IDENTIFICATION OF PHENOLS OF 7,12-DIMETHYLBENZ[a]ANTHRACENE BY CONVERSION INTO THEIR METHYL ETHERS

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(Received October 10th, 1984)

SUMMARY

The compound 7,12-dimethylbenz[a]anthracene (DMBA), a powerful chemical carcinogen, undergoes enzymatic oxidation to a variety of metabolites. Phenols, the monohydroxylated derivatives of DMBA, are an electrophilic species capable of binding covalently with DNA. Although phenols may play a role in carcinogenesis, little information is available regarding these metabolites, many of which are chemically unstable and elusive to quantitative analysis.

In this study, a method is described for the methylation of phenolic products to their chemically stable methyl ether derivatives. Application of this procedure to *in vitro* incubations of DMBA with rat liver microsomes resulted in the isolation and identification of 4-hydroxy DMBA, 8-hydroxy DMBA and 3-hydroxy DMBA.

INTRODUCTION

The compound 7,12-dimethylbenz[a]anthracene (DMBA), a polycyclic aromatic hydrocarbon, undergoes oxidation to carcinogenic metabolites by microsomal enzymes. DMBA is metabolized by aryl hydrocarbon hydroxylase (AHH) to epoxides, which can be converted into phenols by non-enzymatic rearrangement or to dihydrodiols by epoxide hydratase[1]. Further oxidation of dihydrodiols by AHH can yield diol epoxides, the presumed ultimate carcinogenic species[2]. DMBA is also directly hydroxylated to phenols by peroxides formed during the reaction cycle of AHH[3].

Phenolic metabolites are elusive to qualitative and quantitative analysis, since these compounds are synthesized in minute (picogram or nanogram) amounts and can be oxidized to quinones. Free radicals are the hypothesized intermediates in both the formation of quinones and the reduction of quinones to hydroquinones by an NADPH-dependent reductase. These free radicals are capable of binding covalently with nucleic acids[2]. These phenols and/or quinones, like the diol epoxides, may have a role in carcinogenesis.

To avoid the problem of degradation of the phenols during workup procedures, this laboratory has developed a technique which permits the quantitative eval-

uation of the levels of phenolic metabolites by quickly trapping them as their methyl ethers, where the phenolic hydroxyl group is no longer as susceptible to the effects of further oxidation.

MATERIALS AND METHODS

Chemical studies

DMBA was purchased from the Eastman Kodak and checked for purity by high-performance liquid chromatography (HPLC). Samples of 1-MeO-DMBA (MeO stands for methoxy), 2-MeO-DMBA and 6-MeO-DMBA were a generous gift from Dr. Melvin S. Newman of the Ohio State University, Columbus, OH, U.S.A. *cis*-5,6-Dihydro-5,6-dihydroxy-7,12-dimethylbenz[*a*]anthracene was prepared by the method of Cook and Schoental[4]. 4-MeO-DMBA was prepared by the method of Flesher[5]. 3-MeO-DMBA, 5-MeO-DMBA and 9-MeO-DMBA were synthesized by the methods of Newman[6,7]. 8-MeO-DMBA and 11-MeO-DMBA were prepared as described previously[8] by this group.

Biological studies

Sprague-Dawley rats, 60–90 days old, were sacrificed with ether, and the liver was rapidly excised and rinsed in cold 1.15% potassium chloride. The tissues were weighed, pulverized in liquid nitrogen, and homogenized in cold 1.15% potassium chloride (1 ml per 250 mg wet weight). Microsomes were prepared by the method of Fry and Bridges[9]. Microsomal protein concentrations were measured by the procedure of Lowry *et al.*[10], with bovine serum albumin as the protein standard.

Incubation mixtures consisted of microsomes, 20 ml of 0.1 M potassium phosphate buffer (pH 7.4), 4 ml of glycerol, 30 mg of NADPH, 5 μmol of magnesium chloride, and 1 mg of DMBA in 1 ml of ethanol. Incubation mixtures were oxygenated for 60 sec and then incubated at 37°C for 30 min in a Dubnoff constant-temperature metabolic incubator. Incubations and subsequent extractions were conducted in the dark to prevent photo-oxidation of DMBA.

Incubation mixtures were extracted three times with 10 ml of peroxide-free ether, and the extracts were centrifuged at low speed for several minutes to facilitate separation of organic and aqueous phases. The ether extract was transferred to a centrifuge tube containing 100 mg of ascorbic acid dissolved in 10 ml of 3 M sodium hydroxide, and the organic phase was evaporated to dryness with nitrogen. The sodium hydroxide solution was then washed twice with 3 ml of hexane which was discarded, and the aqueous layer was treated with 2 ml of dimethyl sulfate as described below.

