HIGH MICROSOME-MEDIATED MUTAGENICITY OF THE 3,4-DIHYDRODIOL OF 7-METHYLBENZ[A]ANTHRACENE IN *S. TYPHIMURIUM* TA 98

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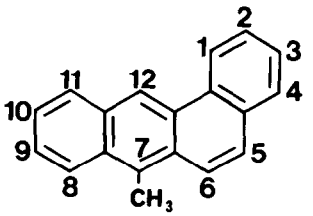
SUMMARY: 7-Methylbenz[a]anthracene and the 1,2-, 3,4-, 5,6- and 8,9-dihydrodiols derived from this hydrocarbon have been tested for mutagenicity towards *S. typhimurium* TA 98 in the presence of rat-liver post-mitochondrial supernatant. At non-toxic concentrations, the mutagenicity of the non-K-region 3,4-dihydrodiol was more than ten-fold higher than that of the other K-region and non-K-region dihydrodiols and more than three-fold higher than that of the parent hydrocarbon. 1,1,1-Trichloropropene 2,3-oxide, an inhibitor of epoxide hydratase, increased the microsome-mediated mutagenicity of 7-methylbenz[a]anthracene but did not alter that of the four related dihydrodiols.

INTRODUCTION: The hydroxylated metabolites formed from 7-methylbenz[a]anthracene by lung and liver microsomal preparations include the 3,4-, 5,6- and 8,9-dihydrodiols and the 3- and 4-phenols[1-4]. The observation that the further



metabolism of non-K-region dihydodiols to vicinal diol-epoxides[5] is important in the metabolic activation of carcinogenic polycyclic hydrocarbons[6-8] has led us to examine dihydrodiols derived from 7-methylbenz[a]anthracene in a bacterial microsome-mediated mutagenicity test. Tissue-mediated mutagenicity tests of this type[9] have been used in studies on the metabolic activation of a variety of chemical carcinogens[10-12] and, with the polycyclic hydrocarbons, can be useful in helping to pinpoint which particular dihydrodiols are pre-

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cursors of biologically-active vicinal diol-epoxides[13,14]. In this paper, we report results obtained when 7-methylbenz[a]anthracene and the 1,2-, 3,4- 5,6- and 8,9-dihydrodiols derived from this hydrocarbon were tested for mutagenicity towards *S. typhimurium* TA 98 in the presence of a rat liver post-mitochondrial supernatant. The effect of an epoxide hydratase inhibitor on the microsome-mediated mutagenicity of these compounds was also investigated.

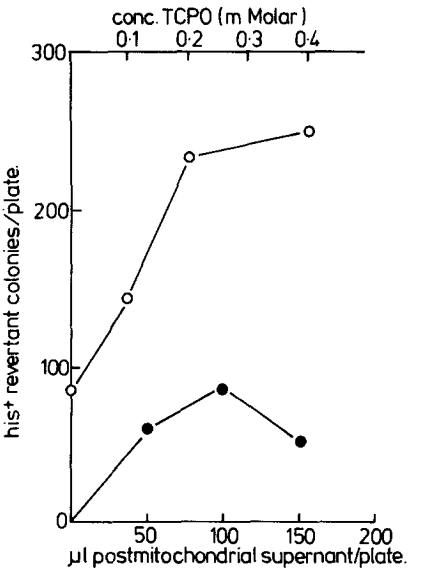
MATERIALS AND METHODS

7-Methylbenz[a]anthracene was prepared from benz[a]anthracene[1] and *trans*-5,6-dihydro-5,6-dihydroxy-7-methylbenz[a]anthracene was prepared from the corresponding *cis*-isomer[15]. 8,9-Dihydro-8,9-dihydroxy-7-methylbenz[a]anthracene was obtained from the hydrocarbon in large scale metabolic experiments[2] and 1,2-dihydro-1,2-dihydroxy-7-methylbenz[a]anthracene and 3,4-dihydro-3,4-dihydroxy-7-methylbenz[a]anthracene were also prepared[16]; these compounds were characterized by their chromatographic, u.v. and mass spectral properties. TCPO* was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin, USA. All compounds were stored at -30° and solutions were prepared in anhydrous DMSO (spectrograde, E. Merck, Darmstadt, Federal Republic of Germany) immediately prior to use in mutagenicity experiments.

Post-mitochondrial supernatants. Adult female BD-IV rats (120-130g) bred in the IARC laboratory, were kept on a Charles River CRF diet; groups of 2-4 animals received an intraperitoneal injection of 3-methylcholanthrene (40 mg/kg) two days before they were killed. Post-mitochondrial supernatants were prepared at 0-4° from pooled rat livers by centrifugation of an homogenate (3 ml of 0.15% KCl/g liver). The resulting post-mitochondrial supernatants were usually kept at 0-4° for 2-3 hrs and then used in mutagenicity experiments although storage of these tissue preparations for up to 4 days at -70° did not appear to affect the microsome-mediated mutagenicity of 7-methylbenz[a] anthracene. All procedures were carried out using sterile glassware and solutions.

