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PHOTOMETRIC ASSAY OF HEPATIC EPOXIDE HYDROLASE ACTIVITY WITH SAFROLE OXIDE (SAFO) AS SUBSTRATE

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Abstract—A simple and sensitive method for the assay of hepatic microsomal epoxide hydrolase activity by using safrole oxide (SAFO) as the substrate is described. It involves differential extraction of the unreacted substrate with *n*-hexane from the incubation medium containing the reaction product, safrole glycol (SAFG) followed by measurement of absorbancy at 288 nm of the separated hexane layer. A stoichiometric relationship between the SAFO disappearance and SAFG formation was confirmed in the enzymatic reaction.

EPOXIDE hydrolase[1,2] (epoxide hydrase;[3,4] epoxide hydratase; EN, 1972, 4.2.1.63), the enzyme that catalyzes the conversion of a wide variety of olefin oxides[1–3,5–13] and arene oxides[4,14–19] to the corresponding glycols, has come into light for its important role in detoxifying toxic or carcinogenic olefins and aromatic hydrocarbons since they are converted by the microsomal monooxygenase, epoxidase, to epoxides,[2,4,20–24] some of which, e.g. respective epoxides of ethylene,[25] *p*-nitrophenoxypropyrene,[26] dibenzanthracene,[22] benzpyrene,[23] 7-methylbenzanthracene and 7,12-dimethylbenzanthracene[24] have recently been demonstrated to form covalent bonds with nucleic acids, nucleotides, or protein. Moreover, the K-region epoxides of the polyaromatic hydrocarbons were found to produce malignant transformations of cells *in vitro*.[21,22,27] In the animal body, the enzyme activity is present predominantly in the liver and to a small extent in the kidneys[28] and the lung.[29] In hepatic cells, the hydrolase is localized almost exclusively in the microsomal fraction and partly in the soluble supernatant fraction.[1,2,28] In hepatic microsomes, the hydrolase is closely associated with cytochrome P-450,[30,31] which plays a vital role in monooxygenation of foreign compounds, as well as hydroxylation of olefins and aromatic compounds such as steroids and fatty acids. Despite the difficulty in separating epoxide hydrolase from epoxidase,[30,31] only the activity of the latter is markedly affected and decreases by lipid peroxidation of microsomes.[32]

Gas–liquid chromatography[1,2,7,29,33] and radiometric methods[29] have been used to determine hepatic epoxide hydrolase activity. However, both methods are tedious and time consuming. In the present paper, a simple method for the assay of hepatic epoxide hydrolase activity using safrole oxide (SAFO: 1-(3′,4′-methylenedioxyphenyl)-2,3-epoxypropane) as the substrate is reported. The outline of the assay method is differential extraction of the unreacted substrate with *n*-hexane from the incubation medium containing the reaction product, safrole glycol (SAFG: 1-(3′,4′-

methylenedioxyphenyl)-2,3-propanediol), followed by the measurement of absorbancy at 288 nm of the separated hexane layer.

MATERIALS AND METHODS

Materials. *n*-Hexane for photometry and spectrophotometry (Spectrosol-hexane which is free from unsaturated aliphatic and aromatic hydrocarbons) and safrole were purchased from Wako Pure Chemicals Co., Tokyo.

Preparation of safrole oxide (SAFO). To a solution of safrole (0·25 moles) in chloroform (200 ml), kept at 0–2°, was added a cold solution of perbenzoic acid (0·35 moles) in chloroform (250 ml) during 30 min with mechanical stirring. The mixture was left standing for 40 hr at room temperature and then shaken successively with 5% Na_2CO_3 (600 ml) and water, and dried over anhydrous sodium sulfate. A crude oil obtained after the removal of the solvent was subjected to silica gel column chromatography by which SAFO, eluted with hexane and benzene (1:2), was separated from unreacted safrole, eluted first with hexane, and from polar by-products to give a thin-layer chromatographically pure oil (36 g). For the complete removal of the solvent, especially benzene, from the isolated oxide, it was distilled at b.p.$_6$ 130°: *m/e* 178 (M^+), 147, 135, 122, 105, 91, 77, 57: $\delta_{TMS}^{CDCl_3}$ ppm; 2·52 (1H, quartet, one of two hydrogens at 3-position), centered at 2·78 (2H, unresolved signals, 1-H and the other hydrogen at 3-position), 3·10 (1H, multiplet, 2-H), 5·94 (2H, singlet, methylenedioxy hydrogens), 6·67 and 6·77 (2H, double doublets, *J* 8 Hz, 5′- and 6′-H), 6·73 (1H, singlet, 2′-H): ν_{film} cm^{-1}; 1486, 1440, 1245, 1034, 925, 832, 808: $\lambda_{max}^{n\text{-hexane}}$ nm (ϵ); 235 (4600), 283 (3870), 288 (3970), 294 (2880).