Products of the dimethyl sulfate reaction were extracted twice with 6 ml of hexane. The hexane extract was washed twice with 5 ml of distilled water, dried with sodium sulfate, and evaporated to a 3-ml volume from which aliquots were removed for HPLC analysis.

The necessity of eliminating unreacted DMBA and membrane lipids during the extraction procedure was demonstrated. When contaminants were not removed, metabolite peaks were obscured by extraneous, fluorescing material. Successive washings with hexane prior to the dimethyl sulfate reaction resulted in reduction of interfering contaminants without concomitant loss of metabolites.

Analytical procedures

Four extraction procedures were tested with a mock incubation mixture (4 µg of DMBA-3-ol, 1 mg of DMBA in 1 ml of ethanol, 20 ml of 0.25 *M* sucrose, and 20 ml of 0.1 *M* potassium phosphate buffer, pH 7.4) for quantitative recovery of the DMBA-3-ol and conversion into 3-MeO-DMBA. In the first procedure, the solution of DMBA and DMBA-3-ol was extracted twice with 10 ml of peroxide-free ether, the organic phase was extracted twice with 3 *M* sodium hydroxide (1 × 8 ml and 1 × 2 ml), and the extract, after extracting twice with 3 ml of hexane (which was discarded) was treated with 2 ml of dimethyl sulfate with constant stirring for 15 min. The reaction mixture was extracted twice with 6 ml of hexane, the hexane extract was washed twice with 5 ml of distilled water, and the washed hexane extract was dried with sodium sulfate. In the second procedure, the ether extract was extracted five times with 2 ml of 3 *M* sodium hydroxide rather than twice with 8 ml and 2 ml of sodium hydroxide. The third procedure was unchanged from the first, except that the ether extract was transferred to a centrifuge tube containing 100 mg of ascorbic acid dissolved in 3 *M* sodium hydroxide, and the organic phase was evaporated to dryness with nitrogen. The fourth procedure was similar to the third, except that ethyl acetate was substituted for ether in the extraction of the DMBA and DMBA-3-OL solution.

The yield of 3-MeO-DMBA from each of the extraction procedures was compared with the 3-MeO-DMBA product when 4 µg of DMBA-3-ol were combined with 10 ml of 3 *M* sodium hydroxide and treated with dimethyl sulfate. It was determined that losses were completely avoided when procedure 3 was used.

A DuPont Model 848 liquid chromatograph was fitted with a Zorbax-Sil column (25 cm × 4.6 mm I.D.). The column was eluted at ambient temperature with 0.01% (v/v) isopropyl alcohol in hexane. Fluorescence spectra of the metabolites and methoxy-DMBA standards were measured with a DuPont Model 836 fluorescence detector.

RESULTS

Quantitative recovery of 3-MeO-DMBA from the phenol was observed using an extraction system in which the ether extract containing the phenol was evaporated onto a solution of sodium hydroxide containing ascorbic acid prior to the dimethyl sulfate reaction. Excess base is necessary not only to insure quantitative conversion into the methoxy derivatives but also to prevent dehydration of dihydrodiols to phenols. This concern was addressed due to the facile conversion of these types of compound into the corresponding esters of the phenols under relatively mild conditions by anhydrides[11]. There was an absence of any LC peaks following the reaction of DMBA-5,6-dihydro-5,6-diol with the reagent system, showing that the conditions of the assay do not promote the degradation of diols to phenolic compounds. An identical sample of diol exposed to acid was converted into a mixture of DMBA-5-ol and DMBA-6-ol, as was evidenced by conversion into the corresponding methoxy derivatives upon reaction with dimethyl sulfate.

The retention times of the individual methoxy DMBA compounds are shown in Table I and were used to identify and quantitate the metabolites in the biological studies. This application was demonstrated by the *in vitro* 30-min incubation of

TABLE I

CHROMATOGRAPHIC BEHAVIOR OF DERIVATIVES OF 7,12-DIMETHYLBENZ[a]AN-
THRACENE

Derivative	Retention time (min)	Peak height/2 ng
1-MeO-DMBA	11.9	3.6
2-MeO-DMBA	14.2	3.0
3-MeO-DMBA	14.5	2.3
4-MeO-DMBA	9.0	4.5
5-MeO-DMBA	8.4	3.3
6-MeO-DMBA	7.2	6.6
8-MeO-DMBA	7.8	3.4
9-MeO-DMBA	13.3	3.4
10-MeO-DMBA	13.0	4.4
11-MeO-DMBA	7.8	4.3

