THE METABOLISM OF 3-METHYLCHOLANTHRENE BY RAT LIVER MICROSOMES - A REINVESTIGATION

T. A. Stoming*, W. Bornstein†, Edward Bresnick†

*Department of Cell and Molecular Biology
Medical College of Georgia
Augusta, Georgia 30902

†Department of Biochemistry, University of Vermont
School of Medicine, Burlington, Vermont 05401

Received October 3,1977

Summary: Metabolites of 3-methylcholanthrene (3-MC) formed by rat liver microsomes were analyzed by high pressure liquid chromatography. The metabolic profile is significantly different from previous studies using thin layer chromatography. The major metabolites include 1-and 2-hydroxy-3-MC. Use of the high pressure liquid chromatographic system allows for the separation of at least seven new metabolites. The amounts of three of these new metabolites are substantially decreased when the potent epoxide hydrase inhibitor 3,3,3-trichloropropene oxide is added to the incubation system. These results then suggest the formation of epoxides of 3-methylcholanthrene other than the K-region oxide.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread throughout our environment. These compounds are formed from the incomplete combustion of organic materials (1). Many of the PAHs isolated to date are known to produce tumors when applied to experimental animals and are probably carcinogenic in man. One of these compounds, benzo[a]pyrene (B[a]P), has been extensively studied. There is now a wide acceptance that B[a]P requires metabolic activation in order to elicit its carcinogenic response (2-5). This metabolism is known to occur mainly by the cytochrome P_{450} monooxygenase system yielding a wide variety of metabolites. The large number of metabolites has complicated the study of the carcinogenicity of B[a]P.

Recent studies have indicated that secondary metabolism of B[a]P catalyzed by the P_{450} system may be required for the formation of ultimate carcinogens (6-11). Along these lines, Borgen et al (12) have shown that trans 7,8-

ISSN 0006-291X

dihydro-7,8-dihydroxybenzo [a]pyrene binds more extensively to DNA in an *in vitro* incubation system than B[a]P. These and other studies have led to the proposal that a diol-epoxide is the ultimate carcinogen of B[a]P (5,13-16).

We have been interested in the metabolism of another potent carcinogen, 3-methylcholanthrene (3-MC). None of the metabolites of 3-MC reported to date indicate that this compound is activated by the same mechanism as proposed for B[a]P, a diol-epoxide. The results from the present study suggest that the metabolism of 3-MC is much more complex than previously reported (17-19) and that 3-MC gives rise to at least four possible arene oxides.

MATERIALS AND METHODS

Chemicals: The [6-^{14}C] 3-MC (60.2 mCi/mmole) was obtained from New England Nuclear Corporation. The material was purified by thin layer chromatography (silica-gel) in benzene-hexane (1:1 v/v). The material migrating as 3-MC was scraped from the plate, extracted with ether, filtered, evaporated to dryness, redissolved in 0.6 ml acetonitrile and purified by high pressure liquid chromatography (HPLC) as previously described (20). The purified material was adjusted to 20 mCi/mmole with non-radioactive 3-MC, Aldrich Chemical Co. (recrystallized from benzene-ether). NADPH was obtained from Sigma Chemical Corp.; spectral grade, glass distilled acetonitrile from Burdick and Jackson Laboratories, Muskegon, Michigan. All other solvents were of reagent grade.

Animals: Rats were obtained from Holtzman Co., Madison, Wisconsin. Four male animals (80-100 g) were injected with 3-MC in corn oil (40 mg/kg) for three consecutive days. Control animals received corn oil alone. Twenty-four hours after the last injection, the animals were sacrificed, the livers from each group pooled, and microsomes were prepared according to the method of Seifried et al. (21).

Assay Conditions: Each tube contained 0.5 mg of microsomal protein, 50 μmoles Tris (Cl) pH 7.4, 3 μmoles $MgCl_2$, 0.5 μmoles NADPH, and 100 nmoles [^{14}C] 3-MC in 20 μl of acetone. When used, 1.0 μmole of 3,3,3-trichloropropene oxide (TCPO) was added in 20 μl acetone. The total incubation volume was 1.0 ml. All samples were incubated at 37°C for 15 min in reduced light. Boiled microsomes served as a control. Reactions were terminated by the addition of 0.5 ml of acetone, and 2.0 ml of ethyl acetate were added to each tube. The tubes were vortexed for 30 seconds and then centrifuged to separate the layers. The organic phase was removed and the aqueous phase was re-extracted with 1.0 ml of ethyl acetate. The ethyl acetate extracts were combined and dried over sodium sulfate. The sodium sulfate was then washed with 0.5 ml of ethyl acetate. The entire organic material was filtered through glass wool and evaporated to dryness under N_2. The samples were stored at -70°C until analyzed.

