THE MECHANISM OF MICROSOMAL HYDROXYLATION OF 7-METHYLBENZ[a]-ANTHRACENE AND 7,12-DIMETHYLBENZ[a]ANTHRACENE BY OXYGEN-18 STUDIES

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Summary: The carcinogenic 7-methylbenz[a]anthracene and 7,12-dimethylbenz[a]anthracene were converted by rat liver microsomes into the corresponding hydroxymethyl derivatives and other metabolic products. The 7-methylbenz[a]anthracene incubation was carried out in $H_2{}^{18}O$, and no incorporation of oxygen-18 was found in the hydroxymethyl metabolite isolated and purified by high pressure liquid chromatography, and analyzed by mass spectrometry. When 7-methylbenz[a]anthracene or 7,12-dimethylbenz[a]-anthracene was incubated with $^{18}O_2$, isotope incorporation was observed in the corresponding hydroxymethyl derivatives, indicating that such hydroxylation is a true oxygenase reaction.

The benzyl hydroxylation reaction has been frequently observed in the microsomal metabolism of exogenous aromatic compounds, e.g., p-nitrotuolene (1), tolbutamide (2) and p-methyl-phenylalanine (3). The carcinogenic methylbenzanthracenes do not represent an exception to this metabolic pathway, and the corre-

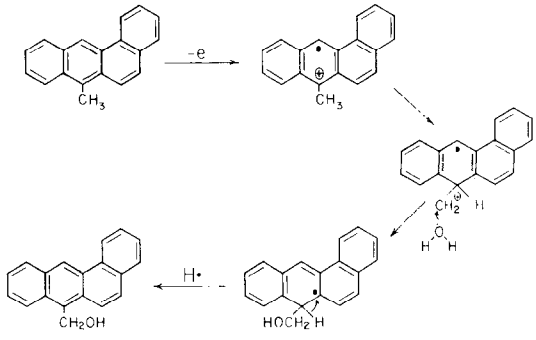

Figure 1. Possible formation of 7-$HOCH_2$BA by nucleophilic attack of H_2O to the MBA radical cation induced by one-electron oxidation.

sponding hydroxymethyl derivatives have been described among the several metabolites (4-11). However, the mechanism by which the oxygen is incorporated in these carcinogenic hydrocarbons is not yet known. The alkyl group can be hydroxylated by the active oxygen induced by the hydroxylating enzyme system as in the case of the microsomal hydroxylation of ethylbenzene to yield methylphenylcarbinol (12) or, plausibly, by the nucleophilic attack of water on the reactive radical cation of the hydrocarbon as illustrated for MBA[1] (Figure 1). In fact, accumulating evidence suggests that one-electron oxidation (13-15) might be an elective mechanism of activation for these compounds. The radical cation generated by such oxidation would react with cellular nucleophiles and presumably constitute the first essential step in the tumor initiation process.

In order to prove whether H_2O or O_2 was responsible for the metabolic hydroxylation of the methyl group, the hydrocarbons MBA and DMBA were converted by rat liver microsomes to their metabolic products in the presence of $H_2{}^{18}O$ or ${}^{18}O_2$.

Materials and Methods: Male Wistar rats (200-250 g; Eppley Colony) were maintained ad libitum on Wayne Lab-Blox diet (Allied Mills, Chicago) and tap water. MBA was synthesized by the method of Wood and Fieser (16), and 7-$HOCH_2$BA was prepared by the method of Badger and Cook (17). DMBA was purchased from Eastman Kodak Co., chromatographed on a silica gel (70-230 mesh) column using benzene-petroleum ether (b.p. 60-90°) (5:95, v/v) and recrystallized from benzene-methanol. 7-$HOCH_2$-12-MBA and 12-$HOCH_2$-7-MBA were prepared by alka-

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[1]Abbreviations: MBA, 7-methylbenz[a]anthracene; DMBA, 7,12-dimethylbenz[a]anthracene; 7-$HOCH_2$BA, 7-hydroxymethylbenz[a]anthracene; 7-$HOCH_2$-12-MBA, 7-hydroxymethyl-12-methylbenz[a]anthracene; 12-$HOCH_2$-7-MBA, 12-hydroxymethyl-7-benz[a]anthracene; NADPH, reduced nicotinamide adenine dinucleotide; DMSO, dimethylsulfoxide.

line hydrolysis of a mixture of the corresponding bromomethyl derivative (15). The two products were separated on alumina (activity I) by eluting with benzene and purified by recrystallization from ethanol (5). Oxygen-18 enriched water at two different concentrations, 98 atom % and 20 atom %, was obtained from Bio-Rad Laboratories. Molecular oxygen-18 (91.1 atom % enrichment) was purchased from Miles Laboratories.

