Rapid Papers

(Pages 803–824)

Transannular Dioxygenation of 9,10-Dimethyl-1,2-benzanthracene by Cytochrome *P*-450 Oxygenase of Rat Liver

By CHIADAO CHEN and MEI-HWA TU

Department of Biochemistry, Northwestern University Medical and Dental School,
Chicago, IL 60611, U.S.A.

(Received 13 September 1976)

9,10-Dimethyl-1,2-benzanthracene is oxygenated by rat liver microsomal cytochrome *P*-450 oxygenase to its 9,10-epidioxide. This transannular 1,4-peroxide is converted further into the diol by the microsomal preparation and NADPH. These two products constitute the majority of the metabolites found under the conditions described.

Rat liver microsomal cytochrome *P*-450 oxygenase has been shown to produce hydroperoxides as the initial oxygenation products with aliphatic (Chen & Lin, 1968, 1969) and aromatic (Shaw & Chen, 1972; Chen & Shaw, 1974) substrates. Peroxide production by the same enzyme system is a possibility. The present paper describes the transannular 1,4-peroxidation of 9,10-dimethyl-1,2-benzanthracene (I), a well-known chemical carcinogen, to yield 9,10-dimethyl-1,2-benzanthracene 9,10-epidioxide (II). The latter is further reduced to *cis*-9,10-dihydroxy-9,10-dimethyl-1,2-benzanthracene (III) (Scheme 1).

Materials and Methods

9,10-Dimethyl-1,2-[9-¹⁴C]benzanthracene (compound I) was purchased from Amersham/Searle, Arlington Heights, IL, U.S.A., and used as such. It contained about 0.2% of 9,10-dimethyl-1,2-[9-¹⁴C]benzanthracene 9,10-epidioxide (compound II) when purchased, as detected by t.l.c., probably owing to photo-oxidation during the manufacturing and/or the t.l.c. process. As the quantity of 9-¹⁴C-labelled compound (II) in 9-¹⁴C-labelled compound (I) was small and as all experiments were performed with a control, the final results should not be

Scheme 1

adversely affected. Unlabelled compound (I) was from Eastman Kodak, Rochester, NY, U.S.A. Compound (II) and 9-¹⁴C-labelled compound (II) were synthesized by the method of Cook & Martin (1940). Compound (III) was synthesized by refluxing compound (II) in methanolic 10% (w/v) KOH with an excess of zinc dust, giving a product with m.p. 186.5–187.5°C [given, 183–184°C (Cook & Martin, 1940), by a catalytic reduction method]. 10-Hydroxymethyl-9-methyl-1,2-benzanthracene was synthesized as described by Boyland & Sims (1965). These samples were purified by repeated recrystallization from more than two solvent systems. Their authenticity and purity were further confirmed by t.l.c., mass spectroscopy and u.v. spectrophotometry.

Microsomal preparations were obtained from young male rats as described by Shaw & Chen (1972). Except where indicated, the basic incubation mixture contained: 300 µg of 9-¹⁴C-labelled compound (I) (5×10^5 c.p.m.), or 410 µg of 9-¹⁴C-labelled compound (II) (8×10^4 c.p.m.); 2 µmol of NADPH; 10 µmol of EDTA; 10 mg of microsomal protein; and 500 µmol of sodium phosphate buffer, pH 7.4; total volume 5 ml. The incubations were performed in Erlenmeyer flasks in the air for 15 min at 30°C. For incubation in a CO atmosphere, Thunberg tubes were used instead of Erlenmeyer flasks. The substrate, NADPH and buffer were placed at the bottom of the tube, and the microsomal fraction in the side reservoir. The tubes were evacuated (2 kPa; 15 mmHg), and refilled with CO+O₂ (80:20). The process was repeated twice. The control tube was refilled with air instead of CO+O₂ after the third evacuation. After the last gas filling, the microsomal preparation in the reservoir was tilted into the tube, when incubation commenced. After incubation, 0.5 ml of methylene chloride was added, thoroughly mixed, and centrifuged at 100g-min to obtain clear separation of two layers. Then 0.2 ml of methylene chloride extract was spotted on a

10cm-long silica-gel-coated aluminium sheet (EM, silica gel 60 F-254) and chromatographed with chloroform until the solvent front reached the top of the plate. The spots, which were detected by u.v. illumination, were cut out and extracted with diethyl ether. Portions of extract were added to vials containing 10 ml of scintillation fluid [0.4% 2,5-diphenyloxazole and 0.005% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene] and counted for radioactivity in a scintillation counter (Isocap 300, Nuclear-Chicago). All the operations before the completion of chromatographic separation were conducted with a minimum amount of light or in total darkness.

