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Oxidation of cycloalkenes in liver microsomes*

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LIVER microsomes have been shown to mediate the oxidation of aromatic[1] and aliphatic[2–4] unsaturated hydrocarbons with the eventual production of glycols. Such reactions require NADPH† and oxygen, are enhanced in activity after treatment of animals with phenobarbital, and in other respects behave as mixed-function oxidations. Benzocycloalkenes such as 1,2-dihydronaphthalene and indene are also oxidized in such systems, and the diols produced are of the *trans*-configuration.[5,6]

Preliminary experiments indicated that the simpler cycloalkene cyclohexene is also oxidized to a diol product.[2] In the present report, experiments are described that demonstrate that cyclopentene, cyclohexene, and cycloheptene are converted to *trans*-diols in liver microsomes.

Cyclopentene (K & K Laboratories), cyclohexene (Eastman Organic Chemicals), and cycloheptene (K & K) Laboratories were commercial products. *cis*-Cyclopentane-, *cis*-cyclohexane-, and *cis*-cycloheptane-1,2-diols were prepared by permanganate oxidation of the respective cycloalkenes.[7] *trans*-Cyclopentane-1,2-diol was obtained by treatment of 2-chlorocyclopentanol with sodium carbonate, following the method described for the hydration of indene bromhydrin by Porter and Suter.[8] *trans*-Cyclohexane-1,2-diol was synthesized by alkaline hydration of cyclohexene oxide,[9] and *trans*-cycloheptane-1,2-diol was recrystallized from a commercial product (K & K Laboratories).

Livers of male Holtzman rats (100–150 g body wt.) or male New Zealand White rabbits (1·5–2·5 kg) that had been pretreated with phenobarbital (rats, 75 mg/kg/day; rabbits, 15 mg/kg/day) intraperitoneally for 3 days were homogenized in 4 vol. of 0·1 M tris buffer, pH 7·5. The supernatant fraction derived from centrifugation for 15 min at 9000 *g* was used immediately, or was lyophilized and stored at −15°. Microsomes were prepared from the 9000 *g* supernatant fraction by centrifugation at 105,000 *g* for 1 hr, followed by resuspension in tris buffer and recentrifugation. Lyophilized 9000 *g* supernatant fraction was reconstituted as needed by suspending in water.

The incubation mixtures were, except where noted, identical with those described for the oxidation of indene.[6] In experiments with cyclohexene, inclusion of nicotinamide, $MgSO_4$, EDTA, and ATP were required for optimum yields of *trans*-diol product; these compounds were also used in experiments with cyclopentene and cycloheptene, although the requirements were not tested with the latter substrates. Substrates were introduced as 10 per cent solutions in absolute ethanol (0·2 ml). Incubation time was 1 hr at 37° in experiments designed for identification of oxidation products, and 30 min in those described in Table 1. At the end of this time, 2·5 g NaCl was added, and the mixture was extracted with 5 ml of *n*-propanol (when the substrate was cyclohexene) or ethyl acetate (when the substrate was cyclopentene or cycloheptene). After evaporation of the extract to dryness at room temperature, the residue was taken up in 0·2 ml ethyl acetate and subjected to gas or thin-layer chromatography.

Gas chromatography was performed with F & M 400-series instruments, using 4 ft glass U-columns, a hydrogen flame ionization detector, and helium carrier gas at 100 ml/min.

For preparation of benzoyl derivatives, the dry reaction product (or a 25-μl sample of a reference diol) was treated with 20 μl benzoyl chloride and heated in a glass-stoppered tube in a boiling water bath for 1 hr. After addition of 0·2 ml water, the tube was again heated for 1 hr. After the contents were cool, 0·2 ml of 1 M NaOH were added and the derivative was extracted into 0·5 ml benzene.

