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INDUCTION OF CYTOCHROME P-450 AND RELATED DRUG-METABOLIZING ACTIVITIES IN THE LIVERS OF DIFFERENT RODENT SPECIES BY 2-ACETYLAMINOFLUORENE OR BY 3-METHYLCHOLANTHRENE

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Abstract—In general, large differences in the control levels of different cytochrome P-450-catalyzed activities (aminopyrine *N*-demethylase, benzo(*a*)pyrene monooxygenase, ethoxyresorufin *O*-deethylase, ethoxycoumarin *O*-deethylase and total 2-acetylaminofluorene metabolism and metabolite pattern) and in the inducibility of these activities in different rodent species (rat, hamster, guinea pig and mouse) and sexes were observed.

(1) For all the activities measured the lowest levels were observed in untreated rats.

(2) With a few minor exceptions, the only species tested in which cytochrome P-450-catalyzed activities were induced by treatment with 2-acetylaminofluorene was the rat.

(3) A larger number of the species tested were susceptible to induction by 3-methylcholanthrene. However, this xenobiotic proved also to induce most potently in the rat.

(4) There are relatively large differences between the male and female rat both in terms of control cytochrome P-450-catalyzed activities and in the inducibility of these activities by 2-acetylaminofluorene and 3-methylcholanthrene. In general, both of these xenobiotics proved to be more potent inducers in the female than in the male.

Thus, it is quite clear that in quantitative terms the hepatic microsomal cytochrome P-450-catalyzed activities and their inducibility by 2-acetylaminofluorene or 3-methylcholanthrene in the male Sprague–Dawley rat are not representative for other rodent species or even for the female of the same species.

Metabolism by the cytochrome P-450 monooxygenase system is the first step in the biotransformation of numerous xenobiotics to water-soluble conjugates which can be excreted via the urine and/or bile (e.g. [1]). The reactions catalyzed by this system may give rise to electrophilic, reactive intermediates—for example, epoxides, carbonium ions and free radicals—which can bind covalently to nucleophilic groups in cellular macromolecules and are thought thereby to directly cause many of the toxic and genotoxic effects of foreign compounds (e.g. [2]). Thus, the level of different isozymes of cytochrome P-450 in an organ or organism can be expected to have far-reaching consequences for, for example, the dose of a given drug required to obtain the desired effect, the side-effects of this drug and the potential toxicity and genotoxicity of chemicals present in our work and general environments.

This situation has led to an enormous, still growing interest in the control of cytochrome P-450 levels at the genetic level. The most extensive efforts in this respect have been concerned with the process of induction, whereby organisms from bacteria to man adapt to exposure to a xenobiotic by selectively increasing the total cellular content of a certain protein(s), most often a protein involved in metabolism of this same xenobiotic (e.g. [3–7]). A model for the induction of cytochrome P-450 has been proposed on the basis of studies with liver from responsive and non-responsive mouse strains exposed to TCDD [8, 9]: aromatic compounds such as TCDD and 3-methylcholanthrene, upon entering the cell, bind to a protein receptor and, following translocation of the inducer–receptor complex into the nucleus, structural genes for cytochrome P-450 are activated in some as-yet unexplained manner. However, it is still uncertain whether this model is also valid for other inducers such as phenobarbital [7].

Much less is known about other forms of genetic control, i.e. the processes which regulate the levels of different isozymes of cytochrome P-450 in various organs (differentiation) (e.g. [10–12]) or organisms [13–15].

We have demonstrated that 2-acetylaminofluorene is an inducer of cytochrome P-450 in the liver of male Sprague–Dawley rats [7, 16–18]. The total microsomal content of cytochrome P-450 is only increased about 50% by intraperitoneal administration of 2-acetylaminofluorene to these animals; but, as demonstrated by immunochemical quantitation, the levels of individual isozymes is affected much more dramatically [7]. Cytochrome P-450c (the major isozyme induced by 3-methylcholanthrene ([7]; see also this reference for a discussion of the nomenclature employed here) is induced approximately 20-fold by 2-acetylaminofluorene; cytochromes P-450b+e (which cannot be distinguished immunochemically and which are the major forms of cytochrome P-450

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induced by phenobarbital [7]) are induced about 10-fold; and the levels of cytochromes P-450d and PB/PCN-E are increased about 2.5–3-fold and 30–40%, respectively.

Of course, these inductions result in changes in the relative rates of different cytochrome P-450-catalyzed reactions in microsomes [17]. The altered metabolic pattern resembles qualitatively in a number of respects that seen after induction with 3-methylcholanthrene, although the effects obtained with the latter inducer are quantitatively much larger. We have also obtained evidence that 2-acetylaminofluorene itself and/or a metabolite other than those formed via 2-aminofluorene or *N*-hydroxy-2-acetylaminofluorene induces cytochromes P-450b+e; whereas 2-aminofluorene, formed by the enzymatic deacetylation of 2-acetylaminofluorene *in vivo* [19], induces cytochrome P-450c. Consistent with this latter conclusion is our finding that 2-aminofluorene inhibits the binding of TCDD to rat liver cytosolic protein, but 2-acetylaminofluorene does not.