Results and Discussion

Incubation of 9-¹⁴C-labelled compound (I) yielded four well-defined spots on t.l.c.: substrate, R_f 0.8; compound (II), R_f 0.65; 10-hydroxymethyl-9-methylbenzanthracene, R_f 0.35; and compound (III), R_f 0.18. The compound-(II) spot appeared to be a pure single component, but trace contaminants may have been present in the compound-(III) spot. The three product spots accounted for over 90% of the radioactivity not contained in the original substrate spot; of this, 20–25% was 10-hydroxymethyl-9-methylbenzanthracene. The formation of these products was decreased substantially by SKF-525A, metyrapone and CO, all known inhibitors of cytochrome *P*-450 oxygenase (Table 1). Enzymic activity, localized in the microsomal fraction, was abolished by pretreatment with Pronase. The small amount of product obtained on incubation with Pronase-treated enzyme was about the same as that obtained on incubation with buffer alone, and was attributable to impurities in the substrate and light-sensitized oxygenation during manipulations. Since it was impossible to conduct experiments in total darkness, some photosensitized

Table 1. Conversion of compound (I) into compound (II) and compound (III)

9-¹⁴C-labelled compound (I) (300 µg; 4.95×10^5 c.p.m.) was incubated with 10 mg of rat liver microsomal proteins and 2 µmol of NADPH in 5.0 ml of phosphate buffer, pH 7.4, containing 2 mM-EDTA at 30°C for 15 min in air. Oxygenase inhibitors used were SKF-525A or metyrapone (0.2 mM). The incubation products were extracted with methylene chloride and subjected to t.l.c. on an aluminium-supported silica sheet (EM; silica gel 60 F-254), with chloroform as the developing solvent. The spots were cut out and counted for radioactivity. For the Pronase treatment, microsomal fraction was pretreated with 2 mg of Pronase for 10 min at 30°C before the addition of compound (I) and NADPH.

Products	Additions to microsomal fraction						Buffer only
	No addition	NADPH	NADPH, SKF-525A	NADPH, metyrapone	NADPH, CO+O ₂ (80:20)	Pronase, NADPH	
Compound (II) (c.p.m.)	2348	4327	1530	703	1370	1319	1412
Compound (III) (c.p.m.)	5018	9350	1091	2549	4646	897	900
Total (c.p.m.)	7456	13677	2621	3252	6016	2216	2312
(% of that with NADPH)	54	100	19	24	44	16	17

peroxidation was unavoidable. Both compound (II) and 10-hydroxymethyl-9-methyl-1,2-benzanthracene have been reported by others. The former, however, was considered a photo-oxygenation product rather than an enzyme product (Boyland & Sims, 1965). The latter will not be further discussed here. Products reported by Sims's group (Boyland & Sims, 1965; Sims, 1970*a,b*), if present, would remain at or near the origin in our t.l.c. system and accounted for 10% or less of the total products when the incubations were done between 20° and 30°C for 15 min, as opposed to 37°C for 30 min or longer. They were not included in our results.

Addition of NADPH to microsomal preparations enhanced oxygenation. Presumably some of the cytochrome *P*-450 oxygenase was present in the reduced active form, and, since no net gain or loss of electrons is involved in peroxidation, either exogenous NADPH is not needed or ferrihaemoprotein can catalyse some oxygenation. In our experiments, moderate amounts of NADPH were added to increase oxygenase activity. Addition of large amounts of NADPH or an NADPH-generating system led to a greater production of compound (III), largely at the expense of compound (II).