Gas chromatographic evidence for the production of *trans*-1,2-cyclohexanediol by oxidation of cyclohexene in reconstituted lyophilized 9000 *g* supernatant fraction of rat liver is shown in Fig. 1. A peak identical in retention time to that produced upon injection of a reference sample of *trans*-1,2-cyclohexanediol was found in the experimental chromatogram, in an area devoid of peaks in a corresponding chromatogram of an extract of a similar reaction mixture in which the substrate was not added until the end of the incubation, immediately preceding extraction. No peak was found corresponding in reaction time to *cis*-1,2-cyclohexanediol.

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† Abbreviations used in this paper are as follows: ATP, adenosine triphosphate; EDTA, ethylene diamine tetraacetate; G6P, glucose 6-phosphate; NADH, reduced nicotinamide-adenine dinucleotide; NADP and NADPH, oxidized and reduced forms, respectively, of nicotinamide-adenine dinucleotide phosphate; tris, tris(hydroxymethyl)aminomethane.

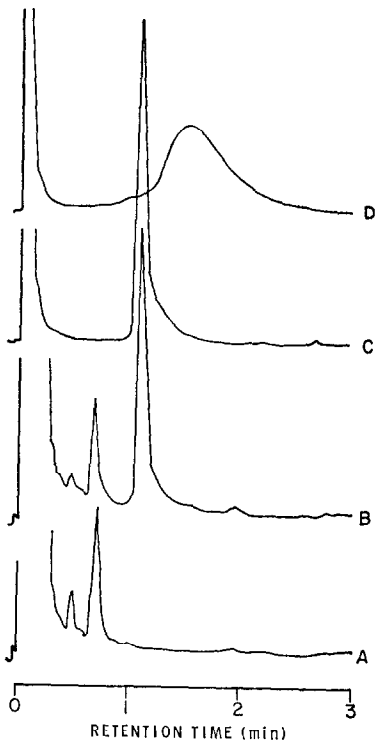

FIG. 1. Gas chromatogram of extracts of incubation mixture of rat liver 9000 *g* supernatant fraction with cyclohexene. Chromatographic conditions: 20 per cent Carbowax 20M on Chromosorb W, 60/80 mesh; column 225°, flash heater 340°, detector 260°. (A) Blank incubation mixture to which cyclohexene was added at end of incubation, immediately before extraction; (B) complete reaction mixture; (C) *trans*-cyclohexane-1,2-diol; (D) *cis*-cyclohexane-1,2-diol.

Oxidation of cyclohexene to the *trans*-diol was also demonstrated in rabbit liver preparations. As shown in Table 1, the diol was formed in freshly prepared 9000 *g* supernatant fraction of rabbit liver as well as in the reconstituted lyophilized preparation produced from the same fraction. The lyophilized preparation contained, in this case, 45 per cent of the activity of the freshly prepared supernatant fraction. Activity was also found in the microsomes prepared from the fresh supernatant fraction. About one-third of the activity was exhibited when NADH was substituted equimolarly for NADPH. Very little activity was found when NADP was substituted for NADPH or when no pyridine nucleotide was present. In no case was any peak corresponding to *cis*-1,2-cyclohexanediol found in the gas chromatogram.

The activity of the oxidative system was quite stable in the lyophilized 9000 *g* supernatant fractions. Thus, an enzyme solution reconstituted from 75 mg of a 63-day-old lyophilized preparation from rat liver catalyzed the formation of 285 nmoles cyclohexane-1,2-diol in the standard incubation system while, after another 71 days' storage at −15°, a similar amount mediated the production of 225 nmoles diol.

trans-Diol products were also found from the oxidation of cyclopentene and cycloheptene in reconstituted lyophilized 9000 *g* fractions from rabbit liver, as shown in Figs. 2 and 3. In each case a peak was found in the chromatogram of the experimental extract identical in retention time to that found for the respective *trans*-1,2-cycloalkanediol. In neither case was a peak found corresponding in retention time to a *cis*-1,2-cycloalkanediol.

