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USE OF A DIMETHYLATED OXIRANE TO CHARACTERISE MICROSOMAL MONO-OXYGENASES IN RAT LIVER

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Abstract—A newly-synthesised dimethylated chlorocyclodiene epoxide is metabolised by NADPH-supplemented rat liver microsomes to produce either one or both of two major metabolites, M1 and M2. Microsomes from adult male rat liver produce both metabolites. By contrast, microsomes made from the livers of mature or immature female rats make only metabolite M1, as do hepatic microsomes from pre-pubertal male rats. Male rats castrated when 7 days old provide liver microsomes that make little of metabolite M2 upon killing at 3–5 months *post partum*. After induction with phenobarbital, microsomes from adult male and female rat liver make metabolites M1 and M2, together with a further unidentified metabolite. After induction with 3-methylcholanthrene, hepatic microsomes from mature female rats continue to make only metabolite M1 but to make it in larger quantities. These differences, which appear to result from the activity of three or more mono-oxygenases, could possibly be exploited to monitor the mono-oxygenase status of rat liver microsomes.

INTRODUCTION

Rats differ from other common laboratory animals in that suitably-supplemented liver microsomes from adult males metabolise many xenobiotics more rapidly than those from livers of adult females [1–3]. Quantitative differences in the primary metabolism of aminopyrine, ethylmorphine and hexobarbital by hepatic microsomes of rats have also been observed in relation to the effect on drug metabolising activity of age, of enzyme induction and of castration [4, 5].

Much effort has recently been directed towards the characterisation of microsomal mono-oxygenases. It now seems probable that cytochrome P_{450} and cytochrome P_{448} are distinct entities [6, 7] and the term cytochrome P_{450} could itself be a generic name embracing a group of related haem proteins [8, 9]. Unless several substrates are studied simultaneously, quantitative differences in metabolism are of limited use for investigating the possible presence of closely similar enzymes with overlapping specificities. If, for example, induction stimulates substrate disappearance, the extra activity might be due to the presence of more of the mono-oxygenase present in non-induced liver or to the action of a new, more potent enzyme.

Most truly xenobiotic substances are unsatisfactory substrates in this respect, although chlorphentermine is metabolised in a qualitatively different way by different species [10]. An intermediate situation is represented by biphenyl, which is hydroxylated to different relative extents in the 2- and 4-positions by microsomes from different sources [11]. Some steroids, however, apparently show differences in the position of hydroxylation when exposed to mono-oxygenases of different origins. Thus [4-^{14}C]testosterone has been used to demonstrate that hepatic microsomes from immature male rats, and from female rats of any age, are apparently unable to hydroxylate this compound in the 16α position, i.e. if such hydroxylation occurs, the amount of the product formed is too low for it to be detected by current methods [12].

A study of the biodegradability of some chlorinated cyclodienes related to dieldrin showed that there was an apparent qualitative difference in the way one such compound was metabolised by hepatic microsomes prepared from adult male and from adult female rats. This substance, 1,2,3,4,9,9-hexachloro-1,4,4a,5,6,7,8,8a - octahydro - 6,7 - dimethyl - 6,7 - epoxy-1,4-methanonaphthalene (Fig. 1), is a dimethylated epoxide and is referred to below as DME. It can be readily synthesised from hexachlorocyclopentadiene, vinyl chloride and dimethyl butadiene [13].

Statistically significant differences in the rate of metabolism of DME by microsomes from different sources have been obtained in 80 independent experiments. The present communication, however, is principally concerned with apparent qualitative differences. We present these data believing that the metabolism of DME could be used to investigate the types, as well as the activities, of mono-oxygenases present in hepatic microsomes derived from different sources.

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Abbreviations employed: DME, 1,2,3,4,9,9-hexachloro-1,4,4a,5,6,7,8,8a - octahydro - 6,7 - dimethyl -6,7 - epoxyl,4-methanonaphthalene; M1, M2, two metabolites of DME, partial structures as in Fig. 1; g.l.c., gas–liquid chromatography; r.r.t., relative retention time; t.l.c., thin layer chromatography.

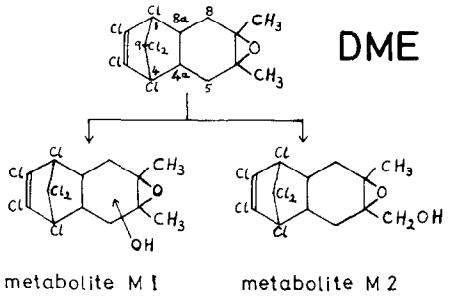

Fig. 1. Formula of 1,2,3,4,9,9-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-6, 7-dimethyl-6, 7-epoxy-1, 4-methanonaphthalene (DME) and the tentative structures of metabolites M1 and M2 based on mass spectrographic evidence.