DMBA with microsomes obtained from rat liver, giving 4-MeO-DMBA, the prin-
cipal product, 4.7 ng/mg protein; 8-MeO-DMBA, 0.9 ng/mg protein and 3-MeO-
DMBA, 2.7 ng/mg protein. A blank experiment using boiled microsomes was void
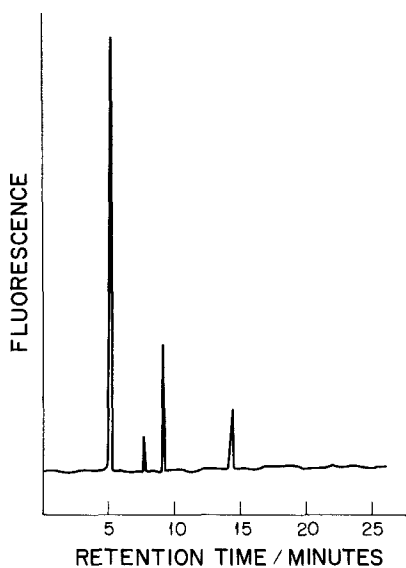of any peaks in these zones (Fig. 1).

Fig. 1. Chromatogram of extract from rat liver microsomal metabolites of DMBA. Order of elution is:
DMBA; 8-MeO-DMBA, 0.9 ng; 4-MeO-DMBA, 4.7 ng; and 3-MeO-DMBA, 2.7 ng. Zorbax-Sil column,
25 cm × 4.6 mm I.D., eluted with 0.01% isopropyl alcohol in hexane.

Since 8-MeO-DMBA and 11-MeO-DMBA have the same retention time in
this system, the identity of the metabolic peak in this zone can be questioned. The
assignment of 8-MeO-DMBA is based primarily on the apparent preferential metab-
olism of DMBA at the 8,9-double bond of this polycyclic aromatic hydrocarbon[12].

Elimination of unreacted DMBA and membrane lipids was accomplished by extraction of the sodium hydroxide solution of the phenol with hexane. When contaminants were not removed. metabolite peaks, although obvious and measureable, were obscured by extraneous, fluorescing material. Successive washings with hexane prior to the dimethyl sulfate reaction resulted in a dramatic reduction of interferring contaminants without concomitant loss of metabolites.

DISCUSSION

The great volume of work dealing with the diol–epoxide theory of carcinogenesis leaves little doubt that in certain systems, this route of metabolism is very likely the main one responsible for the transformation of normal cells to malignancies. But phenols can act as carcinogens through alternate routes, possibly by further metabolism to diol–epoxides in remote parts of the molecule, since 2-hydroxybenz[a]pyrene (2-HOBP) is a very potent skin carcinogen[13].

Interest in phenolic metabolites is heightened by the fact that they are often formed in relatively great amounts. In the case of benz[a]pyrene (BP), the 3-hydroxy and 9-hydroxy derivatives represent the major primary metabolites of this hydrocarbon[14]. The phenolic compounds 1-HOBP and 3-HOBP are several times more mutagenic than BP itself in the Ames test, suggesting that further metabolism to diol–epoxides at the 7,8,9,10 positions may be responsible for this activity. Nevertheless, it is difficult to explain why 2-HOBP or 12-HOBP are not mutagenic under the same conditions, unless deactivation via quinone formation or conjugation is responsible. The lack of mutagenicity of 7-HOBP is understandable, since further oxygenation in this molecule is expected to occur at other ring positions where diols or their epoxides are known to be inactive[15].

One unusual property of the polycyclic hydrocarbon phenols is the ability of some of them to influence dramatically metabolism of the parent hydrocarbon. 3-HOBP competitively inhibits the metabolism of BP by lung microsomes[16]. In the benz1a]anthracene (BA) series, phenols are strong inhibitors of metabolism. 5-HOBA shows an inhibition of the microsomal metabolism of BA by 71% at the extremely low concentration of 1 nanomolar/ml. Similar results were obtained with 6-HOBA, 8-HOBA and 9-HOBA[17]. Gentil et al.[18] have studied the kinetics of metabolism of DMBA in cell culture and have concluded that phenols are produced early (within 8 h) and further metabolized. It is not surprising that some reports which specified the lack of phenolic metabolites have been reported since cultures of the cells were carried out for several days. Thus, the importance of the phenols has probably been overlooked, not only because they are difficult to detect but also because they still have double bonds in other areas of the polycyclic hydrocarbon molecule which are susceptible to epoxide formation with the subsequent formation of diols.

The procedures outlined here utilizing the conversion of phenolic metabolites into their methoxy derivatives will permit the isolation and quantitative identification of these elusive compounds in a variety of biological systems. Although this study has involved the metabolism of DMBA, it is possible that the same procedures can be applied to other polycyclic aromatic hydrocarbons.

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