Animals were killed by cervical dislocation, and the livers were immediately removed and homogenized in 7 volumes of 0.25 M sucrose. Microsomes were isolated according to the method of Shenkman and Cinti (18). The final microsomal pellets were resuspended in 0.9 M Tris buffer, pH 7.5, and used for the $H_2{}^{18}O$ studies. Alternatively, the final microsomal pellets were resuspended in 0.5 M Tris buffer, pH 7.5, for the $^{18}O_2$ studies.

Protein determinations were made according to the method of Lowry et al (19). Incubations involving $H_2{}^{18}O$, 0.75 ml total volume, were carried out in 5 ml volumetric flasks open to the atmosphere. Each flask finally contained 0.75 mg of microsomal protein (in 44 µl Tris buffer), 0.53 mM NADPH, 0.165 mM MBA (in 7.5 µl DMSO), 0.6 ml $H_2{}^{18}O$ (98 atom %) and 0.15 ml $H_2{}^{18}O$ (20 atom %). The samples were preincubated for 10' at 37°C in a shaking water bath. MBA and NADPH were then added and the samples incubated for 20'. Incubations involving molecular oxygen-18 were carried out in screw cap Erlenmeyer flasks, 30 ml, which were sealed with silicon rubber septa. A total volume of 5 ml was used for each incubated sample which contained the same final concentrations of the various components as described above. Similar incubations were also carried out when DMBA was used as substrate. Each sample containing only buffered microsomal suspension was repeatedly evacuated under water aspiration followed by flushing with N_2 and finally filled