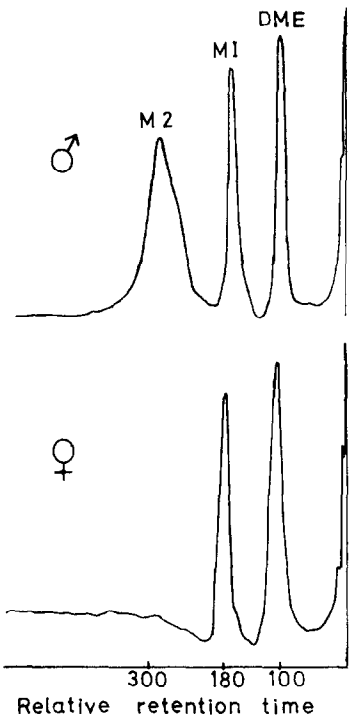

Fig. 2. Gas chromatograms showing solvent front, residual DME and metabolites produced by hepatic microsomes from adult male and from adult female rats. The results are typical of those obtained from 12 independent experiments. The g.l.c. results were obtained using a column (2 m) of SE 52 at an oven temperature of 185°C. The relative retention times of DME, M1 and M2 are 100, 180 and 303, respectively, the last with minor breakdown to its olefinic counterpart. Incubation conditions are described in the Methods section.

METHODS

Microsomes were prepared from livers of CD strain rats. Liver samples were homogenised in ice-cold 0.15M KCl and the homogenate was centrifuged at 11,000*g* for 30 min. The supernatant was removed and re-centrifuged at 105,000*g* for 60 min. The microsomal pellets were washed by re-suspending them in 0.15M KCl, re-centrifuged at 105,000*g* for 30 min and finally re-suspended in 0.15M KCl. A quantity of 0.5 ml of this suspension, equivalent to 0.25 g wet wt of liver, was added to a mixture containing 2.5 ml of 0.14M NaH_2PO_4/0.10M NaOH buffer, pH 7.2, and 0.8 ml of 0.15M KCl. Where applicable, the microsomal suspension was supplemented by the addition of an NADPH-generating mixture comprising 6.3×10^{-4}M $NADP^+$, 3.0×10^{-3}M glucose 6-phosphate and 2.4 units of glucose 6-phosphate dehydrogenase (EC 1.1.1.49). The DME was added as 10 μl of an ethanolic solution containing 3 μg/μl. Incubations were for 10 min at 37° in glass-stoppered flasks shaken at 100 strokes/min. The reaction was stopped by adding 2 ml of acetone and the reaction products were partitioned into *n*-hexane. Portions (5 μl) were examined by g.l.c. using a SE52 column at 185° and a ^{63}Ni source electron capture detector. A more detailed account of the chemical analysis appears elsewhere [13].

Phenobarbital induction involved three daily intraperitoneal injections of a solution containing 50 mg/ml phenobarbital in physiological saline. The dosage was 75 mg/kg body weight. 3-Methylcholanthrene was dissolved in corn oil to give a solution containing 20 mg/ml. This was injected intra-peritoneally on three successive days at a rate of 25 mg/kg body weight.

RESULTS

Hepatic microsomal enzymes from sexually mature male rats convert DME to two major metabolites, M1 and M2, which are detectable by g.l.c. (Fig. 2). Mass spectrographic evidence is consistent with these metabolites having the structures indicated in Fig. 1, although precise identification is not essential for their use as 'markers' to characterise mono-oxygenases. Of importance are the facts that neither metabolite is formed unless an NADPH-generating system is added to the microsomes [13] and that metabolite M2 is not formed when M1 is incubated, in the presence of NADPH-generating mixture, with hepatic microsomes from mature male rats.

To study the metabolism of DME by liver microsomes from male rats of different ages, litter mates were killed at 14, 21, 28, 35 and 42 days after birth. For those killed when 14 days old, two independent experiments were done, in each case with bulked liver prepared from four individuals; for each of the other ages, three or more independent experiments were done using liver from a single individual. Figure 3 shows that liver enzymes from males younger than about 30 days metabolise DME in a qualitatively similar manner to those from liver of young or mature female rats. The characteristic ability of liver enzymes from the male to make metabolite M2 is first manifest approx 35 days after birth. This corresponds to the time of onset of puberty. In a parallel set of experiments, with similar replication, liver microsomes from female litter mates were used as the enzyme source. Although lower rates of metabolism of DME were evident using microsomes from

