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Oxidative cleavage of the ethylenic linkage of stilbene by rabbit liver microsomes

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A variety of ethylenic compounds such as stilbene [1], safrole [2], isosafrole [2], and quinine [3] have long been known to undergo oxidative cleavage in the animal body and excreted as carboxylic acids, e.g. in 1939 Stroud [1] demonstrated benzoic acid to be the major urinary metabolite of stilbene in the rabbit. Similarly, double bonds of carcinogenic hydrocarbons with olefinic character such as $C_{1,2}$ of acenaphthylene [4] and the K-region of dibenz(*a,h*)anthracene [5] are also known to be oxidatively cleaved into dicarboxylic acids *in vivo*. In microorganisms, aromatic double bonds such as those of benzene, naphthalene, and anthracene are cleaved by a dioxygenase via catechols [6]. Although nothing is known as yet of the cleavage mechanism of ethylenic double bonds, including that of stilbene, an attempt to approach this problem has been made by Hopkins [7] by using acenaphthene-1,2-diols as substrates. During the course of the investigation on hepatic microsomal interconversion of *cis*- and *trans*-acenaphthene-1,2-diols both of which are urinary metabolites of acenaphthylene and of either *cis*- or *trans*-acenaphthene-1,2-diol, a hypothetical ketol intermediate was thought to be unstable and spontaneously rearrange to the chemical equivalent, 1,8-naphthalic aldehyde, being enzymatically [8] or non-enzymatically [9] converted to 1,8-naphthalic acid. Further investigations carried out by Drummond *et al.* [10], however, failed to detect the aldehyde in their microsomal reaction system. The procedure used by Hopkins for the isolation of the dicarboxylic acid was found later to be so drastic that acenaphthene quinone formed from the diols by the microsomes was autoxidized and failed to prove whether or not the acid formation was enzymatic [10, 11]. In view of oxidative cleavage mechanism involving a new system other than dioxygenation, the Hopkins' assumption is worthy of reconsideration since a wide variety of olefins [12–15] and arenes [16, 17] have recently been shown to be oxidized by hepatic microsomal monooxygenase to epoxides and subsequently hydrolyzed to glycols. This metabolic reaction is strongly suggested to be closely related to a carcinogenesis mechanism involving aromatic hydrocarbons such as benzo(*a*)pyrene, dibenz(*a,h*)anthracene, and 7-methylbenzanthracene which are converted by hepatic microsomes into the corresponding K-region epoxides as proximal active carcinogens [18–21].

Very recently, we have demonstrated that *cis*- and *trans*-stilbenes are also converted by rabbit liver microsomes stereospecifically to *threo*- and *meso*-1,2-diphenyl-1,2-ethanediols, respectively, via the corresponding epoxides [22, 23]. Furthermore, it was shown that a 9000 *g* supernatant of a rabbit liver homogenate catalyzed the interconversion of the *threo*- and *meso*-glycols [23]. This fact would reasonably indicate that the ketol, benzoin (benzoylphenylcarbinol) which is considerably stable, is an intermediate for the interconversion. A promising approach to the problem of the oxidative cleavage of ethylenic double bonds by hepatic microsomes could be to use the stilbene glycols as model substrates and to prove the intermediacy of the ketol and whether it is a precursor of benzoic acid or not.

For the isolation and identification of the intermediate,

benzoin, both *threo*- and *meso*-glycols (10 μmoles each dissolved in 0·2 ml acetone) were separately incubated at 37° and pH 7·4 (0·1 M phosphate buffer to make a final volume of 10 ml) for 10 min with a 9000 *g* supernatant reconstituted by twice washed microsomes and dialyzed soluble supernatant (both equivalent to 2 g of the rabbit liver), in the presence of either NADP or NAD (5 μmoles) and magnesium chloride (50 μmoles). The reaction mixture was then extracted with peroxide free ether. After the evaporation of the solvent under a nitrogen stream, the residue was subjected to preparative t.l.c. [silica gel, benzene–acetone (4:1)] for eliminating ether-extractable subcellular components and the substrates. Chromatographic zones at R_f 0·25 and 0·65 were separately collected and eluted with ethanol. After the evaporation of the solvent from the eluates under a nitrogen stream, the R_f 0·25 material was trimethylsilylated in the standard manner. Both samples thus obtained were analyzed by g.l.c.-mass spectroscopy using a Hitachi GC–MS system. The trimethylsilylated R_f 0·25 material was identified as benzoin from results of chromatographic data (retention time: 6·1 min on a 3% OV-17 column used at 170°) and of the spectrum which showed ion peaks at *m/e* 179 [base peak, Ph(TMSO) = CH^+] and 105 (Ph – CO^+) and the R_f 0·65 material as benzil (bibenzoyl, retention time: 7·5 min on the above-mentioned column used at 165°) the spectrum of which showed ion peaks at *m/e* 210 (M^+) and 105 (base peak, Ph – CO^+). Both NADP and NAD were effective as acceptors for hydrogens arising from the glycols. A boiled 9000 *g* supernatant or either washed microsomes or dialyzed soluble supernatant alone had no ability to yield benzoin as well as benzil from the glycols.