Further evidence for the nature of the diol products of oxidation of the three cycloalkenes was afforded by thin-layer chromatography. Table 2 shows that in each case, only one metabolite spot was found, corresponding in R_f value to the *trans*-1,2-cycloalkanediol. The product of cyclohexene

TABLE 1. CYCLOHEXENE OXIDATION IN RABBIT LIVER PREPARATIONS

Preparation	Nucleotide*	Diol produced†
9000 *g* Supernatant fraction (freshly prepared)	NADPH	3·23
9000 *g* Supernatant fraction (freshly prepared)	NADP + G6P	3·14
9000 *g* Supernatant fraction (lyophilized)	NADP + G6P	1·42
Microsomes	NADPH	1·19
Microsomes	NADH	0·41
Microsomes	NADP	0·04
Microsomes	None	0·09

* Concentrations when used: NADH or NADPH, 0·3 mM; NADP, 0·06 mM; G6P, 5 mM.

† Micromoles of diol formed in 30 min per amount of preparation derived from 1 g (wet weight) liver. Amounts were estimated from gas chromatographic peak heights.

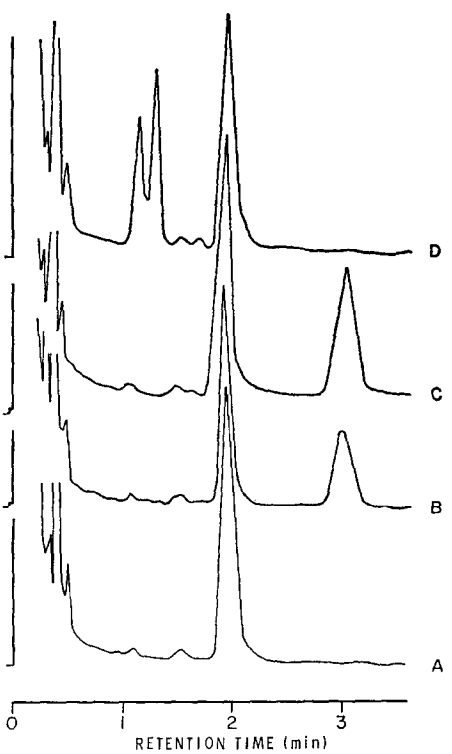

FIG. 2. Gas chromatograms of extracts of incubation mixtures of rabbit liver 9000*g* supernatant fraction with cyclopentene. Chromatographic conditions; 20 per cent Carbowax 20M on Chromosorb W, 60/80 mesh; column 200°, flash heater 195°, detector 315°. (A) Blank incubation mixture to which cyclopentene was added at end of incubation, immediately before extraction; (B) complete reaction mixture; (C) blank prepared as in A with addition of *trans*-cyclopentane-1,2-diol as well as of cyclopentene before extraction; (D) as in C with *cis*-cyclopentane-1,2-diol in place of *trans*-isomer.