HPLC Analysis: To each sample were added reference standards of synthetically prepared 3-MC derivatives (17). The sample volume was adjusted to 75 μl, and 50 μl was injected onto a μBondapak C_{18} column. High pressure analysis was conducted according to previously published procedures (20) or with the aid of a model 660 Waters Assoc. solvent programmer. We have since found that best separation can be achieved using a 35-95% linear gradient of acetonitrile/H_2O

Table 1: Microsomal Metabolism of 3-methylcholanthrene

Conditions	% Metabolism (-TCPO)	% Metabolism (+TCPO)
Control microsomes	7.46 ± 0.37	6.76 ± .24
Induced microsomes	29.60 ± 3.34	24.36 ± 3.78

Animals were pretreated with either corn oil or 3-MC in corn oil (40 mg/kg). Incubations were conducted as outlined in Materials and Methods. The incubation mixtures contained either acetone alone (-TCPO) or 1 μmole of TCPO in 20 μl of acetone (+TCPO). The percent metabolism was determined by dividing the radioactivity of the peaks eluting from the HPLC column prior to 3-MC by the total radioactivity. The values are the average of duplicate determinations.

over 70 min with the use of the model 660 gradient maker. Fractions of 0.5 ml were collected directly into Beckman Bio-vials. To each vial was added TT-21 scintillation cocktail (Yorktown), and radioactivity was determined by scintillation spectroscopy.

RESULTS

The total metabolism of 3-MC by microsomes from induced animals was linear for at least 15 min. The pretreatment of rats with 3-MC resulted in a 4-fold increase in the metabolism of labeled 3-MC over that observed with liver microsomes from control animals (Table 1). The inclusion of the epoxide hydrase inhibitor, TCPO, caused only a slight decrease in total metabolism. In our studies incubation with boiled microsomes was used to obtain "blank" values.

The nature of the 3-MC metabolites was investigated by high pressure liquid chromatography (HPLC). A typical HPLC profile of 3-MC metabolites is presented in Figure 1A. The retention times for the various 3-MC metabolites are presented in Table 2.

The metabolic profile obtained after incubation of radioactive 3-MC with liver microsomes from 3-MC induced rats is presented in Figure 1B. The major

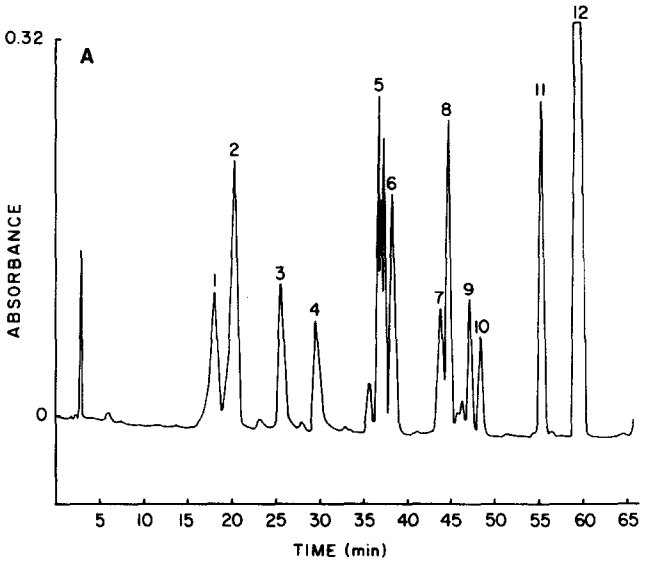

Figure 1A

High pressure liquid chromatographic separation of 3-MC metabolites (A) Ultraviolet profile obtained with reference compounds added to the extraction residue. Metabolism was conducted as outlined in Materials and Methods section. Separation was obtained using a μBondapak C_{18} column as previously described (20). Retention time is depicted on the abscissa and relative absorbance is given on the ordinate. The order of elution is: (1) trans 11,12-dihydro-11,12-dihydroxy-3-MC; (2) trans 1,2-dihydroxy-3-MC; (3) cis 11,12-dihydro-11,12-dihydroxy-3-MC; (4) cis 1,2-dihydroxy-3-MC; (5) 2-hydroxy-3-MC; (6) 1-hydroxy-3-MC; (7) unknown - arises during incubation; (8) 3-MC 11,12-oxide; (9) 2-oxo-3-MC; (10) 1-oxo-3-MC; (11) 3-methylcholanthrylene; (12) 3-MC.