Reaction rates of conversion of benzoin to the *threo*- and *meso*-glycols were estimated using a 15% succinate polyester column by g.l.c. as previously reported [22]. The enzyme catalyzing the reduction of benzoin was localized in the soluble supernatant but not in the microsomes and yielded both *threo*- and *meso*-glycols with a ratio of 10:1. NADPH was a six times more effective cofactor than NADH for the enzyme reaction. The same system catalyzed the reduction of benzil to the glycols and the ratio of both glycols formed was almost the same as that in the reduction of benzoin. No glycol formation was observed when they were incubated with a boiled soluble supernatant in the presence of the reduced pyridine nucleotides.

To determine whether or not benzoin or benzil is a precursor of benzoic acid, the ketol and the diketone, which were recrystallized several times from ethanol before use until they were free from benzoic acid and benzaldehyde, were incubated with washed microsomal preparations. After the addition of hydrochloric acid for terminating the reaction and restraining biologically formed benzoic acid from dissociation, the mixture was extracted with light petroleum, and the extract analyzed by g.l.c., following treatment of it with a solution of diazomethane in light petroleum. Both benzoin and benzil yielded benzoic acid by the incubation with the microsomes in the presence of an NADPH-generating system (Table 1). The benzoic acid formation was not observed when both substrates were incubated in the buffer alone, indicating that they are stable in the buffer and during subsequent analytical procedures. Use of boiled microsomes in the presence and in the absence of the NADPH-generating system yielded only small amounts of benzoic acid. These data indicate that the cleavage reactions are clearly enzymatic. Formation of small amounts of the acid was also observed when the substrates were incubated with either boiled or untreated microsomes alone. This could indicate that interaction of the substrates with microsomal components during incubations or/and subsequent analytical procedures also leads to the oxidative cleavage of the ketones to some extent. Thus, the oxidative cleavage of the ethylenic linkage of stilbene in the rabbit is now depicted as illustrated in Scheme 1. Further investigation on the cleavage mechanism is now in progress in our laboratory.

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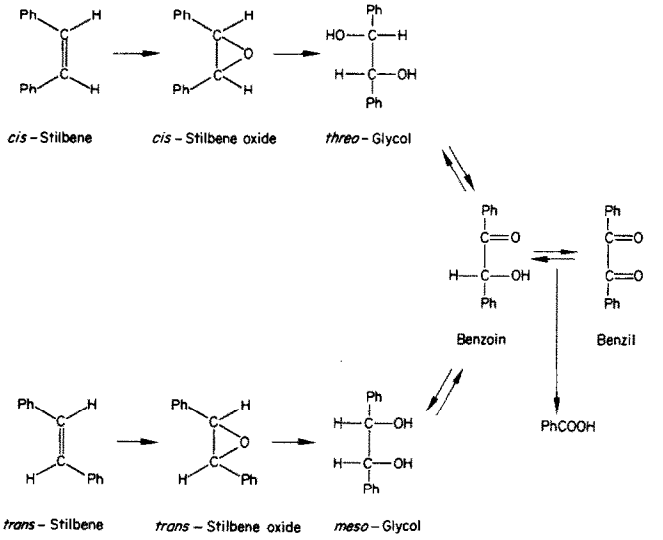

Scheme 1

Table 1. Benzoic acid formation from benzoin and benzil by rabbit liver microsomes

Reaction system	Benzoic acid formed (nmoles)	
	Substrates	
	Benzoin	Benzil
Ms + NADPH-G	206	197
Boiled Ms + NADPH-G	12	11
Ms + NADP	16	15
Ms	16	14
Boiled Ms	9	8
No Ms	0	0

Abbreviations used—Ms: twice-washed microsomes and NADPH-G: NADPH-generating system. The complete reaction system, Ms + NADPH-G, consisted of twice-washed microsomes equivalent to 2 g of rabbit liver, 10 μmoles of the substrate dissolved in 0·2 ml acetone, 5 μmoles of NADP, 50 μmoles each of glucose 6-phosphate, magnesium chloride, nicotinamide, 5 i.u. of glucose 6-phosphate dehydrogenase, and 0·1 M phosphate buffer, pH 7·4, to make a final volume of 10 ml. In the system Ms + NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were omitted from the complete system; in the system Ms, cofactors and all the other fortifying agents were omitted, and in the system No Ms, the substrates were incubated with the buffer alone. The reaction was carried out in air at 37° for 2 hr and terminated by the addition of 1 ml of concentrated hydrochloric acid. Benzoic acid was extracted into 30 ml of light petroleum (bp 34–35°) at a recovery rate of 83 per cent, following saturation of the mixture with sodium chloride. Twenty ml of the petroleum extract separated by centrifugation was transferred into a 50-ml test tube and the solvent removed during a period of 1 hr on a water bath maintained at 40°. The residue was treated at 2° for 30 min with a solution of excess diazomethane in 1 ml of light petroleum. After the removal of unreacted diazomethane together with the solvent on the water bath, the residue obtained was redissolved in *n*-hexane and subjected to g.l.c. Chromatographic conditions: gas-chromatograph—Shimadzu Model GC-1C equipped with a flame ionization detector; column—15% succinate polyester on Shimalite (60–80 mesh, 4 mm × 180 cm); nitrogen as carrier gas—35 ml/min; column temperature—180°; retention time of methyl benzoate—6·2 min.

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