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MICROSOMAL *N*-DEMETHYLASE ACTIVITY IN DEVELOPING RAT LIVER AFTER ADMINISTRATION OF 3-METHYLCHOLANTHRENE*

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Abstract—Microsomal *N*-demethylase activity was examined during various stages of liver development. Its specific activity in fetal and in 1-day neonatal liver was significantly lower than in the liver of the adult female. Between day 1 and 3 *post partum*, the sp. act. of *N*-demethylase rose to the adult female value. The enzymatic activity in the gravid and nonpregnant rat was similar; that of the adult male was 1·7-fold higher. 3-Methylcholanthrene administration was attended by an increase in liver enzyme activity in adult female, neonatal, preweanling and weanling rats. The enzyme activity could not be induced in fetal liver after administration of the drug to the pregnant rat or directly into the amniotic sac. In the former case, the possibility of the inability of the drug to be transported across the placenta was excluded. It was concluded that some aspect of the maternal environment prevented the elaboration of the drug-metabolizing enzymes after administration of 3-methylcholanthrene.

A GROUP of enzymes which function in the biotransformation of physiologic as well as pharmacologic agents is localized within the microsomal fraction of a rat liver homogenate (reviewed in refs. 1–3). The activity of these enzymes varies with species, sex, genetic background and the physiological state of the animal.[1] In addition, the activity of the microsomal drug-metabolizing enzymes is minimal in the livers of fetal rats and rabbits, but increases rapidly *post partum*.[4–7]

A marked increase in the effective activities of several of the liver microsomal enzymes which function in biotransformation occurs after a single i.p. injection to young rats of the polycyclic hydrocarbon, 3-methylcholanthrene.[8–14] Treatment of newborn rabbits with phenobarbital[15,16] or of newborn rats with 3,4-benzpyrene[17] does result in an elevation in the activities of some of the drug-metabolizing enzymes. Administration of barbital to pregnant rats was attended by an increase in the activities of the drug-metabolizing enzymes in the maternal liver.[18] However, treatment of the pregnant animal with an "inducing" agent, in almost all cases, exerted little effect upon the activity of these enzymes in the fetal liver.[17,19] Fouts and Hart[16] have noted a small increase in the activity of the drug-metabolizing enzyme system upon administration of phenobarbital to pregnant rabbits, but not when the drug is given before the last 4 days of fetal life.

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The results of these studies indicated that a more complete investigation of the effects of an inducing stimulus upon the microsomal enzymatic complement of fetal liver would be warranted. In the present investigation, the effects of 3-methylcholanthrene administration upon *N*-demethylase activity have been examined at various stages of liver development. It was concluded that some aspect of the maternal circulation prevented the induction of this microsomal enzyme in fetal rat liver.

EXPERIMENTAL

Animals. The rats employed in this study were obtained from the Cheek–Jones Co., Houston, and included the following groups: male and female rats, 200–300 g in wt; pregnant rats; preweanling rats, 25 g; neonates, 5–7 g; male weanling rats, 40 g; rat fetuses, 16–18 days of gestation. The rats were allowed free access to both Purina rat diet and water.

3-Methylcholanthrene was administered as a single injection i.p or directly into the amniotic sac at a dose level of 1 mg in 0·5 ml corn oil/50 g body wt. (the fetuses were assumed to weigh 5 g). The rats were sacrificed periodically thereafter.

Chemicals. Thin-layer chromatography (TLC) sheets consisted of layers of silica gel on plastic foils and were purchased from the Eastman Kodak Co. The silica gel foils were activated prior to use by heating at 80° for 1 hr. 3-Methyl-4-monomethylaminoazobenzene was generously supplied by Dr. A. H. Conney of the Wellcome Research Laboratories. 3-Methylcholanthrene-6-^{14}C (5 μc/μmole) was obtained from the New England Nuclear Corp.

Enzyme assay. The animals were sacrificed by exsanguination after aortic transection; the livers were removed and a 20% homogenate in 1·15% KCl was prepared at 4°. The homogenate was centrifuged at 9000 *g* for 10 min in a Sorvall centrifuge and the resultant supernatant fraction was carefully withdrawn by means of a Pasteur pipette. The latter served as the source of the microsomal enzymes.