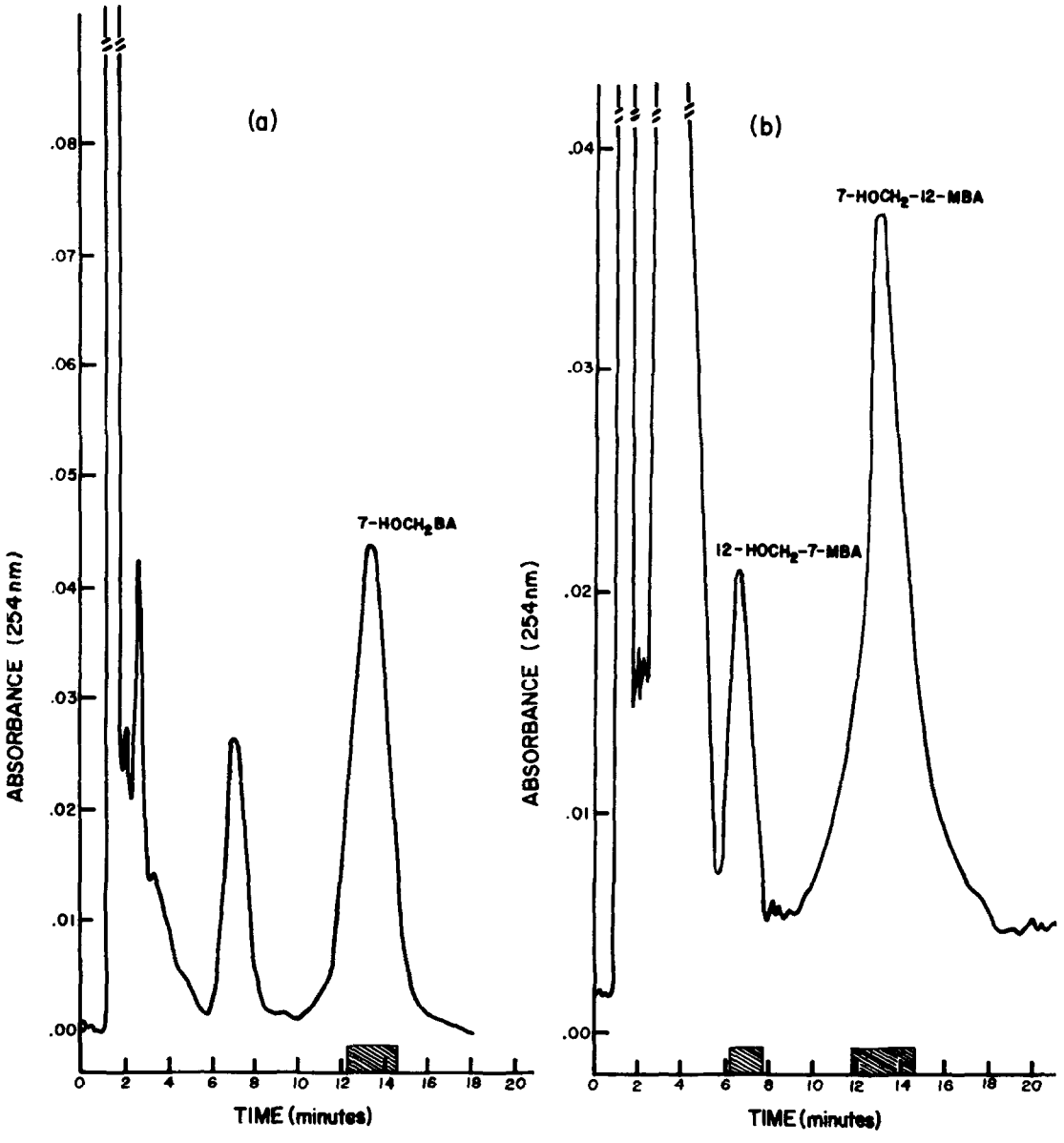

Figure 2. Profile of metabolites and starting material by high-pressure liquid chromatography after microsomal incubation of MBA (a) and DMBA (b). Shaded areas indicate portions of eluent collected for mass-spectral analysis.

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with N_2 at approximately 1 atm. The gas composition above each solution was adjusted approximately to 20% of O_2 and 80% of N_2 by syringe withdrawal of 5 ml of N_2 and by its replacement with $^{18}O_2$

(91.1 atom %) or $^{16}O_2$. Substrate and NADPH were left out of the mixture during the 10' of preincubation. After that time they were added by syringe and then incubated for 20'. All incubations were terminated by adding an equal volume of $CHCl_3$. The metabolites were then extracted twice with $CHCl_3$, and the organic layer was evaporated to dryness with a stream of N_2 at room temperature. The dried residues were redissolved in 10-20 µl of CH_2Cl_2 and injected into a high-pressure liquid chromatograph (Water Associates, Milford Mass.). The metabolites were resolved on a column 1.5 m in length and 2 mm I.D. packed with Corasil II; the solvent system used was CH_2Cl_2-hexane 60:40 at a flow rate of 1.5 ml/min. Eluting peaks were monitored at 254 nm and those corresponding to the metabolites of interest were collected manually as they were eluted. The relative position of the metabolites were determined previously by chromatography of the synthesized authentic compounds under identical experimental conditions. The fractions collected were evaporated to approximately 0.1 ml and analyzed in a mass spectrometer (AEI, Model MS-9) by direct probe inlet at 200°C and 70 e ionization energy. The samples, though limited to the ng range, were sufficient to obtain several representative spectra by repetitive scanning of the molecular ion region. The isotope distribution in the metabolites was calculated by the customary procedure (20).

Results and Discussion: The isolation of pure metabolites depended heavily on the high-pressure liquid chromatograph. Excellent resolution is observed for the metabolites 7-$HOCH_2$BA (Figure 2a) and 7-$HOCH_2$-12-MBA (Figure 2b) from the unaltered substrates and other metabolic products. The product 12-$HOCH_2$-7-MBA is not totally separated from other metabolites, but by delaying the collection of elutant as indicated in Figure 2b a relatively pure product was obtained.