metabolite was 2-hydroxy-3-MC, peak K, although 16 additional radioactive regions were observed. At least three of these radioactive substances exhibited retentior times which were less than that of our first metabolite standard, trans 11,12-dihydro-11,12-dihydroxy-3-MC (peak D).

Other major metabolites included peak L and M which together with the 2-hydroxy 3-MC (peak K) accounted for 70% of the total metabolism. Peak L had a retention time similar to 1-hydroxy 3-MC. Peak M exhibited a retention time similar to K-region oxide. However, this radioactivity may be associated with peak 7 (Figure 1A) and not the K-region oxide. Additional information was obtained from the incubation systems in which TCPO had been included. These data are presented in Table 3.

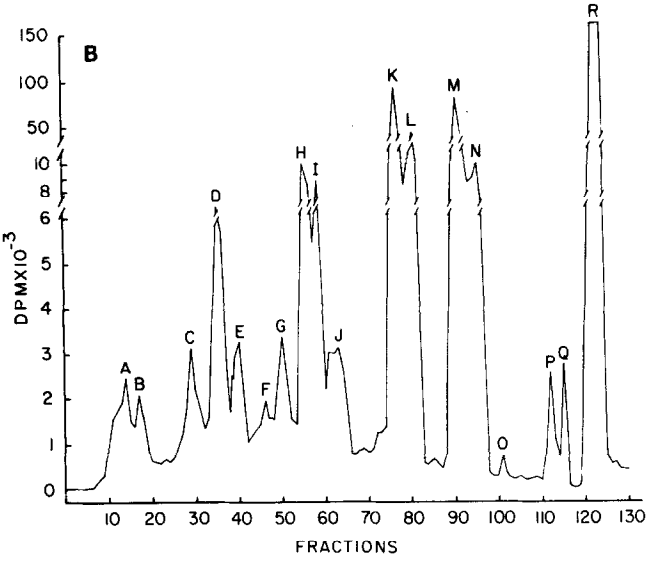

Figure 1B

(B) Chromatographic distribution of radioactive metabolites. One-half ml fractions were collected and subjected to scintillation spectrometry as outlined in Materials and Methods section. Fraction number is represented on the abscissa and total dpm on the ordinate. All values are subtracted from boiled controls. The following tentative assignments have been made. (A, B, and C unknown diols); (D) trans 11,12-dihydro-11,12-dihydroxy-3-MC; (E) trans 1,2-dihydroxy-3-MC; (F) unknown; (G) cis 11,12-dihydro-11,12-dihydroxy-3-MC; (H) unknown; (I) cis 1,2-dihydroxy-3-MC; (J) unknown; (K) 2-hydroxy-3-MC; (L) 1-hydroxy-3-MC; (M) unknown, may contain 3-MC 11,12-oxide; (N) probably ketones; (O) unknown; (P & Q) unknown - probably phenols. Also, (P) has same retention time as 3-methylcholanthrylene; (R) 3-MC.

Five of the radioactive peaks, i.e., peaks A, B, C, D and E, are reduced by more than 50% when TCPO was added to the incubation mixture (Table 3), indicating that these metabolites arise from epoxides. It is interesting to note that peak D (which corresponds to the K-region diol) is reduced by only 51% when 3-MC is used as substrate. We have previously shown that TCPO at equimolar concentrations completely inhibits diol formation if the epoxide is used as substrate (22). It is possible that another metabolite co-migrates with this K-region diol. Also noteworthy is the fact that the radioactive peak co-migrating with trans 1,2-dihydroxy-3-MC (peak E) is reduced when TCPO is added to the incubation system. Again, however, another metabolite may co-migrate with the authentic standard.

