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Enzymatic Hydrolysis of Mono-*n*-alkyl Substituted Ethylene Oxides and Their Inhibitory Effects on Hepatic Microsomal Epoxide Hydrolase

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Hydrolysis of oxides of *n*-1-olefins, heptene, octene, decene, dodecene, tetradecene, and hexadecene, by hepatic microsomal epoxide hydrolase and their inhibitory effects on the hydrolase activity have been described. Enzymatic reaction rates were the highest in heptene oxide, high in tetradecene and hexadecene oxides, and the lowest in decene oxide. The decene oxide inhibited enzymatic hydrolysis of safrole oxide most significantly; much more significant than octene oxide that is recognized as a potent inhibitor. A curve representing relationship between alkyl side chain length of the *n*-1-olefin oxides and their enzymatic reaction rates showed a reciprocal pattern to that for their inhibitory effects on the hydrolase activity.

Epoxides as obligatory intermediates in the microsomal metabolism of olefins and arenes to glycols are, in general, toxic or carcinogenic.[2] K-region epoxides formed from polycyclic aromatic hydrocarbons by hepatic microsomal monooxygenation make covalent binding to nucleic acids and proteins[3,4] and consequently produce malignant transformations of cells.[5] Of olefin oxides, ethylene oxide, a potent toxicant, is known to modify nucleotides[6] and *p*-nitrophenoxypropylene oxide proteins.[7] Epoxides do not always exert their toxicity as mere alkylating agents, *e.g.* heptachlor epoxide, a metabolite of the cyclodiene insecticide, heptachlor, is stable *in vivo* as well as *in vitro* but more toxic than the mother compound.[8] In the animal body, epoxide hydrolase[9,10] (epoxide hydrase,[11] epoxide hydratase, EN 1972, 4.2. 1.63), the enzyme that catalyzes hydrolysis of a variety of olefin oxides and arene oxides to the corresponding glycols, is localyzed almost exclusively in hepatic microsomes[10–12] and plays a key role in regulating epoxide levels *in vivo*.[12] Systematic investigations have been carried out to clarify relationship between rates of enzymatic hydrolysis of epoxides and their structures.[12,13] However, little is known with mono-*n*-alkyl substituted ethylene oxides

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in spite of the important fact that their parent olefins with carbon numbers of 14 to 18 have been utilized as the most common sources for detergents, sodium alkyl sulfates, and 10 to 12 as sources for plasticizers, huge tons of the olefins, therefore, being produced in Japan and the world at the present time. The non-specific hydrolase is inhibited by epoxides, and this makes it possible to trap various epoxy intermediates in the *in vitro* metabolism of both olefins[10,14] and polycyclic aromatic hydrocarbons.[4,15] The inhibition of the hydrolase is significant in mono-substituted ethylene oxides; *n*-1-octene oxide[10,14] and trichloromethyl-ethylene oxide[12] are known to be inhibitors as yet most potent. In the present paper, we wish to report the effect of chain length of *n*-alkyl substituents on the enzymatic hydrolysis of ethylene oxides and on the inhibition of epoxide hydrolase activity.

Experimental

Materials——Epoxides were purchased from Research Organic Inorganic Co., Ltd., Cal., U.S.A. Their homogeniety was confirmed by gas-liquid chromatography (GLC) using a 20% Apiezon L and a 2% OV-17 columns. Glycols[16] were prepared from respective epoxides by the standard method which involved treatment of them in 80% formic acid under refluxing conditions for 1 hr followed by hydrolysis of resulting formates with methanolic sodium hydroxide under monitoring the reaction by thin-layer chromatography (TLC). Crude glycols obtained after the evaporation of the solvent from the reaction mixtures and subsequent extraction of them with ether were recrystallized from isopropyl ether when their carbon numbers were larger than 10. *n*-Heptane- and *n*-octane-1,2-diols were purified by distillation under reduced pressures. Homogeniety of the glycols was also confirmed by GLC using the 20% Apiezon L column after the conversion of them into the corresponding acetonides as mentioned below (Table I).