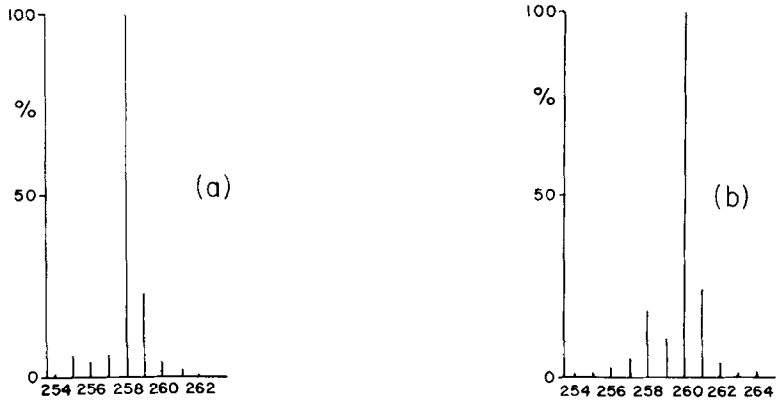

Figure 3. Mass spectra of 7-$HOCH_2$BA (a) and [^{18}O] 7-$HOCH_2$BA (b) in the region of the molecular weight.

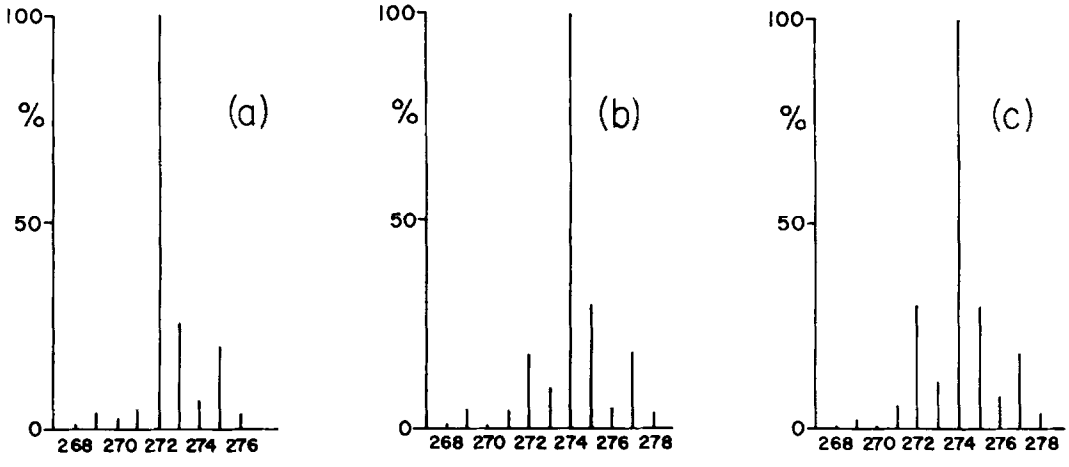

Figure 4. Mass spectra of 7-$HOCH_2$-12-MBA (a), [^{18}O] 7-$HOCH_2$-12-MBA (b) and [^{18}O] 12-$HOCH_2$-7-MBA (c) in the region of the molecular weight.

The mass spectra of 7-$HOCH_2$BA obtained from MBA experiments in the presence of H_2O or $H_2{}^{18}O$ are identical, indicating that no oxygen-18 incorporation from $H_2{}^{18}O$ into 7-$HOCH_2$BA occur. The same metabolite isolated and analyzed from O_2 (Figure 3a) and $^{18}O_2$ (Figure 3b) contains an 88% oxygen-18 incorporation in the latter case. The metabolites 7-$HOCH_2$-12-MBA and 12-$HOCH_2$-7-MBA obtained from DMBA

experiments also contained oxygen-18 when incubated with $^{18}O_2$ (Figures 4b,4c). The percentage of isotope enrichment was 82% and 73%, respectively. Theoretically, the percent incorporation should have been 91.1%. A likely source of error, that can explain the lower incorporation percentages, stems from a partial lack of justification about the assumptions required for quantitative isotope determinations by mass spectra (20), and/or contaminating unknowns eluted and collected along with the hydroxylated products.

The oxygen-18 studies prove that the formation of hydroxymethyl derivatives from methylbenzanthracenes is consistent with a direct insertion of the enzymically induced active oxygen to the methyl group or to the chemically reactive carbon atom (21) adjacent to the methyl group which, in the latter case, would yield the hydroxymethylbenzanthracene by an unknown rearrangement. Hence, the hydroxylation reaction of the methyl group of these compounds is a true oxygenase reaction.

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