Spots on the t.l.c. sheet representing 9-¹⁴C-labelled compound (II) obtained in experiments in which NADPH was added were cut out, extracted with methylene chloride, mixed with 40 mg each of identical unlabelled compounds and recrystallized from various solvent combinations including methanol, chloroform, acetone, benzene, diethyl ether and *n*-hexane. The specific radioactivity reached a constant value of 1038 c.p.m./mg (representing 1.4% conversion into product) for compound (II) and 625 c.p.m./mg (0.85%) for compound (III) on repeated recrystallization, compared with 293 c.p.m./mg (0.4%) and 173 c.p.m./mg (0.23%) respectively in parallel experiments performed with microsomal preparation in the absence of NADPH. Mass-spectroscopic examination of the methylene chloride extract of the compound-(II) spot gave a parent

peak at *m/e* 288, a large peak at 256, which would be due to the loss of one oxygen molecule, and a third peak at 245 due to the loss of CH₃-C-O moiety. Similarly, mass spectroscopy of the compound (III) spot gave peaks at *m/e* 290, 275 and 260, indicating the parent compound and the loss of one or two methyl groups. The presence and identity of these two compounds were further confirmed by t.l.c., u.v.-fluorescence and u.v.-spectrophotometry methods, by using authentic samples for comparison (Cook & Martin, 1940; Bachmann & Bradbury, 1937).

When compound (II) was incubated with liver microsomal preparation in the presence of NADPH, it was converted into compound (I) and compound (III) (Table 2). The formation of the diol is to be expected in view of the peroxidase activity of liver microsomal preparation (Chen & Lin, 1968, 1969; Shaw & Chen, 1972; Van Lier & Smith, 1970; Hrycay & O'Brien, 1971). Considering the reversible nature of enzyme reactions, the small amounts of the deoxygenated compound found were not at all surprising. Interestingly, the inhibition by SKF-525A suggested that cytochrome *P*-450 oxygenase was responsible for all three reactions studied.

Further evidence that a liver microsomal oxygenase is responsible for peroxidation was the observation that, per mg of protein, liver microsomal preparation from phenobarbital-treated rats produces 40% more, and liver microsomal preparations from 3-methylcholanthrene-treated rats 170% more, compound (II) plus compound (III) than did microsomal preparation from untreated rats. Though phenobarbital appeared less effective than methylcholanthrene as an enzyme inducer, microsomal preparation induced by phenobarbital also caused the production of a fourth spot on t.l.c. that amounted to 22% of the total products and had *R_F* 0.43. This was tentatively identified as 9-hydroxymethyl-10-methyl-1,2-benzanthracene. This spot was also observed when the incubation with microsomal preparation derived from untreated animals was

Table 2. *Metabolism of compound (II)*

9-¹⁴C-labelled compound (II) (410 μg; 8.37 × 10⁴ c.p.m.) was incubated with 10 mg of microsomal proteins in 5.0 ml of phosphate buffer, pH 7.4, at 30°C for 15 min. Additions were: NADPH or NADP⁺, 2 μmol; EDTA, 2 mM; or SKF-525A, 0.2 mM. The products were extracted with methylene chloride and treated as in Table 1. For the Pronase treatment, microsomal fraction was preincubated for 10 min at 30°C with 500 μg of Pronase before the additions of NADPH or NADP⁺ and compound (II).

Products	Additions to microsomal fraction					Buffer only
	NADPH	Pronase, NADPH	SKF-525A	NADP ⁺	None	
Compound (I) (c.p.m.)	1394	740	670	753	557	560
(%)	100	53	48	54	40	40
Compound (III) (c.p.m.)	2850	644	996	660	700	670
(%)	100	23	35	23	25	24

carried out at 37°C for 30 min instead of 30°C for 15 min.

