Oxidation of Indene in Liver Microsomes

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SUMMARY

The oxidation of indene to *trans*-1,2-dihydroxyindane in liver preparations *in vitro* has been demonstrated. The reaction was mediated by liver microsomes of rats and rabbits in the presence of NADPH-generating systems. The oxidizing activity of the microsomes was markedly enhanced after pretreatment of the animals with phenobarbital. No *cis*-1,2-dihydroxyindane was found by methods which could have detected it at a level 0.5% of that of the *trans*-diol present in the reaction product. The *cis*-diol was not converted to its *trans*-isomer in the assay system. Indene epoxide was hydrated to *trans*-1,2-dihydroxyindane in the same *in vitro* system.

INTRODUCTION

Dihydrodiol metabolites isolated from the urine of rats or rabbits after administration of naphthalene or of other polycyclic aromatic hydrocarbons have been of the *trans*-configuration (1, 2). Boyland and Sims (3) showed that the rabbit can also oxidize the nonaromatic double bond of 1,2-dihydronaphthalene to the *trans*-diol, but did not report proof of the absence of the *cis*-isomer from the urine. Both *cis*- and *trans*-diol metabolites have been found to be excreted by rats and rabbits, however, after administration of other nonaromatic cyclenes, such as indene (4) and acenaphthylene (5). Whether or not both isomers are primary oxidation products in these cases is unclear, as administration of either the *cis*- or *trans*-isomers of 1,2-dihydroxyindane or of 1,2-dihydroxyacenaphthene was followed by the excretion of both geometric isomers of the respective diol (6, 7).

The oxidations of naphthalene to 1,2-dihydroxy-1, 2-dihydronaphthalene, and of 1,2-dihydronaphthalene to 1,2-dihydroxytetralin have been demonstrated *in vitro* in rat-liver microsomes in the presence of NADPH-generating systems (8, 9). In the latter case, a metabolite chromatographically identified as *trans*-1,2-dihydroxytetralin was obtained, but no evidence for the absence of the *cis*-isomer was cited. We have recently reported the formation of diols from indene and other unsaturated compounds in liver microsomes in the presence of NADPH[1]-generating systems (10). In the present paper, experiments on indene metabolism in such preparations are described, and it is demonstrated that *trans*-1,2-dihydroxyindane is the preponderant or sole geometric isomer formed (Fig. 1).[2]

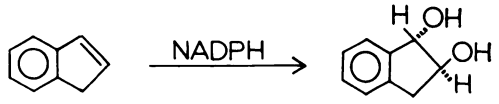

FIG. 1. *Formation of trans-1,2-dihydroxyindane.*

[1] Abbreviations used: ATP, adenosine triphosphate; EDTA, ethylenediamine tetraacetate; G6P, glucose 6-phosphate; NADP and NADPH, oxidized and reduced forms, respectively, of nicotinamide-adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)aminomethane.

[2] Part of this work was reported at a meeting of the American Society for Pharmacology and Experimental Therapeutics at Mexico City in July, 1966.

MATERIALS AND METHODS

Indene and indene epoxide (K & K Laboratories) were distilled and kept at −15°. *cis*-1,2-Dihydroxyindane (m.p. 98–98.5°) was synthesized by permanganate oxidation of indene, after the general method of Clarke and Owen (11) as described by Brimacombe *et al.* (12). *trans*-1,2-Dihydroxyindane was prepared in poor yield (m.p. 155–156°) by the alkaline hydration of indene epoxide, following a suggestion of van Loon (13), or more conveniently (m.p. 154–155°) from indene bromohydrin by the method of Porter and Suter (14).

Male Holtzman rats, 100–150 g, and New Zealand White rabbits, 1.5–2.5 kg, were used. Lyophilized 9000 *g* supernatant fractions of rat liver were prepared as described previously (15). Analogous preparations from rabbit liver were made similarly, except that homogenization was carried out in a Waring blendor rather than in Potter-Elvehjem homogenizers. Microsomal fractions were isolated by reconstituting lyophilized 9000 *g* supernatant preparations in water, centrifuging at 105,000 *g* for 1 hr, and washing the pellet by resuspending in 0.1 M Tris-HCl buffer, pH 7.5, and repeating the centrifugation.