In the present study we have examined the effects of treatment with 2-acetylaminofluorene on the total liver microsomal content of cytochrome P-450, different cytochrome P-450-catalyzed reactions and the pattern of microsomal 2-acetylaminofluorene metabolism in male and female Sprague–Dawley rats, hamsters, guinea pigs and mouse strains responsive and non-responsive to induction by 3-methylcholanthrene. Since induction by 2-acetylaminofluorene resembles that by 3-methylcholanthrene in certain ways (see above), we have investigated the effects of administering the latter inducer to these same animals as well, for comparative purposes. Our purpose has been, in general, to compare the process of induction by 2-acetylaminofluorene in different sexes, strains and species and, in particular, to ascertain whether the male Sprague–Dawley rat is representative in this respect. In addition, the control values obtained provide information about the constitutive cytochrome P-450-catalyzed metabolism of various xenobiotics in these different animals.

MATERIALS AND METHODS

Chemicals. 2-Acetylaminofluorene (m.p. 192–193°) was purchased from Fluka AG (Buchs, Switzerland) and was essentially pure as judged by TLC in two solvent systems (chloroform–methanol, 97:3 (v/v) and petroleum ether (b.p. 40–60°)-acetone, 7:3 (v/v)) and by HPLC [17]. Poly(ethylene glycol) 300 (Kebo, Stockholm, Sweden), benzo(*a*)pyrene, hydroxyresorufin and 3-methylcholanthrene (Eastman Kodak Co., Rochester, NY), hydroxycoumarin (E. Merck AG, Darmstadt, F.R.G.), aminopyrine (4-dimethylaminoantipyrin) (Fluka AG, Buchs, Switzerland) and isocitric acid dehydrogenase (type IV from porcine heart) and $NADP^+$ (Sigma Chemical Co., St. Louis, MO) were obtained from the sources indicated.

Ethoxycoumarin and ethoxyresofurin were synthesized from the hydroxy compounds by ethylation with ethyl iodide [20]. *N*-hydroxy-2-acetylaminofluorene, was synthesized in collaboration with Dr Åke Pilotti, Department of Organic Chemistry, University of Stockholm, and characterized by thin-layer chromatography and NMR; whereas other reference metabolites of 2-acetylaminofluorene were obtained from the National Cancer Institute, DHEW, U.S.A.

G-[^{3}H]-Benzo(*a*)pyrene (701.8 megaBecquerels = 19 Ci/mmol) and 9-[^{14}C]-2-acetylaminofluorene (2.05 megaBecquerels = 55.5 mCi/mmol) were bought from the Radiochemical Centre, Amersham, U.K. The radioactive 2-acetylaminofluorene was purified by TLC (chloroform–methanol, 97:3 (v/v)) and the radioactive benzo(*a*)pyrene by a simple extraction procedure [21] prior to use.

All other chemicals and solvents used were of reagent grade and obtained from common commercial sources.

Animals. Male and female Sprague–Dawley rats weighing 180–200 g (ALAB AB, Sollentuna, Sweden), male Syrian golden hamsters weighing 110–130 g (the Swedish Bacteriological Laboratory, Solna, Sweden), male Dunkin Hartley guinea pigs weighing 350–400 g (HB Sahlins Försöksdjurfarm, Malmö, Sweden) and 6-week-old (approximately 20 g) male mice of the inbred strains C57BL/6N (B6) and DBA/2N (D2) (Banting and Kingman Ltd., Grinston Aldbrough, U.K.) were purchased from the sources indicated. All animals were maintained in our laboratory for several days to a week before treatment to allow acclimatization and given free access to food pellets (Astra-Ewos, Södertälje, Sweden) and water. Guinea pigs were also given ascorbic acid (0.5 g/l) in the drinking water.

Induction. The animals were injected intraperitoneally with 3-methylcholanthrene (20 mg/kg as a 4 mg/ml solution in corn oil) or 2-acetylaminofluorene (50 mg/kg as a 10 mg/ml solution in poly (ethylene glycol) 300) once daily for 5 days. These doses and this schedule of administration have been shown to give maximal induction in the male Sprague–Dawley rat [6, 16].