The metabolism of 3-methyl-4-monomethylaminoazobenzene (3Me4MAB)* was conducted in an assay system which was essentially that of Conney *et al.*,[8] with the modifications outlined below. The composition of the assay system in a total volume of 1·5 ml included: 9000 *g* supernatant fraction, 0·5 ml (8–13 mg protein); fructose-1,6-diphosphate, 6 μmole; NAD, 100 μg; NADP, 100 μg; ATP, 1 μmole adjusted to pH 7·4; nicotinamide, 60 μmole; $MgCl_2$, 5 μmole; KCl, 10 μmole; K-phosphate buffer, pH 7·5, 100 μmole; 3-Me4MAB, 75 μg, dissolved in 25 μl methanol. Under these conditions, the enzyme reaction was linear with respect to time (for at least 15 min) and to the amount of protein (up to 22 mg protein per system). Routinely, 15 min was chosen for the duration of the enzyme reaction.

The reaction was stopped by the addition of 1·5 ml acetone and then 2·0 ml benzene was added. The 3Me4MAB and metabolites were extracted into the acetone–benzene phase and a 2·0-ml aliquot of the latter was evaporated to dryness in 25-ml Erlenmeyer flasks in a vacuum oven. The material was dissolved in 0·2 ml benzene and the amount of the metabolites in this phase was determined by TLC of 50 μl on silica gel–plastic foils with n-hexane:benzene (1:1, v/v) as the developing

* Abbreviations used in this paper are: NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; 3Me4MAB, 3-methyl-4-monomethylaminoazobenzene; ATP, adenosine-5′-triphosphate; 3-MC, 3-methylcholanthrene; RNA, ribonucleic acid.

solvent. The chromatography was conducted in an Eastman Kodak sandwich chamber until the solvent front had reached 15 cm. Under these conditions, the R_f of authentic 3Me4MAB is 0·49; of the demethylated product, 3-methyl-4-aminoazobenzene, 0·24; of the cleaved products (products of azo bond reductive cleavage), 0·0.

The readily visible yellow to yellow-orange spots were cut out, placed in test tubes and 1·0 ml of 7 N HCl were added. The silica gel was dislodged from the plastic foil by shaking and the gel particles were separated by centrifugation. The absorption at 505 mμ of the resultant colored solution was determined. The principle product under the assay conditions was the demethylated product. Excellent agreement was noted between the method originally suggested by Conney *et al.*,[8] i.e. chromatography on alumina columns, and the above TLC procedure.

The protein content of the 9000 *g* supernatant preparation was determined by the Lowry procedure.[20]

Distribution of labeled 3-*MC.* 3-MC-6-^{14}C (5 μc/μmole), 10 μc in 0·5 ml corn oil, was injected i.p. into rats 16–18 days pregnant. The rats were killed 2·5 days later, the maternal and fetal livers were removed, and a 20% homogenate was prepared in cold formic acid in a Waring–Blendor. The formic acid homogenate was centrifuged at 10,000 rpm (Sorvall) for 10 min. The radioactivity present in the supernatant fraction was determined by liquid scintillation techniques with Bray's phosphor system.[21] The efficiency of the counting was 57 per cent.

RESULTS

The *N*-demethylase activity in developing liver and in the liver of pregnant rats is presented in Table 1. The specific activity of liver *N*-demethylase was significantly

TABLE 1. LIVER *N*-DEMETHYLASE ACTIVITY IN PREGNANCY AND DURING DEVELOPMENT*

Rat	Liver *N*-demethylase activity (mμmoles product/mg protein)	Relative activity†
Adult female	3·2 ± 0·4 (4)‡	1·0
Adult male	5·3 ± 0·4 (4)	1·7
Pregnant, 18–20 days	2·5 ± 0·3 (6)	0·8
Fetus, 18–20 days	0·6 ± 0·1 (6)	0·2
Neonate, <1 day (mixed sex)	1·3 ± 0·3 (5)	0·4
Neonate, 3 days old, 7 g (mixed sex)	2·8 ± 0·5 (4)	0·9
Preweanling, 25 g (mixed sex)	3·0 ± 0·4 (4)	0·9
Male weanling, 40–50 g	3·2 ± 0·3 (4)	1·0
Female weanling, 40–50 g	3·4 ± 0·3 (4)	1·0