Enzyme Assay——Microsomes were prepared by the previously reported method[17] from the liver of three male albino rabbits (2.3—2.5 kg) and suspended in 0.1 M phosphate buffer, pH 7.4, so that 9.8 ml of the suspension was equivalent to 0.1 g of the tissue. Each mono-*n*-alkyl substituted ethylene oxide (10 μmoles) dissolved in acetone (0.2 ml) was added to the suspension and incubated at 37° in the region of zero-oder kinetics (5 to 30 min). The reaction was terminated by the addition of 5 N NaOH (1 ml) and the mixture was extracted with ether (30 ml) containing an internal reference (2 μmoles of the other 1-olefin glycol listed in Table I), following saturation of it with sodium chloride. The solvent was removed from the ethereal layer (20 ml), separated, on a water bath maintained at 42°. The residue was redissolved in a minimum volume of ether and then subjected to TLC (silica gel plates: 20×5 cm^2, 0.5 mm in thickness; C_6H_6–acetone (2: 1)). The chromatographic zone corresponding to the glycols was eluted with ethanol (10 ml) by the usual preparative TLC technique. *Rf* values of glycols were between 0.28 and 0.34 under the above mentioned conditions and indicated an increasing tendency according to the increase in their alkyl carbon numbers. However, no difference was observed in the values of a pair of glycols with the closest alkyl side chains. After the evaporation of the solvent from the ethanolic eluate under a nitrogen stream, the residue was dissolved in an acetonide reagent (0.2 ml), prepared by mixing dry acetone (10 ml) with 60% perchloric acid (2 drops), and left to stand for 30 min. The solution was then treated with finely powdered anhydrous sodium carbonate (50 mg) by vigorous agitation to destroy the acid catalyst. After the sedimentation of the inorganic salts, the supernatant containing glycol acetonides was subjected to GLC. GLC conditions used are described in Table I. Recovery of each glycol from the thin-layer adsorbent varied from 70 to 95%, depending on plates. However, its relative recovery to the other glycol added as the internal reference was always constant and estimated with an accuracy of more than 98%.

For the estimation of inhibitory effects of mono-*n*-alkyl substituted ethylene oxides on epoxide hydrolase activity, safrole oxide (SAFO) was used as the substrate.[18] SAFO (0.5 μmoles) was dissolved in ethanol (0.6 ml) containing the ethylene oxide (0.35 μmoles) and Tween 80 (3 mg) and diluted with 0.1 M phosphate buffer, pH 7.4, to make a final volume of 3 ml. To the substrate solution was added a suspension of microsomes in 0.1 M phosphate buffer, pH 7.4 (3 ml, equivalent to 3.5 mg of the rabbit liver), and the mixture was incubated at 37° for 10 to 30 min. The reaction was terminated by the addition of 5 N NaOH (1 ml), and the unreacted SAFO was extracted into Spectrosol-hexane (5 ml; Wako Pure Chemical Industries, Ltd., Tokyo). SAFO disappearance was determined by measuring absorbancy (*E*) at 288 nm of the hexane solu-

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tion separated after the centrifugation. Incubation mixtures containing boiled microsomal preparations and SAFO served as control reference. E value of a 0.1 mM solution of SAFO in the hexane was 0.398.

Result and Discussion

Hydrolysis of Mono-*n*-alkyl Substituted Ethylene Oxides by Hepatic Microsomes

The glycol resulted from the incubation of each mono-*n*-alkyl substituted ethylene oxide with rabbit liver microsomes was extracted into ether containing such an internal reference as the other ethylene glycol with close carbon number, *e.g.* authentic *n*-octane-1,2-diol to enzymatically formed *n*-heptane-1,2-diol (Table I). The extract was then treated on a silica gel thin-layer for separating them from the unreacted oxide. Both glycols formed an inseparable zone on the layer, being eluted, converted into the corresponding acetonides, and analyzed by GLC. An *S*-shaped curve was obtained by plotting rates of the enzymatic glycol formation vs. the alkyl carbon number of the substituted ethylene oxides; the rates were high in C_5-, C_{12}-, and C_{14}-substituted ethylene oxides and the lowest in the C_8-substituted one (Fig. 1).

Table I. Retention Times of Mono-*n*-alkyl Substituted Ethylene Glycol Acetonides in GLC

n-Alkyl substituent[a]	Retention times (min)[b] Column temperatures (°C)				
	150	170	200	230	260
C_5	7.2	—	—	—	—
C_6	14.7	6.1	—	—	—
C_8	—	14.4	8.8	—	—
C_{10}	—	—	17.1	7.6	—
C_{12}	—	—	—	15.8	7.6
C_{14}	—	—	—	—	14.3

a) Either of each pair of two ethylene glycols with closest carbon numbers was used as the internal reference for determining the enzymatically formed counterpart.
b) GLC conditions used: column—20 % Apiezon L coated on Chromosorb W (60—80 mesh), 180 cm × 4 mm; N_2 as carrier gas—34 ml/min. A Shimadzu Model GC-1C gas-chromatograph equipped with a flame ionization detector was used.