Incubation mixtures[3] contained, unless stated otherwise, 100 μmoles of nicotinamide, 50 μmoles of $MgSO_4$, 6 μmoles of EDTA, 3 μmoles of ATP, 25 μmoles of G6P, 0.3 μmole of NADP, 1 mmole of Tris-HCl buffer, pH 7.5, and 75 mg of lyophilized 9000 *g* supernatant fraction in water q.s. 4.5 ml, to which was added 42.5 μmoles of indene in 0.5 ml of dimethylformamide. After gentle shaking in open flasks for 2 hr at 37°, the mixture was deproteinized by the addition of 0.5 ml of 4 M $HClO_4$, neutralized with 4 M KOH, and centrifuged. The supernatant solution was extracted with 5 ml of ethyl acetate, and 4 ml of the organic layer was removed; the remainder was shaken with another 5-ml portion of ethyl acetate, and a 5-ml sample of the organic phase was taken. The combined ethyl acetate extracts were placed in a 20-ml beaker and evaporated to dryness overnight at room temperature. The contents of the beaker were dissolved in 0.5 ml of ethyl acetate and the solution was transferred to a 12-ml glass-stoppered conical centrifuge tube and evaporated to dryness under a stream of nitrogen. The residue was used for the preparation of a derivative, or was dissolved in 0.1 ml of ethyl acetate for gas-liquid chromatography or thin-layer chromatography, or in 2 ml of water for colorimetric assay by a method described elsewhere (16).

Control flasks contained all components except for the substrate solution; these were incubated side-by-side with experimental flasks, and substrate solution was added immediately prior to deproteinization. Subsequent handling was identical to that given experimental reaction mixtures.

"Pesticidequality" ethyl acetate (Matheson Coleman & Bell) was used in all procedures. Efficiency of extraction was determined by adding measured amounts of *cis*- or *trans*-1,2-dihydroxyindane to incubated blank reaction mixtures and carrying out the extraction and gas chromatographic analyses. Cumulative recovery from 1, 2, 3, and 4 extractions were 34, 54, 62, and 64%, respectively, for the *cis*-diol, and 33, 49, 55, and 57% respectively, for the *trans*-isomer. These values were used for correction of the experimental assay data.

Gas-liquid chromatography was carried out in an F & M model 400 instrument, using 4-ft glass columns containing Carbowax 20M, 20% on Chromosorb W, 60/80 mesh. Column temperature was 225°, flash heater 340°, and hydrogen flame ionization detector 260°. Helium carrier flow rate was 100 ml/min. Quantitation was by measurement of peak area (disc integrator) or peak height; *trans*-1,2-dihydroxyindane gave quite symmetrical peaks, and the results from these two measurements were very similar.

For preparation of acetyl derivatives,

[3] The incubation mixture described is one which was developed for use with other substrates and was used for most experiments reported here. We have found that the separate omission of nicotinamide, $MgSO_4$, EDTA, or ATP has no significant effect upon the oxidative activity toward indene.

the dry incubation product (or 0.1-mg sample of a reference diol) was treated with 0.5 ml of acetic anhydride and heated in a glass-stoppered tube in a boiling water bath for 1 hr. The tube was opened after cooling, 0.5 ml of water was added, and the mixture was evaporated to near-dryness on a steam bath and then to dryness at room temperature. The residue was dissolved in 0.5 ml of benzene preparatory to thin-layer chromatography.

Trimethylsilyl ether derivatives were prepared as described elsewhere (17).

RESULTS

Identification of the Product as trans-1,2-dihydroxyindane

Aliquots (5 μl) of the ethyl acetate solution of metabolites derived from an experiment in which indene was incubated with the reconstituted lyophilized 9000 *g* supernatant fraction of the homogenized liver of a rabbit, which had been treated for 4 days with 15 mg/kg/day i.p. of sodium phenobarbital, were subjected to gas-liquid chromatography. Conditions and results are shown in Fig. 2. A peak was observed at a retention time of 13.2 min, which was identical with that obtained on injection of a solution of *trans*-1,2-dihydroxyindane in the gas chromatograph. Addition of *trans*-1,2-dihydroxyindane to a control extract gave a chromatogram identical to that of an experimental extract. Finally, the gas chromatogram of a "spiked" mixture, obtained by adding *trans*-1,2-dihydroxyindane to an extract of an experimental incubation mixture, showed only one peak in the area of *trans*-1,2-dihydroxyindane. *cis*-1,2-Dihydroxyindane gave a broad peak of low sensitivity between 2 and 3 min retention time in this system, and therefore would not be detected unless present in amounts equivalent to those of the *trans*-isomer.

Despite the many steps and transfers required in the procedure, peak heights in chromatograms of extracts of replicate experiments agreed to within 10%.

