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MICROSOMAL AND NUCLEAR METABOLISM OF 3-METHYLCHOLANTHRENE

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Abstract—Hepatic nuclei and microsomes prepared from rats pretreated with 3-methylcholanthrene, or hepatic nuclei prepared from untreated rats, were incubated with generally ^{3}H-labeled 3-methylcholanthrene in the presence of an NADPH-generating system. The metabolic products formed by these three systems were analyzed by high pressure liquid chromatographic techniques and the products were comparable, both qualitatively and quantitatively. The metabolic formation, from both nuclei and microsomes, of radioactive products corresponding to the *cis*-1,2- and 11,12-diol, the *trans*-4,5-, 9,10- and 11,12-dihydrodiol, and the 1- and 2-hydroxy and 1- and 2-ketone derivatives of 3-methylcholanthrene was observed when these metabolites were compared, on high pressure liquid chromatographs, with authentic compounds. Pretreatment of the rats with 3-methylcholanthrene resulted in a marked increase in nuclear metabolism, particularly in the formation of 2-hydroxy-3-methylcholanthrene. These studies further reinforce the importance of the nucleus in the metabolic activation of polycyclic hydrocarbons.

When polycyclic hydrocarbons are metabolized by cytochrome P-450-mediated mono-oxygenase systems, the formation of reactive intermediates, such as diol-epoxides, is necessary for the covalent interaction of the polycyclic hydrocarbons with nucleophilic biological macromolecules [1–3]. In oxidative metabolism studies, the microsomal fraction from rat liver has generally been used as the source of enzyme activity [4–8], but evidence has been presented recently that nuclear fractions have a cytochrome P-450-dependent mono-oxygenase system [9–16] which is also inducible upon administration of appropriate agents to rats. Rat liver nuclei can metabolize benzo(a)pyrene to *trans*-dihydrodiols [17] and, furthermore, the stereospecificity of the 7,8-dihydrodiol product is similar in nuclear and microsomal systems [18]. The rat liver nuclei were also able to form the *syn* and *anti* diol-epoxides from [^{3}H]benzo(a)pyrene 7,8-diol [17] which may well be involved in the metabolic activation of the parent hydrocarbon. As indicated above, the formation of dihydrodiol derivatives of benzo(a)pyrene provides indirect evidence [17] for the presence of epoxide hydrase in nuclei. The direct demonstration of this enzyme activity has been made in our laboratory with the nuclear hydration of benzo(a)pyrene-4,5-oxide [19] and in Orrenius' laboratory with the formation of styrene glycol [14].

In the present paper, we extend the evidence for nuclear metabolism to include another polycyclic hydrocarbon, 3-methylcholanthrene, a potent methylated carcinogen [20] whose microsomal metabolism has been shown to yield a complex mixture of products in which oxidation at both aromatic and aliphatic sites can occur [21–25].

MATERIALS AND METHODS

Materials. Generally ^{3}H-labeled 3-methylcholanthrene ([^{3}H]-3-MC) (9 Ci/m-mole) was purchased from the Amersham-Searle Co. (Arlington Heights, IL, U.S.A.) and was purified before use by high pressure liquid chromatography (h.p.l.c.) using a C_{18} μBondapak column (0.39 × 30 cm) and a linear gradient of 70–100% acetonitrile in water over a duration of 30 min. The *trans*-dihydrodiols [24] and *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene derivatives as well as 1- and 2-hydroxy-3-methylcholanthrene and 3-methylcholanthrene-1- and 2-one [21] were prepared as standards. Spectral grade glass-distilled acetonitrile was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Water, for h.p.l.c. use, was obtained by redistillation of double distilled water to which potassium permanganate was added. All other solvents were reagent grade. NADPH was obtained from the Sigma Chemical Co. (Saint Louis, MO, U.S.A.).

Animals and pretreatment. Male Sprague–Dawley rats, 80–100 g in weight, were used as the source of liver microsomes and nuclei. These rats had been maintained on Purina Rat Chow *ad lib.* under alternating light–dark conditions. Forty-eight and 24 hr prior to death, the rats were injected intraperitoneally with 3-methylcholanthrene in corn oil (20 mg/kg body wt). Control animals received the vehicle alone. Nuclei were prepared from rat liver as described previously [9,11], and microsomes were prepared by the method of Seifried *et al.* [26]. In previous publications [11,16,17], we have addressed the problem of microsomal contamination of our nuclear preparation. The amount of protein

present in the nuclear and microsomal preparations was determined by the method of Lowry *et al.* [27].

