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IDENTIFICATION OF 8,9-DIHYDRO-8,9-DIHYDROXY-7,12-DIMETHYLBENZ(A)ANTHRACENE AS A RAT LIVER METABOLITE OF 7,12-DIMETHYLBENZ(A)ANTHRACENE

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7,12-Dimethylbenz(a)anthracene (DMBA) has been used extensively to induce hormone-dependent breast tumors in the rat (1-5). One theory of the possible mechanism of action of DMBA which has been explored in depth involves the study of the possible formation and subsequent reactions of epoxides of this hydrocarbon. The transient nature of epoxides in aqueous media and in the presence of molecules containing nucleophilic groups has precluded their isolation in many instances (6-10). Identification of further metabolic products and hydrolysis derivatives serves as an indication of the probable areas of the DMBA ring system which have undergone intermediate epoxide formation (9,11-13).

The results presented in this paper provide definitive data to support the concept that a prominent zone of the DMBA molecule involved in metabolism by rat liver is the 8,9-bond. Previously, Boyland and Sims (14) had speculated on the sole basis of the fluorescence of some chromatographic spots, that the 8,9-dihydrodiol of DMBA might be a metabolite of the hydrocarbon. At that time none of the phenols or dihydrodiols of the 8,9,10 and 11 positions of the DMBA molecule had been prepared, so more exact comparison of their data was impossible.

The metabolites of DMBA fall into three main categories: non-polar epoxides, monohydroxy compounds and polyhydroxy compounds. Quantitative separation of these three classes of compounds has been demonstrated by preferential elution of DMBA-5,6-epoxide, DMBA-5-ol and trans-5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz(a)anthracene (DMBA-5,6-diHdiol) (15). The recent acquisition of authentic samples of DMBA-8-ol, DMBA-10-ol and DMBA-11-ol in these laboratories has made possible the study of their chromatographic behavior, and it has been shown that these phenols also elute exclusively in the same fraction as authentic DMBA-5-ol. This phenomenon illustrates the general application of the alumina column technique in the separation of DMBA metabolites of varying polar character.

Electron-capture gas chromatography (g.c.) has been used to identify the heptafluorobutyrate esters of the DMBA phenols and dihydrodiols at the nanogram level (15). Utilizing column chromatography and g.c., we have identified the primary metabolites of DMBA by rat liver to be DMBA-5,6-diHdiol and DMBA-8,9-diHdiol.

Methods: DMBA-5-ol was prepared as reported previously (15,16). DMBA-8-ol and DMBA-11-ol were prepared from 3-methoxyphthalic anhydride, and DMBA-10-ol was prepared from 4-methoxyphthalic anhydride by methods which are described in detail elsewhere*.

Liver microsomes were prepared by the method of Fry and Bridges (17). The microsomal pellet was suspended in 25 ml of 0.1 N phosphate buffer, pH 7.4, and divided into two equal portions, one of which was boiled for 1 min to provide a blank experiment. To both suspensions were added 5 mg NADPH, 2 μmoles $MgCl_2$ and 200 μg DMBA in 0.4 ml ethanol. After incubation for 10 min at 37°, the solutions were extracted with 3 x 10 ml CH_2Cl_2. The CH_2Cl_2 extracts were dried with Na_2SO_4, evaporated, taken up in 50 μl benzene and applied to 0.5 cm x 5.0 cm columns of 5% deactivated Al_2O_3. Elution was carried out with 8 ml each of 3% dioxane in hexane, 5% acetone-1% acetic acid in hexane and ether which was saturated with water. The fractions containing the DMBA metabolites were evaporated and derivatized with 100 μl of 50% heptafluorobutyric anhydride in acetone for 30 min at room temperature (24°), evaporated with N_2 and diluted to 2 ml with heptane. Aliquots (1 μl each) of the blank and metabolic pools were injected

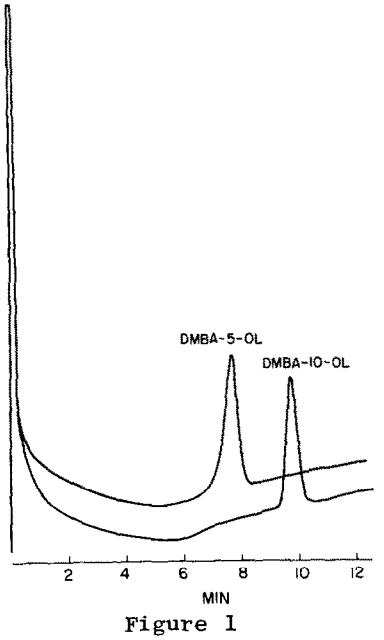

Figure 1

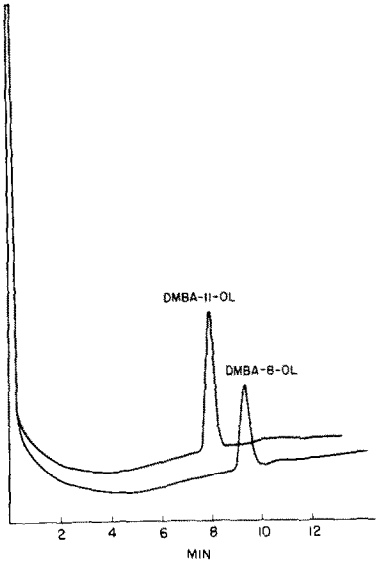

Figure 2

Fig. 1. Gas chromatography scans of the heptafluorobutyrate esters of 2.5 ng DMBA-5-ol and 3.0 ng DMBA-10-ol.
Fig. 2. Gas chromatography scans of the heptafluorobutyrate esters of 1.0 ng DMBA-8-ol and 0.5 ng DMBA-11-ol.