Thin-layer chromatograms of the metabolite and of its derivatives are described in Table 1. Diols were visualized under ultraviolet (2537 Å) light, or with Schiff's reagent after oxidation by sodium periodate (4), or with ammoniacal silver nitrate (prepared by treating 5% aq. $AgNO_3$ with concentrated NH_4OH dropwise until the precipitate redissolved); in the latter case, the chromatogram was heated at 110° to bring out brown spots on a tan background. In each case, spots were found with R_F corresponding to those obtained by subjecting *trans*-1,2-dihydroxyindane to the same treatment. Other spots appeared, near the origin and solvent front, in all chromatograms derived from complete reaction mixtures, including those from nonincubated control mixtures. No spot appeared in any experimental chromatogram with the R_F of *cis*-1,2-dihydroxyindane or an acetyl or trimethylsilyl derivative thereof. Experiments employing the most sensitive method (direct chromatography with visualization

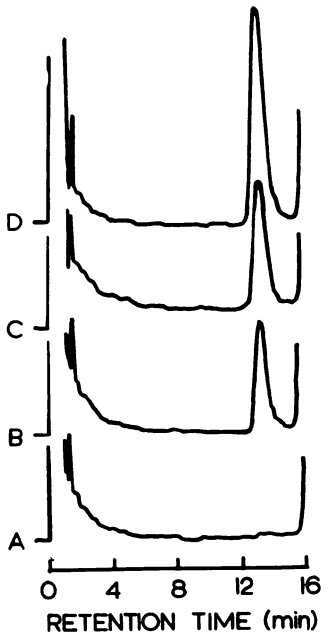

FIG. 2. *Gas chromatograms of reaction product extracts*

Chromatography of 5-μl aliquots carried out as described in the Materials and Methods section. *A*, control extract; *B*, experimental extract; C, control extract + 2 μg of *trans*-1,2-dihydroxyindane; *D*, experimental extract + 2 μg of *trans*-1,2-dihydroxyindane.

TABLE 1
Thin-layer chromatography of metabolite and derivatives

Experiment	Chromatography[a,b]	R_F 1,2-Dihydroxyindane *cis*-	1,2-Dihydroxyindane *trans*-	Metabolite
Direct chromatography	1	0.37	0.23	0.23
Acetyl derivative	2	0.38	0.50	0.50
Trimethylsilyl derivative	3			
Run I		0.10	0.22	0.22
Run II		0.19	0.36	0.33
Run III		0.28	0.45	0.44

[a] All chromatography was performed on Eastman Chromagram sheets No. 6060 (silica gel coating with fluorescent indicator).

[b] Conditions of chromatography: (1) Solvent system: Organic phase from benzene:water:ethyl acetate:glacial acetic acid (4:4:1:1, v/v), shaken vigorously for 2 min and allowed to stand for 30 min before separation. Visualization as described in text. (2) Chromagram sheet was activated at 110° for 15 min before applying samples. Solvent system: hexane:ether (9:1, v/v). Spots were visualized under ultraviolet light. (3) Chromagram sheet activated as above. Solvent system: heptane. Solvent front was run 12 cm from origin, sheet was air-dried, and spots were visualized under ultraviolet light. Sheet was then returned to development tank and solvent again run to 12 cm. After drying and location of spots, process was repeated a third time.

by periodate and Schiff's reagents) on varying amounts of the *cis*-diol showed that the limit of detection was 0.1–0.2 μg. This represents about 0.5% of the amount of *trans*-diol in the experimental extracts which were chromatographed. As noted in the Materials and Methods section, the efficiency of extraction of the two isomers was about the same.

Similar experiments demonstrated that the sole or preponderant diol product formed from indene by analogous preparations from the livers of phenobarbital-treated (75 mg/kg/day i.p. × 4 days) rats is also the *trans*-isomer. Thus, a gas chromatographic peak appeared with the retention time of *trans*-1,2-dihydroxyindane, and when the experimental extract was "spiked" with a known sample of the *trans*-diol, only one sharp peak was found in the chromatogram. Thin-layer chromatograms of the extracts yielded only spots with the R_F of the *trans*-diol, or spots corresponding to those found in nonincubated controls, and no spots with the R_F of *cis*-1,2-dihydroxyindane could be detected by any of the three visualization procedures described above.

In like manner, peaks with the retention time of *trans*-1,2-dihydroxyindane were observed upon gas chromatography of extracts from reaction mixtures using freshly prepared 9000 *g* supernatant fractions of liver homogenates from both rats and rabbits, whether or not the animals had been pretreated with phenobarbital. In one experiment, thin-layer chromatographic spots with the R_F of the *trans*-diol, but none with the R_F of the *cis*-diol, were found from incubation mixtures containing such fresh preparations from untreated and phenobarbital-pretreated rats.