Mutagenicity assays. *S. typhimurium* strain TA 98 derived from the histidine auxotroph TA 1538 by introducing an R factor plasmid (ampicillin resistance, pKM 101) was generously provided by Professor B.N. Ames, Berkeley, California, USA. The presence of the R factor was checked by seeding bacteria on agar that contained ampicillin[17] and the mutability of the TA 98 strain was confirmed using 4-nitroquinoline 1-oxide[18]. Unless otherwise stated, post-mitochondrial supernatant, cofactors ($NADP^+$ and glucose-6-phosphate), bacteria ($1\text{-}2 \times 10^8$ cells/plate) and the substance under test, which was added as a solution in DMSO, were combined in histidine-poor soft agar (0.55% w/v, Difco agar, 0.55% w/v sodium chloride, 45.5 µM histidine and 45.5 µM biotin in Sørensen buffer, 5mM, pH 7.4) and plated in triplicate onto histidine-deficient media as previously described[9]. Freshly prepared plates were kept in the dark at room temperature until the soft agar layer had hardened; they were then inverted and incubated at 37°. Control assays, in which the cofactors for the microsomal mono-oxygenase were omitted, were carried out simultaneously. In the linear parts of the dose-response curves (Fig. 2), there was no obvious toxicity of the hydrocarbon derivatives to the bacteria since background lawns of bacteria were present in each case.

* Abbreviations used are: TCPO, 1,1,1-trichloropropene 2,3-oxide; DMSO, dimethylsulphoxide

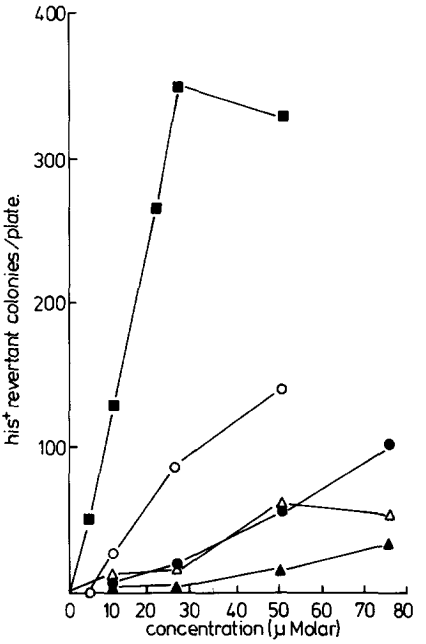


Legend to Fig. 1

The effect on the microsome-mediated mutagenicity of 7-methylbenz[a] anthracene in *S. typhimurium* TA 98 of increasing the amount of post-mitochondrial supernatant added to each plate (●———●) or of adding varying amounts of 1,1,1-trichloropropene 2,3-oxide to plates containing 100 µl of post-mitochondrial supernatant (O———O). The assays were performed as described in MATERIALS AND METHODS with 25 µM 7-methylbenz[a]anthracene added to each plate in DMSO (0.1 ml). Mean values for the number of *his*$^+$ revertants/plate are plotted from groups of from 3-6 plates; the number of *his*$^+$ revertants per plate that appeared when $NADP^+$ and glucose-6-phosphate were omitted or when 7-methylbenz[a]anthracene was omitted, has been subtracted from each value.

RESULTS AND DISCUSSION

In the dose-response study of mutagenicity with 7-methylbenz[a]anthracene and varying amounts of post-mitochondrial supernatant (Fig. 1), maximum mutagenicity was obtained with 100 µl of supernatant per plate; the addition of higher amounts of supernatant decreased the number of mutant colonies of *S. typhimurium* TA 98 that appeared. Neither the hydrocarbon nor any of its dihydrodiol derivatives were mutagenic towards TA 98 when the cofactors for the microsomal mono-oxygenase were omitted. Quantitative comparisons of the mutagenicity of 7-methylbenz[a]anthracene and of four dihydrodiols were carried out at concentrations of up to 75 µM (75 nmoles/ml of soft agar) with 100 µl of post mitochondrial supernatant per plate; these results are shown in Fig. 2. The mutagenic potency of the compounds tested, expressed as the number of histidine revertant colonies per 10^{-7} moles of hydrocarbon per plate, taken from the linear region of the dose response curve, was in the



Legend to Fig. 2

Microsome-mediated mutagenicity of 7-methylbenz[a]anthracene (O——O) and of the 1,2- (●——●), 3,4- (■——■), 5,6- (▲——▲) and 8,9- (△——△) dihydrodiols derived from this hydrocarbon in *S. typhimurium* TA 98. Varying concentrations of the hydrocarbons were incubated in the presence of 100 μl post-mitochondrial supernatant as described in MATERIALS AND METHODS and in the legend to Fig. 1.

following descending order: 3,4-dihydrodiol, 480; 7-methylbenz[a]anthracene, 140; 1,2- and 8,9-dihydrodiols, 40 and the 5,6-dihydrodiol, 20. Thus, the mutagenic metabolite or metabolites formed from the 3,4-dihydrodiol of 7-methylbenz[a]anthracene were some three times more effective than those formed from the parent hydrocarbon and more than ten times more effective in inducing mutations than those formed from the other related dihydrodiols.