*Manuscript in Preparation.

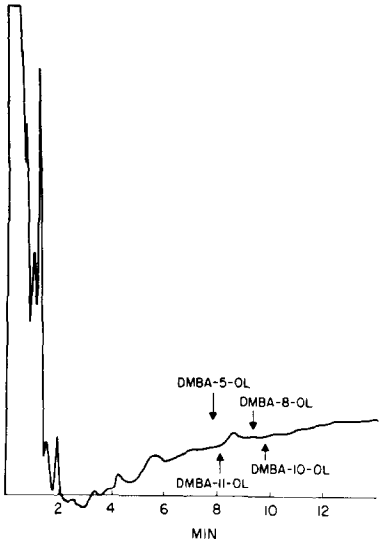

Figure 3a

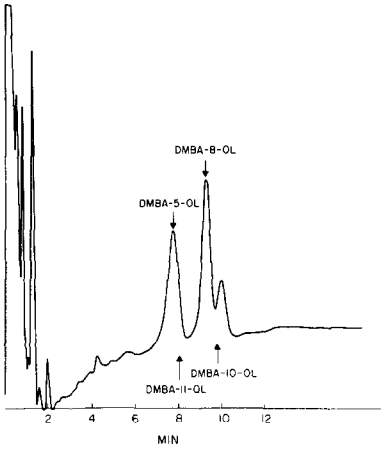

Figure 3b

Fig. 3. Gas chromatography scans representing 1/2000th of the derivatized polar metabolites of DMBA by rat liver microsomes. Panel a: boiled blank; Panel b: regular incubation.

onto 8 ft x 2 mm OV-17 (3%) columns at 260° as reported previously (15). Samples of DMBA-5,6-diHdiol, DMBA-5-ol, DMBA-8-ol, DMBA-10-ol and DMBA-11-ol were run concurrently.

Results: Figures 1 and 2 show the retention times of samples of the heptafluorobutyrate esters of DMBA-5-ol, DMBA-8-ol, DMBA-10-ol and DMBA-11-ol. Figure 3 shows the detector response to the heptafluorobutyrate esters of the liver metabolites of DMBA which are derived from the fraction which contains compounds with more than one hydroxyl function. The blank liver incubation run concurrently showed no peaks even when twice as much sample was injected. No peaks were evident in the 3% dioxane/hexane and 5% acetone/hexane fractions when subjected to derivative formation and g.c. It has been shown that epoxides and monophenols are quantitatively eluted from deactivated alumina by these solvent systems. The absence of both these types of metabolites may be due to their further metabolism in the comparatively long (10 min) incubation period.

Previous work has shown that ether quantitatively elutes dihydrodiols of DMBA from alumina after the less polar compounds have been eluted with 3% dioxane/hexane and 5% acetone/hexane. It has also been demonstrated that dihydrodiols of polycyclic hydrocarbons easily dehydrate to fully aromatic systems in the presence of traces of acid (15,16). Thus, heptafluorobutyric anhydride converts DMBA-5,6-diHdiol to the heptafluorobutyrate ester of DMBA-5-ol. No diesters, which would possess widely different retention times on gas chromatographic analysis, are formed. In keeping with

the established behavior of dihydrodiols with this reagent, the components of the polar fraction derived from the metabolism of DMBA form derivatives whose retention times dictate that they are esters of monophenols. Indeed, two of the three metabolites match exactly the heptafluorobutyrate esters of DMBA-5-ol and DMBA-8-ol. In addition, the peaks maintained their sharpness and there was no shifting of the peak maxima by mixture of the standard phenols with the metabolic derivatives and injection simultaneously into the gas chromatograph. Of the three major metabolic peaks, the one with the lowest retention time was also close in retention time to that of DMBA-11-ol, but a mixture of this peak with authentic DMBA-11-ol caused a shift in the retention time and a broadening of the peak. The metabolic peak which had the longest retention time was also shifted and considerably broadened when mixed with authentic DMBA-10-ol.

Peak area was calculated by multiplying the height of the peak by the width at half-height, and the areas of the standards were compared with those of the comparable peaks in the metabolic pool. From 200 µg DMBA, the amount of DMBA-5,6-diHdiol formed is 6.64 µg representing a 3.32 percent conversion and the amount of DMBA-8,9-diHdiol formed is 5.02 µg representing a 2.51 percent conversion.

In this paper, identification of the primary metabolite described as DMBA-8,9-diHdiol rests exclusively on its conversion in acidic media to the known DMBA-8-ol. Of course, further proof of the structure of this metabolite would be obtained by comparison of physical constants, such as chromatographic behavior, with authentic diol. Efforts toward the synthetic acquisition of this compound are currently underway in this laboratory.

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