Microsomal and nuclear metabolism. Liver microsomes (approximately 2.5 mg of protein) or nuclei (approximately 25 mg of protein) were suspended in 0.05 M Tris buffer (pH 7.4) (3 ml) containing NADPH (2 μmoles) and $MgCl_2$ (1 μmole); [^{3}H]-3-MC (50 μCi) and cold 3-methylcholanthrene (0.1 μmole) in acetone (30 μl) were added to the suspension. Incubations were carried out at 37° for 15 min in the dark. The reaction was stopped by the addition of acetone (1.5 ml) and ethyl acetate (3.0 ml), the tubes were vortexed and then centrifuged to separate the layers. The organic phase was removed and the aqueous phase re-extracted with ethyl acetate (3 ml). The ethyl acetate extracts were combined and dried using sodium sulfate, millipored and evaporated to dryness under N_2.

Each sample was dissolved in 250 μl acetonitrile, and 50 μl was injected onto the high pressure liquid chromatographs and eluted with 40–95% acetonitrile in water (containing 0.2% acetic acid, v/v), using the non-linear gradient No. 8 mode (Waters Associates) over 70 min with a flow rate of 1 ml/min. Fractions of 0.5 ml were collected and the radioactivity was determined by liquid scintillation counting techniques.

RESULTS

The mixture of metabolites obtained after metabolism of [^{3}H]-3-MC by control nuclei, induced nuclei and induced microsomes was examined by h.p.l.c. Their radioactivity elution profiles are shown in Fig. 1, together with the fluorescence profile of synthetic derivatives of 3-methylcholanthrene (Fig. 2). The metabolic patterns obtained from both stimulated nuclear and microsomal incubations were similar, as were the metabolic products from control nuclear preparations, although a marked general decrease in metabolism was observed using control nuclei.

The radioactivity profile, when compared to the fluorescence pattern, can be divided into four regions: an initial very polar area; dihydrodiols; alkyl alcohols; ketones and the parent hydrocarbon. The initial fractions (fractions 1–30), shown in Fig. 1, probably contain highly polar metabolites, such as monohydroxyl dihydrodiols and tetrols. These highly polar metabolites seem to be formed more readily, under the *in vitro* conditions used here, by microsomal rather than nuclear preparations (Table 1). However, as no synthetic standard compounds are available, these metabolites were not investigated further in the present study.

The dihydrodiol region (fractions 31–80) of both microsomal and nuclear metabolites had radioactive peaks which corresponded to the *trans*-11,12- and *cis*-1,2-dihydrodiol as well as the *trans*-4,5-, *cis*-11,12- and *trans*-9,10-dihydrodiol; however, the radioactivity associated with the metabolic *cis*-11,12-and *trans*-9,10-dihydrodiol could not clearly be separated into two distinct peaks due to the similar retention times of these two compounds. Unknown metabolites also eluted in the dihydrodiol region, e.g. a broad radioactive metabolic peak was observed in the region associated with the *trans*-4,5-dihydrodiol and it is probable that this peak contains more than one component. A major unknown metabolite also eluted between the radioactive peaks of the metabolic *trans*-4,5- and *cis*-11,12/*trans*-9,10-dihydrodiols.

The only qualitative difference between microsomal and nuclear metabolic products eluting in the dihydrodiol region was the presence of a minor microsomal metabolite eluting between fractions 63 and 67 which could not be detected with certainty as a nuclear metabolite.