Preparation of microsomes. Animals were starved overnight before decapitation in order to reduce hepatic levels of glycogen. The liver was removed immediately, placed in ice-cold 0.25 M sucrose and weighed, after which, in those species containing a gallbladder, this organ was removed before homogenization. Liver microsomes were subsequently prepared by differential centrifugation according to Ernster and coworkers [22] and washed once in 0.15 M Tris–Cl, pH 8.0, in order to remove adsorbed cytosolic protein [23]. The microsomal pellet was finally resuspended in a volume of 0.25 M sucrose equal in ml to the original liver weight in g.

Assays. All enzyme measurements were carried out on freshly prepared microsomes.

Cytochrome P-450 content was quantitated spectrophotometrically on the basis of the difference spectrum between the carbon monooxide–reduced cytochrome complex and the reduced cytochrome itself, according to Omura and Sato [24].

This measurement was performed first and thereafter microsomes containing 0.05–1.0 nmol cytochrome P-450 were used in the different enzyme assays in order to assure linearity with time and protein. In all assays of activity an NADPH-regenerating system was used [21] and the reaction initiated by the addition of substrate after preincubation

Table 1. Liver weights and protein contents of the microsomal fractions from control, 2-acetylaminofluorene- and 3-methylcholanthrene-treated animals

Animal	Treatment	Liver weight (g)	Liver-somatic index	Microsomal protein (mg/mg)
Male rat	C	7.2 ± 0.1	0.038 ± 0.001	16.1 ± 1.8
	AAF	7.5 ± 0.3	0.039 ± 0.002	14.6 ± 2.7
	MC	9.5 ± 0.7[e]	0.050 ± 0.004[e]	16.7 ± 3.3
Female rat	C	6.6 ± 0.2[b]	0.035 ± 0.001[b]	10.1 ± 0.7[b]
	AAF	6.0 ± 0.7	0.032 ± 0.004	10.2 ± 1.1
	MC	8.6 ± 0.5[e]	0.045 ± 0.003[e]	11.6 ± 1.9
Hamster	C	3.2 ± 0.3[c]	0.027 ± 0.003[c]	17.6 ± 1.7
	AAF	4.6 ± 0.7[d]	0.038 ± 0.006[d]	11.7 ± 0.1[e]
	MC	4.6 ± 0.2[e]	0.038 ± 0.002[e]	22.8 ± 1.5
Guinea pig	C	15.9 ± 3.9[b]	0.042 ± 0.010	20.7 ± 1.8[a]
	AAF	14.1 ± 1.8	0.038 ± 0.005	18.6 ± 1.8
	MC	16.0 ± 1.3	0.043 ± 0.003	21.2 ± 0.9
DBA/2 mouse	C	0.88 ± 0.11[c]	0.044 ± 0.006	13.2 ± 2.5
	AAF	0.78 ± 0.02	0.039 ± 0.001	13.5 ± 3.5
	MC	0.95 ± 0.05	0.048 ± 0.003	14.1 ± 2.0
C57BL/6 mouse	C	1.12 ± 0.06[c]	0.056 ± 0.003[c]	13.9 ± 1.8
	AAF	1.06 ± 0.04	0.053 ± 0.002	12.9 ± 3.0
	MC	1.21 ± 0.12	0.061 ± 0.006	17.6 ± 1.4[d]

Animals were induced, microsomes prepared and protein measured as described in Materials and Methods.

C = control; AAF = 2-acetylaminofluorene; MC = 3-methylcholanthrene.

The values presented are the means ± S.D. for 3 animals.

[a] Significantly different from the control value for male rat at the level of $P < 0.05$.

[b] Significantly different from the control value for male rat at the level of $P < 0.01$.

[c] Significantly different from the control value for male rat at the level of $P < 0.001$.

[d] Significantly different from the control value for the same species at the level of $P < 0.05$.

[e] Significantly different from the control value for the same species at the level of $P < 0.01$.

for several minutes at 37°. In control incubations the NADPH-regenerating system was not present.

Aminopyrine *N*-demethylation was assayed by measuring the amount of formaldehyde liberated using the Nash reagent [17, 25].

Benzo(*a*)pyrene monooxygenase was measured radiometrically using a simple extraction procedure to separate metabolites and remaining substrate, according to DePierre and coworkers [21].

Ethoxyresorufin and ethoxycoumarin *O*-deethylations were monitored continuously at 37° by measuring the formation of the corresponding hydroxy products on an Aminco-Bowman spectrofluorimeter [20].

2-Acetylaminofluorene (20 μM) was incubated for 30 min at 37°, which yielded a 15–20% conversion of the substrate to metabolites [17]. Sodium acetate buffer, pH 6.5, was subsequently added to the reaction mixture and the metabolites extracted with diethyl ether. These metabolites were quantitated by high-pressure liquid chromatography using a modification [17] of the method of Smith and Thorgeirsson [26].

Protein was measured using a slight modification of the Lowry procedure [27] with bovine serum albumin as standard.