Preparation of safrole glycol (SAFG). A solution of SAFO (1 g) in acetic acid (10 ml) was refluxed for 3 hr, and the crude product obtained after the removal of the solvent was then heated for 30 min in 4% (w/v) methanolic NaOH (50 ml). After the evaporation of the solvent to dryness, followed by dissolution of the residue in ether and water, crude SAFG was obtained from the ethereal layer, decolorized by active charcoal, and recrystallized from isopropyl ether and acetone to give prisms (0·6 g), m.p. 79–80°: *m/e* 196 (M^+), 165, 135, 122, 105, 91, 77: ν_{KBr} cm^{-1}; 3210, 1500, 1485, 1438, 1336, 1245, 1189, 1162, 1094, 1072, 1045, 942, 930, 902, 805, 780: λ_{max}^{EtOH} nm (ϵ); 235 (4200), 287 (3920).

Enzyme assay. Rabbit liver microsomes (corresponding to 7 mg of the liver), prepared by the previously reported method[34] and suspended in 0·1 M phosphate buffer, pH 7·4 (2 ml) were incubated at 37° for 10–30 min in a 50 ml glass-stoppered centrifuge tube with a substrate solution (3 ml) prepared by diluting an ethanolic solution (0·6 ml) of SAFO (1 μmole), which contained Tween 80 (3 mg), with 0·1 M phosphate buffer, pH 7·4. The reaction was terminated by the addition of 5N NaOH (1 ml) and the unreacted substrate extracted with Spectrosol-hexane (5 ml). The absorbancy of the organic layer, separated by centrifugation at 3000 rev/min for 3 min, was measured at a wave length of 288 nm using a Hitachi Model 139 photometer. Incubation mixtures containing boiled microsomes served as a blank.

Isolation and determination of SAFG from incubation mixtures. A large scale reaction mixture, consisting of SAFO (1 mmole), ethanol (12 ml), Tween 80 (60 mg), microsomes (corresponding to 2 g rabbit liver), and 0·1 M phosphate buffer, pH 7·4, to make a final volume of 100 ml, was incubated at 37° for 1 hr, and the reaction was terminated by the addition of 10 N NaOH (10 ml). The mixture was saturated with

sodium chloride and extracted twice with ethyl acetate (200 ml each). A crude product obtained after the removal of the solvent from the combined ethyl acetate extract was dissolved in a minimum volume of benzene and chromatographed on a silica gel column. The column was eluted with a mixture of benzene and acetone (4:1) and the eluates monitored by TLC. SAFG separated was recrystallized from isopropyl ether and acetone.

For determination of SAFG, 5 ml of the above incubation mixture was transfered into a 50 ml centrifuge tube containing 5 N NaOH (1 ml) and Spectrosol-hexane (5 ml) at various intervals. After the photometric determination of the SAFO extracted into the hexane, the aqueous residue was washed with *n*-hexane (20 ml), saturated with sodium chloride and then extracted twice with ethyl acetate. The residue obtained by the evaporation of the solvent from the combined ethyl acetate extract was dissolved in an acetonide reagent (1 ml, prepared by mixing 10 ml of dry acetone and two drops of 60% perchloric acid) containing tolan (diphenylacetylene) as internal reference, and left stand for 30 min at room temperature. The solution was then vigorously agitated with finely powdered anhydrous sodium carbonate (10 mg) to decompose the acid catalyst. After sedimentation of inorganic salts, the supernatant solution containing a SAFG acetonide was analyzed by GLC.

Chromatography. Thin-layer chromatography (TLC) was carried out by using plates coated with Wakogel B-5 UA containing an inorganic phosphor agent. Chromatograms were visualized either under u.v. light (225 nm) or by spraying with concentrated sulfuric acid followed by heating. Both SAFO and SAFG showed intense and characteristically changeable coloration of reddish to dark violet at the beginning of heating the plate after spraying with sulfuric acid. Gas–liquid chromatography (GLC) was carried out on a Shimadzu Model GC-1C gas chromatograph equipped with a flame ionization detector using a column of 2% OV-17 coated on Chromosorb W (60–80 mesh, 4 mm × 180 cm).