* See Experimental section for experimental details. The 9000 *g* supernatant fractions from individual livers from the adult male and female, 40–50 g weanlings and pregnant rats were employed as enzyme sources. The livers from all the fetuses of a pregnant rat or from 6–10 neonates were combined and the supernatant fractions were prepared; livers from 2 preweanlings were employed in the preparation of the supernatants. The time of incubation was 15 min.

† *N*-demethylase activity of liver from adult female rats was assumed to be 1·0.

‡ Mean ± S.E. (No. of rats or groups).

lower in the fetus and in the 1-day-old neonate compared to the adult female. The depressed values were not the result of a lowered capacity of the NADPH-generating system, since bolstering of the assay mixture with either NADPH or NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase exerted little effect upon the formation of the demethylated product. Between the first and third day *post partum*, enzyme activity in the neonatal liver rose to the normal adult value. Enzyme activities in the livers of preweanling, male or female weanling and pregnant rats were similar.

The effect of administration of 3-methylcholanthrene to adult female rats is depicted in Table 2. At 24 hr after injection of the drug, liver *N*-demethylase activity rose

TABLE 2. EFFECT OF 3-MC ADMINISTRATION UPON *N*-DEMETHYLASE ACTIVITY IN ADULT LIVER*

Days after 3-MC injection	Liver *N*-demethylase (mμmoles product/mg protein)
0†	3·2 ± 0·3 (6)‡
1	7·7 ± 0·6 (5)
2	8·6 ± 0·4 (4)
3	8·7 ± 0·6 (3)
4	10·0 ± 0·9 (4)

* Adult female rats were given a single injection of 3-MC, 20 mg/kg, in corn oil, i.p., and were sacrificed at the times indicated in the table. *N*-demethylase activity was determined as described in the text.

† Animals receiving an injection of only corn oil. Preliminary experiments had indicated that the values for *N*-demethylase activity in corn oil-treated rats (1, 3 or 4 days after injection) were essentially identical to those of noninjected controls.

‡ Average ± S.E. (No. of rats).

to 240 per cent of the control value. Thereafter, enzyme activity gradually reached a plateau, until at 4 days after the initial administration of the polycyclic hydrocarbon, the sp. act. of *N*-demethylase represented 310 per cent of the original control value. The effect of 3-methylcholanthrene upon the microsomal *N*-demethylase activity was also apparent in preweanling and in neonatal rats (Table 3). Enzyme activity in liver from neonates was augmented by 210 and 480 per cent, at 2 and 3 days after administration of 3-MC respectively. On the other hand, at 2 and 3 days, the specific activity of liver *N*-demethylase from preweanling rats was enhanced by 280 and 530 per cent, respectively (Table 3).

The magnitude of the response of preweanling rats to the polycyclic hydrocarbon raised the possibility of an equal effect in fetal rat liver. The results of a number of experiments clearly indicated that no elevation in the fetal liver microsomal *N*-demethylase activity was demonstrable after the i.p. administration of 3-methyl cholanthrene to pregnant rats, even after 3 days, although the maternal liver responded in a suitable fashion. In one experiment, an increase in maternal liver *N*-demethylase of 180 per cent of the control value was noted 1 day after injection of the drug.