Table 2: Retention Times for 3-MC Metabolites

Compound	Retention Time (min)
trans 11,12-dihydro-11,12-dihydroxy-3-MC	18
trans 1,2-dihydroxy-3-MC	20.5
cis 11,12-dihydro-11,12-dihydroxy-3-MC	25.5
cis 1,2-dihydroxy-3-MC	29.5
2-hydroxy-3-MC	37.5
1-hydroxy-3-MC	38.5
3-MC 11,12-oxide	44.5
2-oxo-3-MC	47
1-oxo-3-MC	48.5
3-methylcholanthrylene	55.5
3-MC	60

Assays were run as given in Materials and Methods. To each extraction residue was added between 0.5 and 1.0 µg of synthetically prepared 3-MC derivatives. The HPLC was conducted according to previously published procedures (20). Retention times were recorded at maximal peak height.

Since three of the diols have retention times less than that of the K-region diol, it is unlikely that these compounds are all primary metabolites of 3-MC. Our preliminary studies have shown, however, that these substances are rapidly formed, i.e., all are present after only 1 min. incubation (results not shown). The radioactivity associated with the cis K-region diol (peak 3, Fig. 1A), in all probability arises from the on column decomposition of the K-region oxide, which we have demonstrated previously (20). Finally, at least seven radioactive peaks have been isolated which do not have retention times similar to our known 3-MC standards. These include peaks A, B, C, F, H, J and O (Figure 1B).

Table 3: Microsomal Metabolism of 3-Methylcholanthrene - % Metabolism*

Compound	Probable Identity	-TCPO	+TCPO	$\frac{(+TCPO)}{(-TCPO)}$
A	unknown	0.50 ± .03 (1.69)+	0.07 ± .01 (0.29)	(.17)
B	unknown	0.37 ± .02 (1.25)	0.11 ± .02 (0.45)	(.36)
C	unknown	0.57 ± .13 (1.92)	0.10 ± .02 (0.41)	(.21)
D	trans 11,12-dihydro-11,12-dihydroxy-3-MC	1.12 ± .05 (3.78)	0.45 ± .04 (1.84)	(.49)
E	trans 1,2-dihydroxy-3-MC	0.44 ± .08 (1.49)	0.08 ± .02 (0.33)	(.22)
F	unknown	0.26 (0.88)	0.15 ± .02 (0.62)	(.70)
G	cis 11,12-dihydro-11,12-dihydroxy-3-MC	0.55 ± .15 (1.86)	0.40 ± .03 (1.64)	(.88)
H	unknown	1.35 ± .16 (4.56)	0.70 ± .13 (2.87)	(.63)
I	cis 1,2-dihydroxy-3-MC	1.14 ± .21 (3.85)	0.79 ± .02 (3.24)	(.84)
J	unknown	0.69 ± .11 (2.33)	0.46 ± .03 (1.89)	(.81)
K	2-hydroxy-3-MC	8.44 ± 1.08 (28.51)	7.24 ± 1.67 (29.68)	(1.04)
L	1-hydroxy-3-MC	3.90 ± .82 (13.18)	3.77 ± 1.07 (15.46)	(1.17)
M	unknown - 3-MC 11,12-oxide	8.32 ± .48 (28.11)	7.30 ± .81 (30.18)	(1.06)
N	probably ketones	1.49 ± .36 (5.03)	1.80 ± .28 (7.38)	(1.47)
O	unknown	0.07 (0.24)	0.14 ± .07 (0.54)	(2.38)
P+Q	probably phenols	0.39 ± .08 (1.32)	0.83 ± .37 (3.40)	(2.58)
TOTAL		29.60	24.39	

Incubations and HPLC analysis were conducted as outlined in Materials and Methods. The incubation mixture contained either acetone alone (-TCPO) or TCPO in acetone (+TCPO). The values are the average of duplicate determinations The letters refer to the same peaks as in Figure 1B. All values are subtracted from incubations with boiled microsomes.

* percent metabolism was determined by dividing the radioactivity from the specific metabolite by the total radioactivity eluting from the HPLC column.

+ percent contribution of the specific metabolite to the total metabolism.