Characteristics of the Reaction

Table 2 shows the results of a number of experiments in different preparations and under various conditions. Pretreatment of both rats and rabbits with phenobarbital caused an increase in the level of indene-oxidizing activity. Some of this effect may be due to an increased stability of the enzyme system from phenobarbital-treated animals. Thus, the ratio of product formed after 2 hr incubation to that after 15 min was higher for the preparation from phenobarbital-treated rabbits than was that for the control preparation. Nevertheless, the activity even in the first 15 min of the enzyme system from the pretreated animals was about 2.5 times that of the control system. The production of diol from indene in the presence of either rat or rabbit liver preparations was dependent upon the concentration of NADP in the assay system; the small degree of reaction observed in the absence of added NADP was probably due to the presence of cofactor in the enzyme preparation. G6P was included in the usual

TABLE 2
Oxidation of indene in liver preparations

Animal	Pretreatment	Preparation[a]	NADP (μM)	G6P (mM)	Diol formed[b] (mμmole/g liver)
Rat	None	F-9000	12	5	31
Rat	Ph[c]	F-9000	12	5	159
Rabbit	None	F-9000	12	5	210
Rabbit	Ph	F-9000	12	5	603
Rat	Ph	L-9000	0	5	92
Rat	Ph	L-9000	12	5	195
Rat	Ph	L-9000	60	5	304
Rat	Ph	L-9000	120	5	387
Rat	Ph	L-9000	180	5	377
Rat	Ph	L-9000	12	0	56
Rabbit	None	L-9000	60	5	591[d]
Rabbit	None	L-9000	60	5	611
Rabbit	Ph	L-9000	12	5	931
Rabbit	Ph	L-9000	60	5	1503[d]
Rabbit	Ph	L-9000	60	5	2330
Rabbit	Ph	L-9000	120	5	2070
Rat	Ph	Microsomes	—[e]	5	370

[a] F-9000, 2 ml of freshly prepared 9000 *g* supernatant fraction per flask; L-9000, 75 mg of lyophilized 9000 *g* supernatant fraction per flask; microsomes from approximately 95 mg of lyophilized 9000 *g* supernatant fraction per flask.

[b] Calculated on the basis of wet weight of liver from which the preparation was derived. In experiments with rat liver preparations, extracts from 4 flasks were combined for analysis. Analysis was by gas chromatography in all cases.

[c] Ph = sodium phenobarbital, i.p., 4 days, 75 mg/kg/day (rats) or 15 mg/kg/day (rabbits).

[d] Incubation time, 15 min.

[e] NADPH, 1.5 μmole, added at beginning of reaction and every 15 min thereafter for 2 hr.

assay mixture to assist in the maintenance of NADP in its reduced form, via the soluble G6P dehydrogenase contained in the preparation. When G6P was omitted, there was a marked diminution in extent of the reaction. Finally, when isolated microsomal fraction, separated from soluble enzymes, was employed with frequent addition of NADPH, production of *trans*-1,2-dihydroxyindane was demonstrated.

The results obtained by gas chromatography were verified by colorimetric assay of glycols. In one experiment performed in quadruplicate with lyophilized preparation from a phenobarbital-treated rabbit, an average production per gram of liver of 1.40 μmoles of *trans*-1,2-dihydroxyindane was demonstrated by gas chromatography, while 1.30 μmoles of glycol were found colorimetrically. Since the colorimetric method measures both *cis*- and *trans*-dihydroxyindane, this is further evidence that no significant amount of the *cis*-diol was produced.

Test for Diol Interconversion

It seemed possible that the appearance of only the *trans*-isomer of 1,2-dihydroxyindane as an oxidation product of indene could be due to rapid conversion of any *cis*-diol which might be formed to its *trans*-isomer. To test this possibility, amounts of *cis*-1,2-dihydroxyindane from 0.13 to 1.33 μmoles were incubated in the standard assay system containing lyophilized 9000 *g* supernatant fraction from phenobarbital-pretreated rabbits. Thin-layer chromatography of the extracts from these experiments yielded only spots with the R_F of the *cis*-compound, and no spots appeared with the R_F of the *trans*-isomer. In one case, measurement by gas chromatography

showed 94% recovery of *cis*-1,2-dihydroxyindane after a 2-hr incubation. It was concluded that the absence of the *cis*-diol from the indene reaction products was not due to its conversion to its geometric isomer during the course of the incubation or recovery procedures.