Quantitatively, the total amount of radioactive

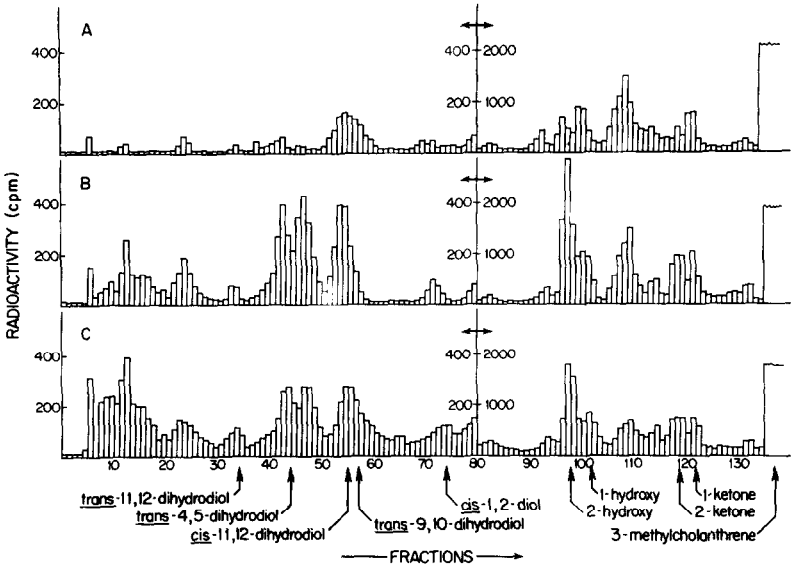

Fig. 1. Radioactive h.p.l.c. elution profiles of the liver metabolism of [^{3}H]-3-methylcholanthrene by control nuclei (A), induced nuclei (B), and induced microsomes (C). Background radioactivity has been subtracted. Fractions 1–80 are shown on a scale increased five times that of fractions 81–140.

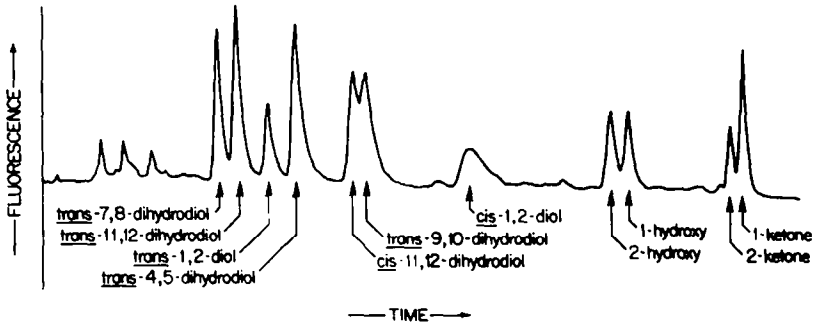

Fig. 2. Fluorescence h.p.l.c. elution profiles of authentic derivatives of 3-methylcholanthrene. The compounds were dissolved in acetonitrile and injected into a C_{18} μBondapak column, and a gradient from 40–95% acetonitrile in water (containing 0.2% acetic acid, v/v) was conducted as described in the text. The eluent was monitored by fluorescence.

Table 1. Nuclear and microsomal metabolism of 3-methylcholanthrene*

Fraction number	Possible structure†	Control nuclei		Induced nuclei		Induced microsomes	
		Metabolite (%)	Product (pmoles)	Metabolite (%)	Product (pmoles)	Metabolite (%)	Product (pmoles)
1–30	Polyols	6.5	0.36	11.7	0.92	14.6	1.23
31–36	*trans*-11,12-Dihydrodiol	1.4	0.08	2.0	0.16	2.3	0.20
40–45	*trans*-4,5-Dihydrodiol‡	2.0	0.11	4.1	0.33	3.4	0.29
46–51	Unknown	1.9	0.11	4.1	0.33	3.4	0.29
52–62	*cis*-11,12- and *Trans*-9,10-Dihydrodiol‡	4.9	0.27	5.7	0.45	5.9	0.50
63–67	Unknown	ND§	ND	ND	ND	1.9	0.16
70–76	*Cis*-1,2-diol	2.2	0.12	2.4	0.19	3.1	0.27
78–88	Unknown	4.4	0.25	4.5	0.35	6.4	0.54
91–95	Unknown	7.3	0.40	3.7	0.29	4.4	0.38
97–100	2-Hydroxy	7.7	0.42	16.6	1.31	9.9	0.84
101–105	1-Hydroxy	11.2	0.62	6.7	0.52	7.4	0.63
106–113	Unknown	24.5	1.36	14.2	1.12	11.6	0.98
114–116	Unknown	6.4	0.35	3.9	0.31	4.6	0.39
117–120	2-one	6.2	0.34	9.4	0.74	7.3	0.62
121–125	1-one	9.9	0.55	6.2	0.49	7.5	0.63
131–135	Unknown	4.6	0.25	5.0	0.40	6.2	0.52

* See materials and methods and legend to Fig. 1 for details.