The question was entertained: Was the 3-methylcholanthrene transported across the placenta into the fetus? The question was answered by injecting i.p. ^{14}C-labeled

TABLE 3. EFFECT OF 3-MC ADMINISTRATION UPON LIVER *N*-DEMETHYLASE ACTIVITY IN NEONATAL AND WEANLING RATS*

Days after 3-MC treatment	Liver *N*-demethylase (mμmoles product/mg protein)
Neonates, 5–7 g 0†	2·8 ± 0·5 (4)‡
2	5·9 ± 0·8 (4)
3	13·5 ± 1·2 (5)
Preweanling, 25 g 0	3·0 ± 0·4 (4)
2	8·5 ± 0·4 (4)
3	16·0 ± 1·0 (5)

* Neonatal and preweanling rats of mixed sex were given either a single injection of 3-MC in corn oil, 20 mg/kg, i.p., or corn oil alone and were sacrificed as indicated in the table.

† Corn oil-treated controls.

‡ Average ± S.E. (No. of groups). Livers from 2 preweanling rats were combined to give 1 group; livers from 3 neonates comprised 1 group.

drug into pregnant rats and determining the distribution of label in maternal and fetal liver. The results of this experiment are indicated in Table 4. The data establish the transport of 3-methylcholanthrene (or derivative) into the fetal liver circulation, although the sp. act. of the label in the latter was only 17 per cent of the specific activity present within the maternal liver.

TABLE 4. DISTRIBUTION OF 3-MC IN MATERNAL AND FETAL LIVER*

Source of liver	Sp. act. (dpm/g dry wt.)
Maternal	26,800
Fetal	4500

* Ten μc 3-MC-6-^{14}C (5 μc/μmole) was injected i.p. into 3 rats 13–16 days pregnant. The rats were sacrificed 2·5 days later, the maternal and fetal livers were removed and pooled, and were homogenized in formic acid to 20 per cent. The homogenates were centrifuged and the radioactivity in an aliquot of the supernatant fraction was determined by liquid scintillation techniques. The efficiency of the counting was 57 per cent. An aliquot of the supernatant fraction was dried at 100° overnight and the dry weight was calculated.

A more direct means of introducing the polycyclic hydrocarbon into the fetal circulation was sought. Based upon previous studies on the incorporation of pyrimidines into fetal liver ribonucleic acid,[22] it was decided to employ the direct administration of 3-methylcholanthrene into each amniotic sac. The results of these experiments are

offered in Table 5. No significant elevation in the specific activity of fetal liver *N*-demethylase was noted at any time after administration of 3-MC into the amniotic sacs. No increase in the activity of maternal liver *N*-demethylase was apparent after administration of the polycyclic hydrocarbon in this fashion.

TABLE 5. EFFECT OF 3-MC ADMINISTRATION UPON *N*-DEMETHYLASE ACTIVITY IN FETAL LIVER

Treatment	When sacrificed (days)	*N*-demethylase activity (mμmoles product/mg protein)	P value†
Sham-operated controls	0	0·6 ± 0·2 (6)‡	
3-MC	1	0·9 ± 0·4 (4)	>0·05
3-MC	3	1·0 ± 0·3 (6)	>0·05
3-MC	4	1·2 ± 0·6 (4)	>0.05
Maternal liver	2–3	2·4 ± 0·1 (2)	

* Rats, 16–18 days pregnant, were anesthetized with ether, the uterine horns were exposed, and a single injection of 3-MC, 0·1 mg/0·05 ml corn oil, was administered into each amniotic sac. The fetuses were replaced in the abdominal cavity and the incision was sutured. The rats were sacrificed periodically thereafter and *N*-demethylase activity of the fetal livers was determined. The fetal mortality rate of the last group was 50 per cent. Usually, the livers from 10 fetuses were combined to form one group. The sham-operated controls represent fetuses that received 0·05 ml corn oil alone. The rats bearing these fetuses were sacrificed 1 and 4 days later. Since the *N*-demethylase activity did not substantially differ, the values have been grouped together.

† Difference between experimental and control value.

‡ Mean ± S.E. (No. of determinations).

An elevation of *N*-demethylase activity was noted in the group of 1-day neonatal rats that had received 3-MC via the amniotic sac method *in utero*, 2–3 days prior to birth, i.e. 3–4 days prior to sacrifice. These data are presented in Table 6. The values for the experimental group were 240 per cent higher than the values for the untreated group, an elevation significant at a level of $P < 0{\cdot}005$. The activity of *N*-demethylase, when only corn oil was injected under the above conditions, did not significantly differ from that of the untreated controls.

