OXIDATION OF TOLUENE BY THE MICROSOMAL MONOOXYGENASE SYSTEM OF RAT LIVER

A. JINDRA JR[a], Z. ŠÍPAL[a] and A. JINDRA[b]

[a]*Department of Biochemistry, Charles University, Prague 2,*
[b]*Department of Biochemistry and Microbiology,*
Faculty of Pharmacy, Komenský University, Bratislava

Received July 8th, 1971

The microsomal fraction of rat liver homogenate hydroxylates the methyl group of toluene to a primary alcohol. The apparent K_m of the process is $8{\cdot}3 \cdot 10^{-4}$M. The reaction is competitively inhibited by methylcyclohexane, its rate is not affected by benzene. Both methyl derivatives are probably hydroxylated by the same system which attacks preferentially the aliphatic side chain.

One of the biotransformation reactions of foreign organic compounds is the oxidation of the methyl group attached to the carbon atom which leads *via* a hydroxymethyl group all the way to a carboxygroup. Numerous examples of this reaction of aliphatic hydrocarbons, as well as of the side chains of aromatic hydrocarbons are known[1,2] where, using both *in vivo*[3] and *in vitro*[4] experiments, the metabolites formed were identified usually as carboxylated derivatives. The behaviour of the methyl group attached directly to the alicyclic, aromatic or heterocyclic ring was studied relatively infrequently *in vivo*[5] and still less so *in vitro* with enzyme preparations. Thus, *e.g.* oxidation of methylcyclohexane by the microsomal monooxygenase system elucidated the differences in sensitivity to attack of primary, secondary and tertiary C—H bonds during hydroxylation of the aliphatic system. Oxidation of methylcyclohexane takes place mostly at the ring while the oxidation of the methyl group is negligible[6]. With the aromatic analogue methylcyclohexane, *i.e.* toluene, Daly and coworkers[7] found in the guinea-pig and rabbit liver homogenates that benzyl alcohol was preferentially formed together with minute amounts of derivatives hydroxylated at the ring. Similarly, it was found[8] that *p*-nitrotoluene was transformed in the microsomal fraction of rabbit liver homogenate *via* *p*-nitrobenzyl alcohol and *p*-nitrobenzaldehyde to *p*-nitrobenzoate.

In agreement with the paper[7] it was possible here to detect benzyl alcohol as the main product of hydroxylation of toluene in the microsomal preparation of rat liver. In view of the difference in the relative sensitivity to attack of the carbons of the methyl group and of the ring in the alicyclic and the aromatic system we were interested in the possibility of mutual interaction of the two substrates during hydroxylation.

EXPERIMENTAL

Preparation of the microsomal monooxygenase system. The microsomal fraction of rat liver (Wistar males, 150 g, Velaz) was isolated according to[9]. The liver was homogenized in 0·25M

sucrose containing 0·001M-EDTA (pH 7·5) and after removing the coarse fractions by sequential centrifugation at 600 *g*, 12000 *g* and 18000 *g* (10 min each), the microsomal fraction was obtained by 60 min centrifugation at 105000 *g* (Janetzki VAC 60, 8 × 10 ml head). The sediment was washed by suspending it in 0·15M-KCl (half volume of the original homogenate) and, after centrifugation (30 min, 105000 *g*), the sediment was suspended in 0·15M-KCl containing 0·06M Tris buffer of pH 7·5 in a ratio of 1 ml per 2 g liver.

The protein content was determind by the biuret reaction according to[10] using bovine serum albumin as standard.

Incubation of the sample. The incubation mixture contained 200 μmol Tris buffer of pH 8·5, 5 μmol NADP (Arzneimittelwerk, Dresden), 50 μmol sodium salt of glucose-6-phosphate, 6 μmol sodium ATP (Reanal), 260 Kornberg units of glucose-6-phosphate dehydrogenase (Sigma, Type V), 1·0 ml suspension of the microsomal fraction and an appropriate amount of toluene in a total volume of 3·8 ml. Before adding substrate and the microsomal fraction the mixture was flushed with oxygen for 1 min. The mixture was incubated under shaking (120 strokes/min) in closed centrifuge tubes in a Dubnoff incubator (Development Workshop, Czechoslovak Academy of Sciences) at 37°C. The reaction was stopped by adding 0·2 ml 20% zinc sulphate and 0·2 ml saturated barium hydroxide; the precipitated protein was separated by centrifugation (4000 g, 10 min) and the supernatant used for analysis.

Isolation of benzyl alcohol. Deproteinized aqueous solution after incubation was shaken in a ground stopper tube with 12·5 ml ether for 1·5 min. The ether phase (10 ml) was separated and replaced with 10 ml pure ether. The mixture was shaken again, another 10 ml of the ether phase were added, the two extracts were combined, dried with anhydrous sodium sulphate and evaporated *in vacuo* at 20°C, the walls of the tube were rinsed with ether which was re-evaporated, and the residue was diluted with methanol to 0·20 ml.

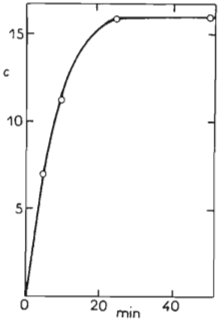

FIG. 1

Dependence of Toluene Oxidation on Incubation Time

Toluene concentration 12·3 mM. *c* μmol benzyl alcohol/mg protein.

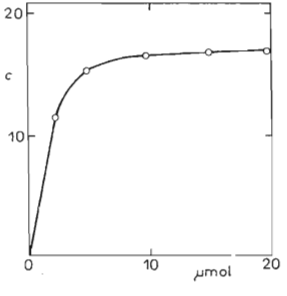

FIG. 2

Dependence of the Rate of Toluene Oxidation on Substrate Concentration

c μmol benzyl alcohol/mg protein min.