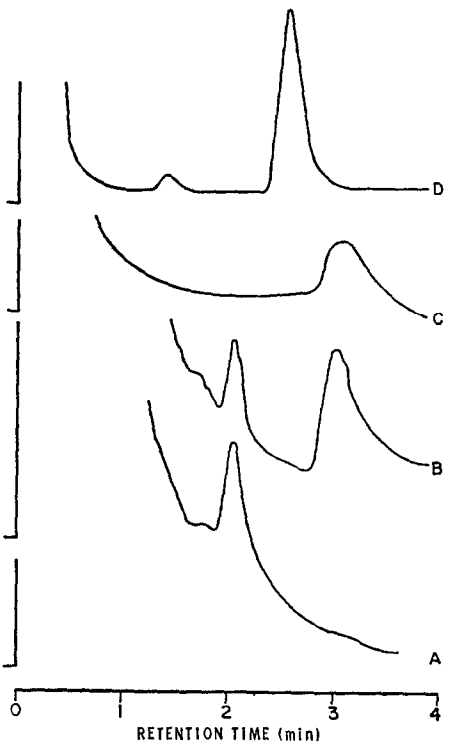

FIG. 3. Gas chromatograms of extracts of incubation mixtures of rabbit liver 9000*g* supernatant fraction with cycloheptene. Chromatographic conditions: 2 per cent OV-1/3 per cent OV-17 on Chromosorb W (AW-DMCS), 60/80 mesh; column 120°, flash heater 160°, detector 185°. (A) Blank incubation mixture to which cycloheptene was added at end of incubation, immediately before extraction; (B) complete reaction mixture; (C) *trans*-cycloheptane-1,2-diol; (D) *cis*-cycloheptane-1,2-diol.

TABLE 2. THIN-LAYER CHROMATOGRAPHY OF METABOLITES AND REFERENCE DIOLS*

		R_f		
		1,2-Diols		
Substrate	System†	*cis*-	*trans*-	Metabolite
Cyclopentene	1A	0·53	0·40	0·40
Cyclohexene	1B	0·64	0·55	0·56
	2	0·47‡	0·65‡	0·65‡
Cycloheptene	3	0·42	0·28	0·29

* All chromatography was performed on Eastman Chromagram sheets No. 6060 (silica gel coating with fluorescent indicator). Diols were located by spraying with ammoniacal silver nitrate;[6] benzoates were visualized under ultraviolet (2537 Å) light. Reference diols were dissolved in blank incubation mixtures and extracted in the same way as were the metabolites.

† (1) Organic phase from benzene–water–ethyl acetate–glacial acetic acid, shaken vigorously for 2 min, and allowed to stand for 30 min before separation; (1A) 3:3:3:1, v/v; (1B) 2:4:3:1, v/v. (2) Hexane–ether, 95:5 (v/v). (3) Organic phase from benzene–water–ethyl acetate, 4:2:4 (v/v).

‡ Benzoyl derivatives.

oxidation was chromatographed both as the free diol and as its benzoyl ester. In no case did a spot appear with the R_f of a *cis*-cycloalkanediol.

The oxidation of benzocycloalkenes in mammalian liver microsomes has been shown to result in the formation of glycols. In the metabolism of 1,2-dihydronaphthalene[5] and of indene,[6] *trans*-diols were found; in the latter case, the *trans*-isomer was shown to be the preponderant or sole product, since no *cis*-diol could be demonstrated by methods which would have detected quite small quantities. In both of these hydrocarbons, the olefinic double bond is situated α, β to the benzene ring. The same is true of styrene, which is also oxidized to a glycol in liver microsomes.[4] The present experiments show that the simpler cycloalkenes, in which there is no aromatic ring, are also oxidized in these organelles, and that the products, as in the cases of the benzocycloalkenes, are predominantly or solely of the *trans*-configuration.

The epoxides of styrene, indene, and cyclohexene are hydrated in liver microsomes.[10] In the cases of the cyclic compounds, the diol products are of the *trans*-configuration, the same isomeric form as that obtained in the overall oxidation of the hydrocarbons. Styrene oxide[11] and octene oxide[12] have been shown to be intermediates in the conversion of the respective unsaturated hydrocarbons to glycols. Very recently, it has been demonstrated that the oxidation of cyclohexene to its *trans*-diol also involves the intermediate formation of an epoxide.[13] The same pathway is probably involved in the oxidations of the other cycloalkenes reported here.

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Modification by psychotropic drugs of the cyclic adenosine monophosphate response to norepinephrine in rat brain*

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THE CATECHOLAMINES are thought to play an important role in brain function, and have been implicated in the mode of action of a number of psychotropic drugs.[1] Many of the peripheral actions of the catecholamines are known to be mediated by changes in the intracellular level of adenosine 3′,5′-monophosphate (cyclic AMP),[2] and there is now evidence to suggest that some of their central

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