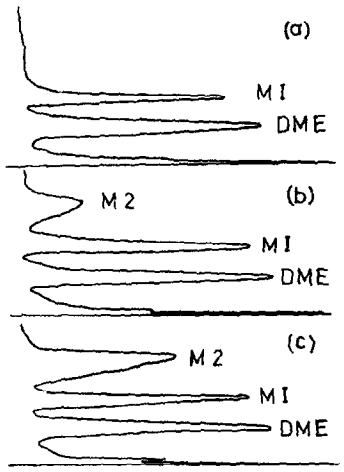

Fig. 3. Gas chromatograms showing the progressive increase, with age, of production of metabolite 2 by supplemented liver microsomes from young male CD rats. Incubation conditions, including the supplementary NADPH-generating system, are described in the Methods section, except that the incubation time was 30 min. (a) 28-day old male rats; (b) 35-day old male rats; (c) 42-day old male rats. The hepatic microsomes of 14, 21 and 28 day old male rats produce no metabolite M2. From about 35 days old, metabolite M2 is clearly visible at r.r.t. 303 and by 42 days old the ratio of the amounts of metabolite M2 to M1 is similar to that for the adult (Fig. 2). Similar experiments were carried out with female rats; at all ages the results were identical to that shown in Fig. 3(a).

livers of younger animals, the qualitative picture was the same at all ages, i.e. microsomal preparations from female rats of all ages gave g.l.c. traces with the same appearance as that of the adult female in Fig. 2 or the 28 day old male in Fig. 3.

Androgen 'imprinting' is known to affect the rate of metabolism of many xenobiotics and, for steroids, may also influence the position of hydroxylation [14–16]. The preceding results are therefore consistent with the possibility that metabolite M2 of DME is produced by an androgen-dependent mono-oxygenase system. This possibility is supported by the results of work done on male rats castrated at different ages (Fig. 4). In three experiments, hepatic microsomes from male rats castrated when 30 days old metabolised DME more slowly than similar preparations from intact male litter mates but they nevertheless produced both metabolite M1 and metabolite M2. Animals castrated 7 days *post-partum* produced relatively much smaller amounts of metabolite M2 than of metabolite M1, and in two of the four experiments only negligible amounts of metabolite M2 were produced.

The effect of phenobarbital induction of liver enzymes in male and female adult rats is shown in Fig. 5. For male rat liver microsomes, the only qualitative effect of phenobarbital induction is the appearance of an additional small peak at a relative retention time (r.r.t.) of 332. This extra peak has not been observed in preparations using microsomes

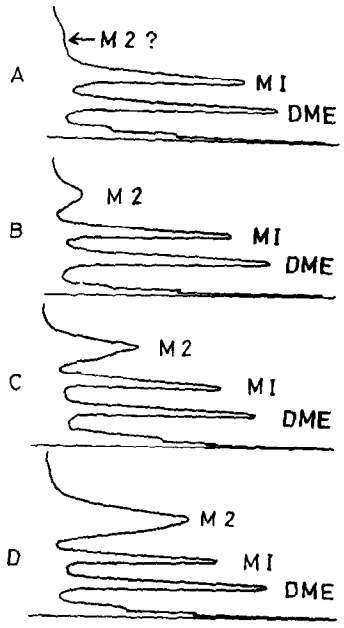

Fig. 4. Gas chromatograms showing the effect of age of castration on the production of metabolite M2 by hepatic microsomes from adult male CD rats. A, castrated at 7 days after birth and killed when 5 m old (triplicates within two experiments); B, castrated at 7 days after birth and killed when 3 m old (two experiments); C, castrated at 30 days after birth and killed when 2–4 m old (three experiments); D, non-castrated 3 m-old control male (typical of six experiments).

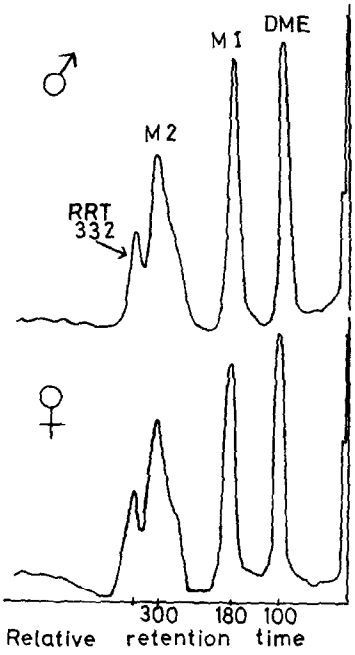

Fig. 5. Gas chromatograms showing the effect of phenobarbital induction of liver enzymes prior to preparing liver microsomes. For non-induced controls (five independent experiments) see Fig. 2. After induction, microsomes from livers of both sexes make metabolite M2 and a new metabolite with a r.r.t. of 332. Details of the induction procedure, of the incubations and of the analytical methods are given in the Methods section.