Qualitative assay and quantitative determination of benzyl alcohol. Benzyl alcohol was estimated by thin-layer chromatography on silica gel (Kieselgel GF_{254} Merck, 20 × 20 cm plates, layer thickness 0·3 mm, activated for 1 h at 105°C), in benzene–ethyl acetate (95 : 5). For detection we used fluorescence quenching under a mercury-discharge tube Mineralight SL 2537 and bleaching of potassium permanganate solution. The R_F value for benzyl alcohol was 0·20. To determine benzyl alcohol we used gas chromatography (Chrom 31, Laboratorní přístroje, Prague) on unglazed tile (0·20—0·315 mm) wetted with 4% polyethylene glycol adipate (length of column 1·2 m, temperature 165°C, temperature of injector 240°C, carrier gas nitrogen, 0·3 kp/cm^2). 6 µl samples were placed on the column with a Jintan Terumo Microsyringe MS-10. The retention time of benzyl alcohol under the conditions was 7·5 min. For the evaluation, a derivation record was used, the area under the peak of benzyl alcohol being measured planimetrically. The amount of benzyl alcohol in sample was read from a calibration graph (linear between 0·1 and 0·5 µmol benzyl alcohol per incubated sample).

RESULTS AND DISCUSSION

The ability of the microsomal monooxygenase system from rat liver to oxidize toluene to benzyl alcohol was demonstrated by thin-layer chromatography. After 60 min incubation at 37°C of a sample containing 50 µmol toluene the benzyl alcohol formed was identified by comparison with a standard. The heat-denaturated (10 min at 100°C) sample was inactive. Dependence of oxidation on time (Fig. 1) is linear for the first 10 min, after 30 min the amount of benzyl alcohol formed attains a constant value. It follows from the dependence of the rate of toluene oxidation (Fig. 2) that above 4·3 mM toluene the amount of benzyl alcohol formed does not depend on substrate concentration. At this concentration of toluene the conversion to benzyl alcohol amounts to 2·5%. Fig. 3 reveals an apparent K_m 8·3 . 10^{-4}M.

It follows from a comparison of the results of Frommer and coworkers[6] and Daly and coworkers[8] as well as from the present results that hydroxylation of aliphatic (and alicyclic) hydrocarbons proceeds differently from that of the aromatic ones.

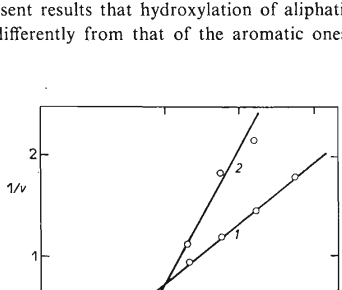

FIG. 3

Effect of Methylcyclohexane on the Rate of Toluene Oxidation

1 No inhibitor, *2* 2·1 mM methylcyclohexane. For experimental conditions see text. 1/v = min. mg protein/nmol benzyl alcohol.

In the former case, preferential attack is observed on the carbon atom with a lower number of hydrogen atoms, in the latter case the methyl group is attacked in preference to the aromatic carbons. It was thus of interest to examine the mutual influence of the two types of substrate. A standard, enzymically active, mixture was combined in one case with toluene alone (2–10 μmol per sample), in another case the sample contained toluene plus 8 μmol methylcyclohexane. As shown in Fig. 3, methylcyclohexane inhibits competitively the formation of benzyl alcohol from toluene. It may thus be assumed that the hydroxylation of both types of cyclic hydrocarbons is catalyzed by a single enzyme system. With aliphatic compounds, greater affinity is shown by carbon atoms with a smaller number of hydrogens attached; on the other hand, the aromatic ring is attacked less strongly than the primary carbon of the methyl group. In agreement with this, benzene which is hydroxylated *in vivo*, did not affect the rate of benzyl alcohol formation from toluene in analogous experiments. This is in agreement with the finding that the affinity of benzene for cytochrome P-450 which is taken for the actual hydroxylation site in the microsomal monooxygenase system (expressed by the magnitude of the so-called spectral dissociation constant of the enzyme-substrate complex[11]) is much lower than the affinity of toluene[12].

REFERENCES

1. Williams R. T.: *Detoxication Mechanisms.* Wiley, New York 1959.
2. Parke D. V.: *The Biochemistry of Foreign Compounds.* Pergamon Press, Oxford 1968.
3. Gillette J. R. in the book: *Progress in Drug Research* (E. Jucker, Ed.), Vol. 6, p. 13. Birkhäuser, Basel 1963.
4. Parke D. V. in the book: *Recent Advances in Pharmacology* (J. M. Robson, R. S. Stacey, Eds), Fourth Edition, p. 29. Churchill, London 1968.
5. Gillette J. R.: Advan. Pharmacol. *4*, 219 (1966).
6. Frommer U., Ullrich V., Staudinger H. J.: Z. Physiol. Chem. *351*, 903 (1970).
7. Daly J., Jerina D., Witkop B.: Arch. Biochem. Biophys. *128*, 517 (1968).
8. Gillette J. R.: J. Biol. Chem. *234*, 139 (1959).
9. Remmer H., Greim H., Schenkman J. B., Estabrook R. W. in the book: *Methods in Enzymology*, (S. P. Colowick, N. O. Kaplan, Eds) Vol. X, p. 703. Academic Press, New York 1967.
10. Cleland K. W., Slater E. C.: Biochem. J. *53*, 547 (1953).
11. Schenkman J. B., Remmer H., Estabrook R. W.: Mol. Pharmacol. *3*, 113 (1967).
12. Šípal Z.: Unpublished results.

Translated by A. Kotyk.