Metabolism of Indene Epoxide in Vitro

In preliminary experiments, amounts of indene epoxide of the same magnitude as those of indene used above, were incubated in place of indene in the standard assay system. Both *cis*- and *trans*-1,2-dihydroxyindane were found at the end of the incubation period, and it was concluded that indene epoxide could not be an intermediate in the oxidation of indene to its *trans*-glycol. It was then discovered, however, that the indene epoxide contained detectable amounts of both *cis*- and *trans*-1,2-dihydroxyindane. In subsequent experiments, smaller amounts (4 μmoles) of the epoxide, freshly vacuum-distilled, were used. Thin-layer chromatograms of the extracts of these reaction mixtures exhibited only spots of R_F corresponding to *trans*-1,2-dihydroxyindane, and none with the R_F of the *cis*-diol. The same result was obtained when the extracted product was acetylated and chromatographed as described previously; the sole diol derivative found was the *trans*-isomer, and the *cis*-compound could not be detected. Acetylation of indene epoxide and subsequent chromatography yielded spots for neither *cis*- nor *trans*-diol acetates. It is therefore apparent that both indene and its epoxide give rise to the same geometrical isomer of 1,2-dihydroxyindane in this *in vitro* system.

DISCUSSION

It has been demonstrated that indene is oxidized to *trans*-1,2-dihydroxyindane by microsomal preparations from rat and rabbit liver in the presence of NADPH. This reaction is similar to the formation of *trans*-1,2-dihydroxytetralin from 1,2-dihydronaphthalene in liver microsomes (9), although in that case, rigorous proof of the absence of the *cis*-diol was not presented. In the present experiments, *cis*-1,2-dihydroxyindane has been shown to be absent or formed, if at all, to an extent less than 0.5% of that of the *trans*-diol, in reconstituted lyophilized preparations from the liver of phenobarbital-pretreated rabbits. In lyophilized and fresh preparations of untreated and phenobarbital-pretreated rats and rabbits, the *trans*-diol was the only geometrical isomer detected.

In vivo, however, excretion of both *cis*- and *trans*-1,2-dihydroxyindane occurs after administration of indene to rats or rabbits (4). When either the *cis*- or *trans*-diol is administered to rats, both geometrical isomers are excreted in the urine (7). From the present work, it is apparent that the primary oxidation product of indene in liver microsomes is the *trans*-diol, and that conversion of *cis*- to *trans*-1,2-dihydroxyindane either occurs elsewhere in the body than in the endoplasmic reticulum of the liver, or is mediated by a microsomal enzyme which is destroyed during the process of enzyme preparation.

Epoxides were proposed by Boyland (18) to be intermediates in the addition of the elements of hydrogen peroxide across aromatic double bonds. Epoxides have also been postulated as intermediates in the production of glycols from nonaromatic cyclenes (3, 5, 9). After administration of indene epoxide to rats, both *cis*- and *trans*-1,2-dihydroxyindane were found in the urine (7). Both of these isomeric diols, however, were excreted when either of them was administered separately; it was therefore impossible to make a conclusion about precursor relationships among these compounds. In none of the above cases has an epoxide intermediate been demonstrated. Halogenated insecticides such as aldrin, however, are converted to epoxides in liver microsomes in the presence of NADPH (19). Hydration of dieldrin, the epoxy metabolite of aldrin, to *trans*-6,7-dihydroxydihydroaldrin has been shown in rabbits *in vivo* (20).

In the current work, no epoxide intermediate has been demonstrated. Nevertheless, a place for indene epoxide in the pathway of oxidation of indene in liver microsomes is not ruled out, as both indene

and its epoxide give rise to the same geometrical isomer of 1,2-dihydroxyindane in this system.

Indene thus joins 1,2-dihydronaphthalene as a class of compounds converted to *trans*-diols in liver microsomes in the presence of NADPH. In both cases, the aliphatic double bond which is oxidized occupies a 1,2-relationship to a benzene ring. The same is true of the aromatic double bonds of naphthalene, anthracene, and phenanthrene which are oxidized to dihydrodiols (1, 2). A dihydrodiol metabolite of benzene, or of other aromatic compounds where the target double bond is other than α to an aromatic ring, has apparently not been discovered. We have, however, reported the oxidation of cyclohexene to its glycol in liver microsomes (10), and have recently determined that the metabolite is of the *trans*-configuration (unpublished). Investigations on similar cyclene systems are in progress.

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