TABLE 6. ELEVATION OF MICROSOMAL *N*-DEMETHYLASE ACTIVITY IN NEONATAL RATS RECEIVING 3-MC WHILE *IN UTERO**

Treatment	*N*-demethylase (mμmoles product/mg protein)	Relative value†	P value
Corn oil-treated, *in utero*, 1-day neonate	1·4 ± 0·3 (5)‡	1·0	
3-MC treated, *in utero*, 1-day neonate	4·4 ± 0·7 (5)	3·4	<0·005§

* 3-MC, 0·1 mg/0·05 ml corn oil, was injected into the amniotic sacs of 20-day pregnant rats. The rats delivered 2–3 days later; the 1-day-old neonatal rats were sacrificed and the *N*-demethylase activity of neonatal liver was determined. The experimental group represents neonatal rats that had received 3-MC 3–4 days previously under these conditions.

† The sp. act. of the microsomal enzyme in the corn oil-treated group is assumed to be 1·0.

‡ Mean ± S.E. (No. of determinations).

§ As determined by Student's *t*-test.

DISCUSSION

The injection of 3-methylcholanthrene is followed by an increase in *N*-demethylase activity in rat liver; the effect appears to be sustained for at least 4 days after a single injection of the polycyclic hydrocarbon. In the present studies, it was not possible to significantly elevate enzymatic activity in fetal rat liver by administering 3-methylcholanthrene either i.p. to the mother rat or directly into the amniotic sac. Elevation of *N*-demethylase activity was noted only when the fetal rats were delivered and not when fetal rats remained within the maternal circulation. It is not known whether the inability to induce these enzymes in fetal liver is due to the stage of fetal development, to the presence of a maternal factor preventing the induction, or to the lack of an active inducer in the fetus.

It is clear from the studies of Schimke *et al.*[23] and of Segal and Kim[24] that the rate of catabolism of an enzyme can very markedly influence the steady-state level of that enzyme. Thus, the effective concentration of an enzyme can be increased not only by changing the rate of synthesis but also by depressing the extent of its catabolism. Unfortunately, investigations of the exact mode of interaction of 3-methylcholanthrene with the drug-metabolizing enzyme system are hampered by the inability to obtain the latter in soluble form and consequently by the inability to purify them. However, evidence has ben presented by Golboin and Sokoloff[14] and by von der Decken and Hultin[25] demonstrating the stimulatory effect of 3-methylcholanthrene upon the incorporation of amino acids into protein both *in vivo* and *in vitro*. These experiments have not been performed in neonatal or fetal rats, and we cannot say at this moment if catabolism of the drug-metabolizing enzyme system is operating at an elevated rate.

The study of the lack of action of 3-methylcholanthrene in fetal liver is further complicated by the reported alterations in the cytology of the rat hepatocyte which occur during liver maturation. Thus, Nadal and Zajdela[26] have noted the occurrence of mononucleated diploid cells exclusively within rat fetal liver. As development proceeded through neonatal life, the number of these cells in the liver declined rapidly and the cells became more polymorphic. In the adult, the hepatocyte was largely mononucleated and tetraploid, with some octoploid and binucleated diploid types. Polyploidy is therefore an aspect of liver maturation. These cytological studies were extended by the biochemical analysis of the macromolecular composition of liver nuclei during development.[27] The mononucleated diploid cell may not be capable of responding to the inducer stimulus of 3-methylcholanthrene while the more adult hepatocyte will give the desired effect.

The question of the elevation in the activity of the enzymes of biotransformation by 3-methylcholanthrene (or derivative) in fetal tissues is of prime importance. The problem fringes upon the aspects of development and differentiation. What is the factor which makes induction possible? How is the induction phenomenon accomplished? Why is the induction restricted to only a few tissues if each cell possesses the identical genotype? These are questions which are under active consideration in this laboratory.

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