These results suggested that the mechanism of activation of 7-methylbenz[a] anthracene is analogous to that described for benzo[a]pyrene[6,8] and benz[a] anthracene[7] and involves the formation of a vicinal diol-epoxide following the sequential actions of microsomal mono-oxygenase and epoxide hydratase enzymes on 7-methylbenz[a]anthracene. In order to investigate the role of the hydratase in the conversion of this hydrocarbon into mutagenic metabolites, microsome-mediated mutagenicity studies were carried out in the prescence of TCPO, a known inhibitor of epoxide hydratase[19]. TCPO alone was not mutagenic to *S. typhimurium* TA 98 at concentrations of up to 0.4mM but did cause a linear increase in the mutagenic effects caused by 7-methylbenz[a]anthracene

when the epoxide hydratase inhibitor was present in concentrations of up to 0.2mM (Fig. 1); a smaller increase in 7-methylbenz[a]anthracene mutagenicity occurred when the TCPO concentration was raised from 0.2 to 0.4mM. At 0.4mM, TCPO caused a three-fold increase in the microsome-mediated mutagenicity of 7-methylbenz[a]anthracene (Fig. 1). In contrast, this concentration of TCPO did not change the microsome-mediated mutagenicity of any of the four dihydrodiols derived from this hydrocarbon and identical dose response curves were obtained with the dihydrodiols either in the absence (Fig. 2) or in the presence of TCPO.

The results of these experiments with the epoxide hydratase inhibitor TCPO can most easily be interpreted as showing that simple epoxides formed from 7-methylbenz[a]anthracene are mutagenic to *S. typhimurium* TA 98 and that the inhibition of the enzymic hydration of these simple oxides to the corresponding dihydrodiols therefore increases the overall yield of mutants obtained from incubations with the hydrocarbon. Data from a different type of experiment in which added epoxide hydratase was found to diminish the microsome-mediated mutagenicity of benzo[a]pyrene[20] support this conclusion. The lack of effect of TCPO on the microsome-mediated mutagenicity of the dihydrodiols seems to indicate that the hydratase is not directly involved in the activation of the diols and supports the idea that the active intermediates are diol-epoxides; the ineffectiveness of TCPO may also mean that the active diol-epoxides formed in this case are not themselves good substrates for epoxide hydratase[21]. This type of experiment also helps to explain why the quantitative correlations between microsome-mediated mutagenicity and carcinogenic potency are so poor within the polycyclic hydrocarbons as a class of compounds[22,23].

With regard to the metabolic activation of polycyclic hydrocarbons, the results reported here show that the microsomal metabolites formed from the 3,4-dihydrodiol of 7-methylbenz[a]anthracene are clearly much more effective as mutagens than are those formed either from the related 1,2-, 5,6- or 8,9-dihydrodiols or from the parent hydrocarbon itself. This indicates that the corresponding vicinal diol-epoxide, 3,4-dihydro-3,4-dihydroxy-7-methylbenz[a]anthracene 1,2-oxide, is likely to be a compound that possesses a high biological activity reminiscent of that associated with certain diol-epoxides related to benzo[a]pyrene[24,25]. Vicinal diol-epoxides derived both from benz[a]anthracene and from 7-methylbenz[a]anthracene have previously been found to be direct acting bacterial mutagens[13]. On theoretical grounds, it has been suggested that diol-epoxides of the type represented by the 3,4-diol 1,2-oxide of 7-methylbenz[a]anthracene might be particularly

effective electrophilic reactants[26] and recent results obtained in bacterial mutagenicity tests with dihydrodiols derived from the weak carcinogen benz[a]anthracene[14] appear to support this proposal.

With 7-methylbenz[a]anthracene, the inference, from the present results, that the 3,4-diol 1,2-oxide is likely to be a biologically important metabolite is supported by other studies, some of which are still in progress. In an *in vitro* malignant transformation system, the 3,4-dihydrodiol has been found to be much more active than the parent hydrocarbon itself or three other dihydrodiols derived from 7-methylbenz[a]anthracene[27] and early results from initiation/promotion experiments on mouse skin point to the same conclusion (I. Chouroulinkov and A. Gentil, personal communication). In addition, the fluorescence spectral characteristics of the 7-methylbenz[a]anthracene moieties that became bound to the DNA of mouse skin treated with this carcinogen have been found to resemble those of anthracene but not those of phenanthrene, which again indicates that metabolic activation involves, in this case the 1,2,3,4-ring rather than the 8,9,10,11-ring[28]. Together the existing evidence indicates therefore that the metabolic activation of 7-methylbenz[a]anthracene involves the further metabolism of a non-K-region diol to a vicinal diol-epoxide and that, with this hydrocarbon, the biologically important vicinal diol-epoxide is most probably 3,4-dihydro-3,4-dihydroxy-7-methylbenz[a]anthracene 1,2-oxide; experiments are in progress which may confirm and extend the results reported here.

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