Spectroscopy. Ultraviolet, infrared, and mass spectra were recorded on Hitachi Models 323 recording spectrophotometer, EPI-G3 infrared spectrophotometer, and RMU 7 high resolution mass spectrometer, respectively, and NMR spectra on a JEOL Model 4H-100.

RESULTS AND DISCUSSION

Assay for epoxide hydrolase. A number of GLC methods have been presented for measuring epoxide hydrolase activity.[1,2,7,29,33] However, they proved too time consuming for routine assay required for investigations such as enzyme purification, enzyme behavior in the animal body, and enzyme distribution. Oesch *et al.*[29] have recently described a convenient radiometric method which involves measurement of a radioactive glycol enzymatically formed from [7-^{3}H]-styrene oxide following removal of the unreacted oxide from incubation media by differential extraction with light petroleum. Their method is useful for routine assay of enzyme activity but is still time consuming and tedious since, for instance, it requires freezing of the aqueous layer when the petroleum one containing the unreacted substrate is separated. A much simpler method could be direct measurement of either the unreacted substrate or the resulting glycol by photometry. Measurement of the reaction product by u.v. absorption photometry is almost impossible since no suitable non-chromophoric solvent is available for quantitative extraction of a polar glycol that

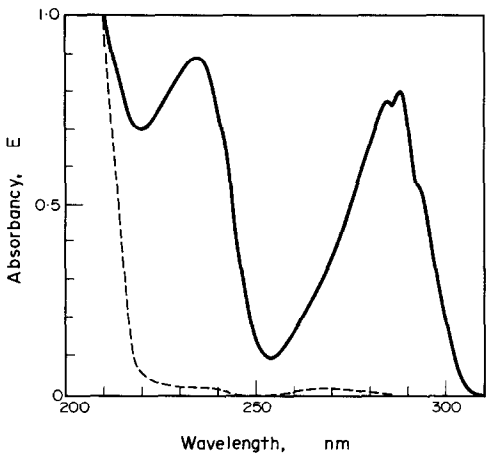


FIG. 1. Ultraviolet absorption spectra of a solution of SAFO in *n*-hexane (solid line) and of a *n*-hexane extract of rabbit liver microsomes (broken line). Concentration of SAFO was 0·2 mM. Microsomes (corresponding to 0·5 g of the rabbit liver) were extracted with Spectrosol-hexane (5 ml) in the presence of 1 N NaOH.

remains in incubation media after the differential extraction of the parent epoxide with a non-polar solvent. For the purpose of direct photometry, the substrate to be used, therefore, should have a peak maximum with a sufficiently large molecular absorption coefficient in the region where no back-ground absorption appears, be stable in water, reactive with the hydrolase, and quantitatively separable from the enzymatically formed glycol by extraction.

SAFO that was synthesized in our laboratory was found to be a suitable substrate for direct u.v. absorption measurement since it not only was quantitatively extract-

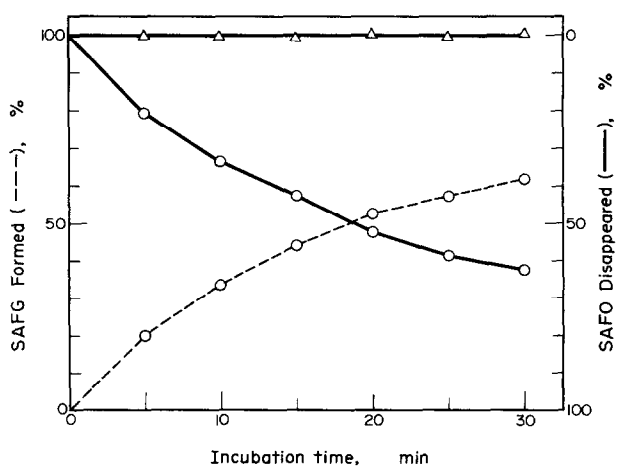


FIG. 2. Stoichiometry of enzymatic SAFO hydrolysis reaction. Incubation media contained boiled (△) or untreated microsomes (○). An aliquot (5 ml) of the large scale incubation mixture (1 mole SAFO and microsomes corresponding to 1 g of the rabbit liver) mentioned in the text was taken at each interval and it was extracted with Spectrosol-hexane (5 ml). The separated hexane layer was diluted 30 times with the same solvent and the amount of unreacted SAFO determined by photometry. SAFG remaining in the aqueous residue was extracted into ethyl acetate and analyzed as the acetonide by GLC.

able with *n*-hexane from incubation media in which the reaction product, SAFG, remained completely, but also showed absorption maxima with sufficiently large values in the wave length region where no absorption due to hexane extractable hepatic microsomal components appeared (Fig. 1).