Peroxides are generally reactive compounds. With this in mind, 9-¹⁴C-labelled compound (II) was incubated for 60 min at 20°C with chicken ovalbumin in 0.1 M-sodium phosphate buffer, pH 7.4. After incubation, the reaction mixture was extracted thoroughly with ether, dialysed against water, and filtered through a Sephadex G-50 column (1.0 cm × 30 cm). Of the radioactivity used, 7.5% was found in the void volume along with the protein. 9-¹⁴C-labelled compound (II), or 9-¹⁴C-labelled compound (I), which contained some 9-¹⁴C-labelled compound (II), was also incubated in the same manner with calf thymus DNA and with ox liver RNA for 2 h. The ether-extracted aqueous portion were precipitated with 0.5 M-HClO₄, filtered through Millipore filters (1.0 μm pore size) and washed with ethanol and ethanol/ether (3:1, v/v). Of the compound (II) used, 2.41 nmol/mg bound to DNA and 2.23 nmol/mg to RNA, whereas in the control experiments with compound (I), 0.62 nmol/mg bound to DNA and 0.24 nmol/mg to RNA. These results indicated, though did not prove, an irreversible binding between compound (II) and cellular macromolecules.

These findings suggest that rat liver microsomal cytochrome P-450 oxygenase catalyses the mono-oxygenation of some substrates and the dioxygenation of others, and is therefore more aptly characterized as simply an 'oxygenase', rather than a 'mono-oxygenase' (Hayaishi, 1974) or a 'mixed-function oxidase' (Mason, 1957). It appears doubtful that the activated oxygen species is either an oxene (Hamilton, 1964) or a superoxide anion, O₂⁻ (Strobel & Coon, 1971), since O₂ would be required and there is no gain or loss of electrons in peroxidation. As 1,4-peroxidation is a Diels-Alder diene or a singlet-oxygen reaction, the two methyl groups of compound (I) should enhance the L-region activity and leave the K-region of benzantracene unaffected. This is contrary to the proposal that K-region activation favours carcinogenesis

(Marquardt *et al.*, 1972; Huberman *et al.*, 1972), since compound (I) is more carcinogenic than benzantracene (Badger, 1954). Under our experimental conditions compound (II), compound (III) and 10-hydroxymethyl-9-methyl-1,2-benzanthracene accounted for the bulk of the products; hence other oxygenated metabolites reported in the literature are probably minor products and become apparent only under conditions requiring higher temperature and longer incubation time.

This investigation was supported by grant no. CA-15030 awarded by the National Cancer Institute, D.H.E.W.

References

- Bachmann, W. E. & Bradbury, J. T. (1937) *J. Org. Chem.* **2**, 175-182
- Badger, G. M. (1954) *Adv. Cancer Res.* **2**, 84-85
- Boyland, E. & Sims, P. (1965) *Biochem. J.* **95**, 780-787
- Chen, C. & Lin, C. C. (1968) *Biochim. Biophys. Acta* **170**, 366-374
- Chen, C. & Lin, C. C. (1969) *Biochim. Biophys. Acta* **184**, 634-640
- Chen, C. & Shaw, Y. S. (1974) *Biochem. J.* **144**, 497-501
- Cook, J. W. & Martin, R. H. (1940) *J. Chem. Soc.* 1125-1127
- Hamilton, G. A. (1964) *J. Am. Chem. Soc.* **86**, 3391-3392
- Hayaishi, O. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.), pp. 6-10, Academic Press, New York and London
- Hrycay, E. G. & O'Brien, P. J. (1971) *Arch. Biochem. Biophys.* **147**, 14-27
- Huberman, E., Kuroki, T., Marquardt, H., Selkirk, J. K., Heidelberger, C., Grover, P. L. & Sims, P. (1972) *Cancer Res.* **32**, 1391-1396
- Marquardt, H., Kuroki, T., Huberman, E., Selkirk, J. K., Heidelberger, C., Grover, P. L. & Sims, P. (1972) *Cancer Res.* **32**, 716-720
- Mason, H. S. (1957) *Science* **125**, 1185-1188
- Shaw, Y. S. & Chen, C. (1972) *Biochem. J.* **128**, 1285-1291
- Sims, P. (1970a) *Biochem. Pharmacol.* **19**, 795-818
- Sims, P. (1970b) *Biochem. Pharmacol.* **19**, 2261-2275
- Strobel, H. W. & Coon, M. J. (1971) *J. Biol. Chem.* **246**, 7826-7829
- Van Lier, J. E. & Smith, L. L. (1970) *Biochem. Biophys. Res. Commun.* **40**, 510-516