† Radioactive metabolites were detected by their high pressure liquid chromatographic behavior relative to authentic compounds.

‡ Uncharacterized radioactive metabolites were also present in this peak.

§ ND, not detected.

metabolites found in the dihydrodiol region from both induced microsomal and nuclear incubations was similar (1.43 and 1.45 pmoles, respectively), while control nuclei produced lower levels of metabolites in this region, i.e. 0.69 pmole.

The third area of metabolic radioactivity, fractions 81–105, contained three major radioactive peaks, two of which correspond with 1- and 2-hydroxy-3-methylcholanthrene on h.p.l.c. There was a marked increase in the formation of 2-hydroxy-3-methylcholanthrene with induced microsomal, and particularly, induced nuclear preparations when compared with that formed from control nuclei (see Table 1).

In the fourth region, fractions 106–145, which contains relatively nonpolar metabolites, the ketones, 3-methylcholanthrene-1-one and 2-one, were detected, as well as unchanged hydrocarbon. The formation of these ketones could be the result of chemical oxidation of the related monohydroxy-metabolites [21] during the incubation process. There was a major radioactive peak (fraction 107–110) eluting just before the ketones, which could be due to phenols. Since no synthetic phenolic compounds were available, this peak could not be characterized more fully in the present study.

DISCUSSION

The metabolites formed from 3-methylcholanthrene by induced microsomal and nuclear systems appear to be virtually identical when examined by h.p.l.c. This lends support to previous observations [11,17,18] in which a qualitatively similar spectrum of metabolites was found when benzo(a)pyrene was incubated in the presence of either nuclei or microsomes. The monooxygenase system present in rat liver nuclei can be induced by prior administration of 3-methylcholan-

threne, as shown by the higher level of metabolites formed with induced compared to control nuclei (Fig. 1 and Table 1), and thus does resemble the cytochrome P-450 microsomal mono-oxygenase system.

A previous examination of diol formation from 3-methylcholanthrene by microsome-mediated incubations had shown the formation of all five aromatic dihydrodiols as well as the *cis* and *trans*-1,2-diols [24], the products being analyzed by h.p.l.c. on a silica column. In the present study using a reverse phase high pressure liquid chromatographic system, it has been possible to separate a wide variety of structurally different 3-methylcholanthrene metabolites of varying polarity. However, some resolution was lost in the dihydrodiol region and only five out of the seven vicinal diols could be detected with certainty. The *trans*-11,12-, and 4,5-dihydrodiols have high pressure liquid chromatographic retention times similar to the two diols not detected here (*trans*-7,8-and 1,2-diol), and these latter diols may well be present but the peaks were not resolved under the present conditions. The metabolic formation of a *cis*-dihydrodiol (the K-region diol) was observed here and has been reported previously [23] as a metabolite. However, as the identities of the products described here are based solely on their high pressure liquid chromatographic retention times relative to authentic compounds, their characterizations must be regarded as tentative until more complete physicochemical data are available.

Monohydroxylation of the methylene bridge of 3-methylcholanthrene by microsomal preparations has been reported previously [4,21,23,25]. However, the reports differ in the relative amounts of 1- and 2-hydroxy-3-methylcholanthrene formed. Whether these conflicting reports may be due to differences in stability of the metabolic products, to differences in induction procedure, or to differences in the strain or ages of animals used is not known at present. The formation of 2-hydroxy-3-methylcholanthrene was found to be increased significantly by the prior induction of the liver in both microsomal and nuclear systems, whereas in control nuclei metabolism the 1- and 2-hydroxy derivatives were found in approximately the same amounts. The role which hydroxylation of the methylene bridge plays in the *in vivo* activation of 3-methylcholanthrene is not yet known, although the bay region diol-epoxide has been reported to be involved in the binding of 3-methylcholanthrene to DNA [28–30]. Selective deuteration in the 1- and 2-positions, however, does lead to lower tumor incidence in skin painting experiments [31].

The evidence presented here clearly shows that both quantitatively and qualitatively the nucleus resembles the endoplasmic reticulum in its mono-oxygenase system. These results also clearly indicate that nuclei can metabolize 3-methylcholanthrene to more active forms and, thus, the data cause doubts about the exclusive involvement of endoplasmic reticulum in the activation process. It is also obvious from these experiments that additional work is necessary to ascertain the mode of *in vivo* metabolic activation of 3-methylcholanthrene.

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