DISCUSSION

Over the past several years, much work has been done to elucidate the mechanism of tumor production by PAHs. The advent of HPLC has provided an extremely powerful tool for the separation of metabolites of these PAHs and should be of great value in this problem. However, most of the work to date has been on one compound, B[a]P. It is now known that B[a]P gives rise to a variety of metabolites and one of these, trans 7,8-dihydro-7,8-dihydroxy-B[a]P, is believed to be a proximate carcinogen.

We have been interested in the metabolism of another potent carcinogen, 3-MC. Until now, the only metabolic studies have been performed using thin layer chromatography which does not have excellent resolution (17-18). We have now applied the resolving power of HPLC to study 3-MC metabolism. This paper clearly shows that 3-MC gives rise to a number of diol-type molecules, the structures of which have as yet not been identified. The basis for this supposition is the marked reduction in the formation of these metabolites upon the addition of the epoxide hydrase inhibitor TCPO. This paper further demonstrates that 3-MC gives rise to a more complex array of metabolites than previously thought (17-19).

Acknowledgements: This investigation was supported by grants CA 21481 and 20711 from the National Cancer Institute. T.A.S. wishes to thank Ms. M. A. Gangarosa for her excellent technical assistance.

REFERENCES

1. Author unknown (1972) Particulate Polycyclic Organic Matter, National Academy of Sciences, Washington, D. C.
2. Grover, P. L. and Sims, P. (1968) Biochem. J. 110: 159-160.
3. Gelboin, H. V. (1969) Cancer Res. 29: 1272-1276.
4. Marquardt, H., Grover, P. L. and Sims, P. (1976) Cancer Res. 36: 2059-2064.
5. Newbold, R. F. and Brookes, P. (1976) Nature 261: 52-54.
6. Conney, A. H., Wood, A., Levin, W., Lu, A.Y.H., Chang, R. L., Wislocki, P., Goode, R. L., Dansette, P. M., Yagi, H. and Jerina, D. M. in D. Vallow, J. Kocsis, R. Snyder and Vainio (Eds.), Reactive Intermediates, Formation, Toxicity and Inactivation, N. Y., Plenum Press.
7. Levin, W., Wood, A. W., Yagi, H., Jerina, D. M. and Conney, A. H. (1976) Proc. Natl. Acad. Sci. 73: 3867-3871.
8. Levin, W., Wood, A. W., Yagi, H., Dansette, P. M., Jerina, D. M., and Conney, A. H. (1976) Proc. Natl. Acad. Sci. 73: 243-247.
9. Slaga, T. J., Viaje, A., Berry, D. L. and Bracken, W. (1976) Cancer Letters 2: 115-122.

10. Grover, P. L., Hewer, A., and Sims, P. (1971) FEBS Letters 18: 76-80.
11. Grover, P. L., Hewer, A., and Sims, P. (1972) Biochem. Pharmacol. 21: 2713-2726.
12. Borgen, A., Darvey, H., Castagnoli, N., Crocker, T. T., Rasmusen, R. E. and Wang, I. Y. (1973)J. Med. Chem. 16: 502-506.
13. Sims, P., Grover, P. L., Swaisland, A., Pal, K., and Hewer, A. (1974) Nature 252: 326-328.
14. Wislocki, P. G., Wood, A. W., Chang, R. L., Levin, W., Yagi, H., Hernandez, O., Jerina, D. M., and Conney, A. H. (1976) Biochem. Biophys. Res. Commun. 68: 1006-1012.
15. Malaveille, C., Bartsch, H., Grover, P. L. and Sims, P. (1975) Biochem. Biophys. Res. Commun. 66: 693-700.
16. Huberman, E., Sachs, L., Yang, S. K. and Gelboin, H. V. (1976) Proc. Natl. Acad. Sci. 73: 607-611.
17. Sims, P. (1966) Biochem. J. 98: 215-228.
18. Sims, P. (1970) Biochem. Pharmacol. 19: 795-818.
19. Bürki, K., Siebert, R. A. and Bresnick, E. (1972) Biochim. Biophys. Acta 260: 98-109.
20. Stoming, T. A. and Gerardot, R. J. (1977) Life Sci. 20: 113-116.
21. Seifried, H. E., Birkett, D. J., Levin, W., Lu, A. Y. H., Conney, A. H. and Jerina, D. M. (1977) Arch. Biochem. Biophys. 178: 256-263.
22. Stoming, T. A. and Bresnick, E. (1973) Science 181: 951-952.