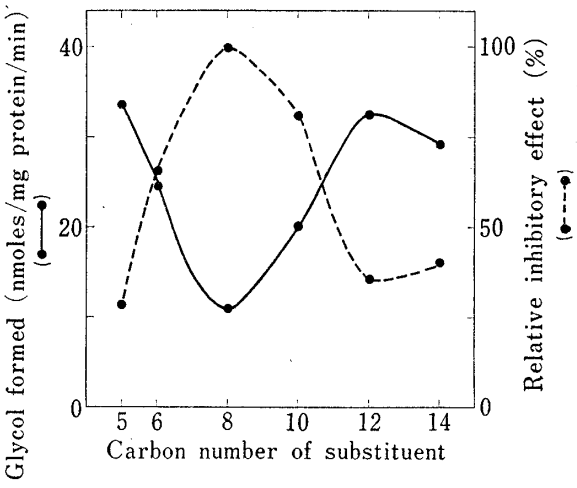

Fig. 1. Reaction Rates of Enzymatic Hydrolysis of Mono-*n*-alkyl Substituted Ethylene Oxides and Their Relative Inhibitory Effects on Epoxide Hydrolase Activity

Data are arithmetic mean values of at least three experiments.

Although it is difficult to interpret the phenomenon observed, this might be a reflexion from a structural moiety of the protein near the protonic active site of the hydrolase[19] through which an epoxide molecule interacts since the presence of hydrophobic binding sites of near the active site has been strongly suggested.[12] Based on this suggestion, the present data indicate that hydrophobic interactions with the substrate molecule through the binding sites decrease according to the elongation of the alkyl side chain and a hydrophilic repulsion occurs to dominate at the alkyl side chain length of C_8. One more hydrophobic site that interacts with the substituent would be present in the direction of chain elongation since an increase in alkyl carbon number up to 12 increases the reaction rate.

Inhibition of Epoxide Hydrolase Activity by Mono-*n*-alkyl Substituted Ethylene Oxides

Evidence for the inhibition of the epoxide hydrolase activity by epoxides has been presented by Watabe and Maynert, leading to the first success in trapping labile epoxy inter-

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mediates in the microsomal metabolism of olefins to glycols.[14,20] This method has been applied to detect labile K-region epoxides formed as intermediates to dihydrodiols during the microsomal metabolism of carcinogenic polycyclic aromatic hydrocarbons, *e.g.* cyclohexe oxide for biologically formed benzo[*a*]pyrene oxide[4] and styrene oxide for pyrene and benzo-[*a*]pyrene oxides.[15] These could indicate that olefins ingested or their epoxides formed in the hepatic metabolism may follow the *in vivo* accumulation of epoxides derived from toxic or carcinogenic olefins or arenes as a result of their inhibitory effects on hepatic epoxide hydrolase. A long term application of the C_{14}-alkyl substituted ethylene oxide to the rat causes carcinoma.[21] The recent work of Oesch, *et al.* has demonstrated a wide variety of olefin oxides to inhibit hepatic microsomal epoxide hydrolase activity and 1-octene oxide and trichloromethyl–ethylene oxide to be the most potent inhibitors.[22] However no systematic investigation has been done with a series of mono-*n*-alkyl substituted ethylene oxides. In the present study, effects of chain length of the epoxides on epoxide hydrolase activity was investigated by using safrole oxide (SAFO) as the substrate, stoichiometric conversion of which to the corresponding glycol, safrole glycol, by hepatic microsomes has been shown.[18]

All the epoxides (7×10^{-5}M each) used had inhibitory effects on the enzymatic hydrolysis of SAFO (10^{-4}M). Inhibition of the reaction was the most significant in the C_8-alkyl substituted ethylene oxide. The curve for relative inhibitory effects of the ethylene oxides on the enzymatic hydrolysis of SAFO showed a reciprocal pattern to that of the enzymatic hydrolysis of them (Fig. 1). The poorer substrates for epoxide hydrolase were the more potent inhibitors. This phenomenon would depend on the lowered actual concentrations of the inhibitors remaining unhydrolyzed since olefin glycols are known to have no inhibitory effect on the hydrolase activity.[9] The microsomes hydrolyzed SAFO at the rate of 0.064 μmoles/mg protein/min in the absence of the epoxides, but the C_8-alkyl substituted ethylene oxide lowered the rate to the extent of 72%.

The present results, especially the result of inhibition of the hydrolase by *n*-1-decene oxide, might provide a powerful tool for further investigations on biotransformation and toxicity mechanisms of olefins. A study on the effect of the pretreatment of animals with 1-decene or its epoxide on toxicity of other olefins simultaneously ingested is now in progress in our laboratory.

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