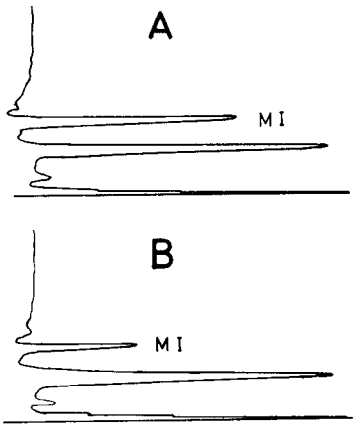

Fig. 6. Gas chromatograms showing the effect of 3-methylcholanthrene induction of liver enzymes of adult female CD rats prior to preparing liver microsomes. For induced male rats, studied simultaneously, no qualitative or quantitative change from the non-induced (Fig. 2) condition was evident. For the induction procedure, and for incubation and analytical details, see the Methods section. Results are typical of triplicates within three independent experiments.

from non-induced livers. Microsomes from phenobarbital-induced livers of female rats made metabolite M2 in large quantities. They also made the extra metabolite with a r.r.t. of 332. Thus the qualitative pattern of metabolism by microsomes from livers of male and female adult rats were identical, and both were different from the pattern obtained using microsomes made from non-induced livers.

In contrast to the effect of phenobarbital, 3-methylcholanthrene induction did not lead to qualitative changes in metabolite production by microsomes from livers of either sex of CD rats (Fig. 6). In particular, 3-methycholanthrene failed to induce in female rat liver a mono-oxygenase capable of producing metabolite M2 (despite an approximate doubling of the amount of metabolite M1 produced by induced compared with corn-oil injected female rats). Other incubations contained aniline as substrate. These showed that, per mg protein present, 30% more aniline was converted to *p*-hydroxyaniline after 3-methylcholanthrene induction than in non-induced microsomal preparations.

DISCUSSION

For many substrates, only quantitative differences in drug-metabolising activity are evident when microsomes are used from livers of male and female rats, from livers of rats of different ages, or from livers of rats pre-treated with different inducing agents [3, 4]. However, DME is a readily-synthesised xenobiotic which shows an apparent qualitative difference in metabolism when added, together with NADPH-generating mixture, to rat hepatic microsomes from animals of different ages, sex or pre-treatments. Its metabolites are readily detectable in sub-nanogram amounts without t.l.c. separation and without radio-labelling. Oxidative reactions are not complicated by hydrolytic action because epoxide hydratase activity is prevented by the steric effect of the two methyl groups on the oxirane ring [13, 17].

At least two groups of mono-oxygenases exist in liver, the terminal cytochrome being P_{450} and P_{448}, respectively. Inducing agents such as 3-methylcholanthrene induce cytochrome P_{448}-dependent mono-oxygenases [7]. The present work with DME suggests that metabolite M1 is made, at least after 3-methylcholanthrene induction, by a cytochrome P_{448}-dependent hydroxylase present in female rat liver microsomes. It is not clear whether this same enzyme is responsible for the metabolite M1 made by microsomes from uninduced liver of male and female rats.

A probable corollary is that metabolite M2 is not made by a P_{448}-dependent hydroxylase. On the contrary, the appearance of metabolite M2 only after the onset of puberty in the male rat (Fig. 3) is consistent with it being produced by an androgen-dependent enzyme system. This possibility is reinforced by the fact that early castration partly or completely suppresses its appearance (Fig. 4). Its production after phenobarbital induction suggests that it is probably formed by a cytochrome P_{450}-dependent hydroxylase [7].

The superior drug-metabolising ability of hepatic microsomes from male rats is often ascribed to an increased affinity of substrate for cytochrome P_{450} arising from some modification brought about by androgens [5]. The results described above throw some doubt upon this interpretation in the present case. The *quantitatively* superior DME-metabolising ability of microsomes from sexually mature male rats, compared to those from mature females, can be accounted for in terms of a *qualitative* difference in the reaction products. As was reported earlier [13], and as is also apparent in Figs. 2, 3(c) and 4(D), metabolite M2 represents 60–75% of the g.l.c.-visible metabolic products produced by male rat liver microsomes. If the greater rate of disappearance of DME using such microsomes is to be ascribed to increased enzyme–substrate binding, the affinity between DME and the enzyme that produces metabolite M2 must be increased from a negligibly low level by androgens in the growing male and also by phenobarbital induction of liver enzymes in mature females.

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