For the assay of epoxide hydrolase activity, SAFO was dissolved in a mixture of 0·1 M phosphate buffer, pH 7·4, and ethanol containing Tween 80 and incubated with rabbit liver microsomes. After termination of the reaction by the addition of a caustic alkali, measurement of the unreacted substrate extracted into *n*-hexane was carried out by direct photometry. The absorbancy (E) of a 0·2 mM solution of SAFO in *n*-hexane was 0·792 at 288 nm. The rate of disappearance of SAFO (0·2 mM) from the incubation medium was 6·38 mM/mg protein/min. Michaelis constant (K_m) obtained in the range of substrate concentrations of 0·008 to 0·4 mM was 0·19 mM and maximum velocity (V_{max}) 12·6 mM mg protein^{-1} min^{-1}.

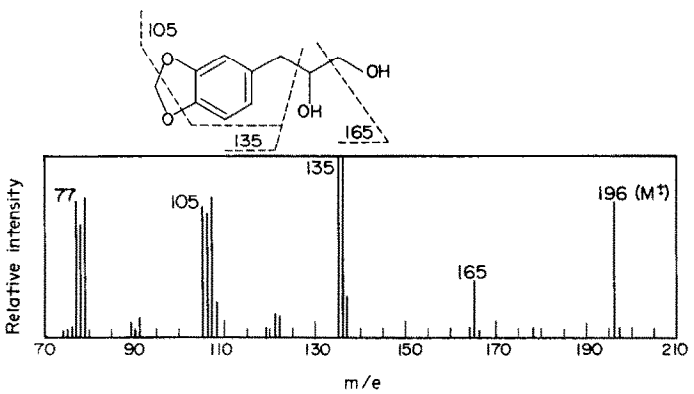
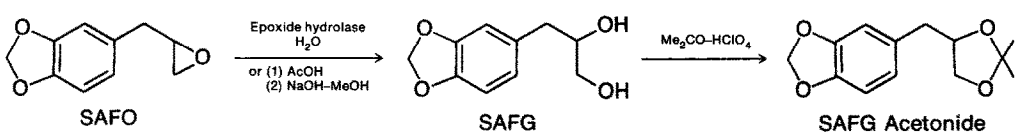


FIG. 3. Mass spectrum of SAFG enzymatically formed from SAFO. It was identical with that of the authentic specimen.

SAFO was stable in water at pH 7·4 so that it did not disappear to any appreciable extent from the incubation medium containing boiled microsomes within the usual incubation time of 30 min (Fig. 2).

Identification of SAFG enzymatically formed from SAFO. An ethyl acetate extract obtained from a large scale reaction mixture containing 1 m-mole (178 mg) of SAFO which was incubated for 1 hr showed the presence of only one metabolite with the same R_f value of 0·20 as that of authentic SAFG in thin-layer chromatograms [benzene–acetone (2:1)]. The R_f value of SAFO was 0·85 under the conditions used. The metabolite was isolated by silica gel column chromatography and recrystallized from isopropyl ether and acetone to give needles (135 mg). The i.r. and mass spectra were identical with those of authentic SAFG (Fig. 3).



SCHEME 1

Stoichiometry of the enzymatic reaction. After the photometric determination of unreacted SAFO in the reaction mixture the aqueous layer was washed with *n*-hexane, and SAFG was extracted quantitatively with ethyl acetate. A crude product obtained on evaporation of the solvent was treated with an acetonide reagent including tolan (diphenylacetylene) as the internal reference for subsequent GLC analysis. The SAFG acetonide formed (Scheme 1) was determined by using a 2% OV-17 column (column temperature 160°; flow rate of nitrogen as carrier gas 43 ml/min). Retention times of the acetonide and tolan were 15·4 and 11·9 min, respectively. A stoichiometric relationship between the SAFO disappearance and SAFG formation was demonstrated at each interval of the reaction (Fig. 2).

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