (50 $\mu$g) was sufficient to saturate the system; a calculated Michaelis constant ($K_m$) for this substrate was $2.8 \times 10^{-4}$ *M*. Enzyme activity was also directly proportional to the protein concentration of liver microsomes, up to 10 mg of protein per incubation.

The distribution of activity with respect to rat tissues is indicated in Fig. 2. Liver microsomes had the greatest activity, whereas those from kidney and lung appeared to be 25 and 5 percent as active, respectively. No demonstrable enzyme activity could be detected in microsomes from either spleen or small intestine. Finally, as reported by Oesch *et al.* (*12*) for the hydration of styrene oxide, equimolar quantities of 3,3,3-trichloropropene oxide completely inhibited the formation of the diol from 3-methylcholanthrene oxide (data not shown).

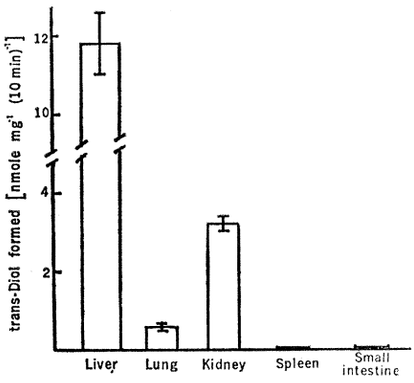


Fig. 2. Epoxide hydrase activity in rat tissues. Microsomes from the tissues of three male rats (approximately 120 g) were prepared as described in the text. Each incubation received 3.0 mg of microsomal protein and the number of nanomoles of *trans*-diol formed were measured. The bars indicate the standard error.

In conclusion, we have demonstrated a relatively simple method for determining epoxide hydrase activity which is based on the conversion of 3-methylcholanthrene-11,12-oxide to the corresponding diol. The gas chromatographic assay is advantageous because (i) it requires only readily available substrates and should be applicable to various arene oxides, and (ii) it is extremely sensitive and can detect less than 0.2 nmole of diol.

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## Regulation of Phosphodiesterase Synthesis: Requirement for Cyclic Adenosine Monophosphate–Dependent Protein Kinase

Abstract. *Endogenous cyclic adenosine monophosphate (AMP) and its dibutyryl derivative increase cyclic AMP phosphodiesterase activity in cultured lymphoma cells. This effect is prevented by cycloheximide. A variant population of cells deficient in cyclic AMP–dependent protein kinase contains lower basal phosphodiesterase activity, which cannot be induced by cyclic AMP.*

Adenosine 3′,5′-monophosphate (cyclic AMP) is well established as an intracellular "second messenger" for many hormones, and plays an important role in controlling growth of cells. However, the molecular basis of cyclic nucleotide action is known for only a few of these processes. Protein phosphokinases specifically activated by cyclic AMP control the moment-to-moment activity of several enzyme systems [for example, glycogen synthetase and glycogen phosphorylase (*1*)]. Although similar cyclic AMP–activated protein kinases are present in most mammalian cells (*2*), they have not been directly implicated as mediators of the other effects of cyclic AMP, including induction of the synthesis of specific enzymes (*3*, *4*) and regulation of cell growth and differentiation (*5*). We have reported the selection of a stable population of cultured lymphoma cells deficient in both cyclic AMP–activated protein phosphokinase and its cyclic AMP–binding subunit, and have presented evidence that these cells may prove useful in defining the role of such kinases in mediating the diverse actions of cyclic AMP (*6*). We now present