Statistical analysis. Statistical analysis was performed using the Student's *t*-test.

RESULTS

Effects on liver weight and microsomal protein

As expected, treatment of both male and female rats with 3-methylcholanthrene led to significant increases in the weight of the liver in these animals; whereas, in agreement with our earlier findings [16], intraperitoneal administration of 2-acetylaminofluorene did not increase liver weight in rats (Table 1). The only other significant effects on this parameter were small increases after treatment of male hamsters with either of the xenobiotics used here.

There are rather pronounced differences in the amount of microsomal protein recovered per gram control liver for some of the different species investigated here (Table 1). The lowest value (10 mg/g) was obtained for the female rat, while the corresponding value for the male guinea pig was twice as high. Whether such differences reflect different contents of endoplasmic reticulum *in vivo* or simply differences in the recovery of this organelle and/or in the contamination of the microsomal fractions obtained remains unclear. In only two cases did the inducer have any effect on the recovery of microsomal protein: treatment of male hamsters with 2-acetylaminofluorene caused a one-third decrease in this parameter, while treatment of male C57BL/6 mice (the responsive strain) with 3-methylcholanthrene increased microsomal protein 27%.

Table 2. Levels of liver microsomal cytochrome P-450 and cytochrome P-450-catalyzed activities in control, 2-acetylaminofluorene- and 3-methylcholanthrene-treated animals

Animal	Treatment	Cytochrome P-450 (nmol/mg protein)	Aminopyrine *N*-demethylase	Benzo(*a*)pyrene monooxygenase	Ethoxyresorufin *O*-deethylase	Ethoxycoumarin *O*-deethylase
				(nmol metabolized/min–nmol cytochrome P-450)		
Male rat	C	0.46 ± 0.10	6.74 ± 0.99	0.36 ± 0.20	0.48 ± 0.08	0.48 ± 0.10
	AAF	0.69 ± 0.09[f]	5.52 ± 1.21[d]	0.64 ± 0.24[d]	3.42 ± 0.70[f]	1.31 ± 0.27[f]
	MC	1.48 ± 0.13[f]	3.49 ± 1.02[f]	4.62 ± 0.93[f]	40.4 ± 13.3[f]	1.91 ± 0.41[f]
Female rat	C	0.22 ± 0.04[b]	5.27 ± 1.59	0.11 ± 0.04	0.77 ± 0.14[a]	1.27 ± 0.14[c]
	AAF	0.25 ± 0.03	7.48 ± 3.00	1.60 ± 0.35[f]	10.0 ± 3.3[e]	2.64 ± 0.64[d]
	MC	0.66 ± 0.06[f]	9.36 ± 1.05[d]	7.59 ± 0.67[f]	39.5 ± 14.4[e]	3.71 ± 0.41[f]
Hamster	C	0.64 ± 0.08	28.9 ± 2.5[c]	2.75 ± 0.42[c]	1.53 ± 0.19[c]	4.09 ± 0.63[c]
	AAF	0.62 ± 0.07	18.8 ± 2.1[e]	1.94 ± 0.19[d]	1.45 ± 0.24	2.95 ± 0.39[d]
	MC	1.57 ± 0.09[f]	15.7 ± 1.2[f]	1.99 ± 0.26[d]	7.90 ± 0.76[f]	2.63 ± 0.03[e]
Guinea pig	C	0.40 ± 0.06	13.8 ± 2.4[b]	4.25 ± 1.35[b]	1.60 ± 0.30[b]	2.55 ± 0.38[c]
	AAF	0.41 ± 0.04	14.4 ± 1.2	3.41 ± 1.05	1.90 ± 0.34	2.71 ± 0.68
	MC	0.71 ± 0.01[f]	12.8 ± 0.6	3.80 ± 0.52	11.4 ± 0.94[f]	1.56 ± 0.39[f]
DBA/2 mouse	C	0.54 ± 0.18	23.7 ± 2.2[c]	1.56 ± 0.20[c]	1.80 ± 0.06[c]	2.74 ± 0.83[b]
	AAF	0.41 ± 0.06	28.5 ± 5.1	2.31 ± 0.31[d]	2.02 ± 0.15	2.78 ± 0.32
	MC	0.32 ± 0.02	24.8 ± 2.2	1.78 ± 0.22	2.13 ± 0.40	2.75 ± 0.25
C57BL/6 mouse	C	0.35 ± 0.03	23.1 ± 2.9[c]	1.42 ± 0.29[b]	2.25 ± 0.14[c]	3.02 ± 0.14[c]
	AAF	0.43 ± 0.01[e]	29.5 ± 1.4	2.21 ± 0.07[e]	2.30 ± 0.16	3.27 ± 0.28
	MC	1.05 ± 0.05[f]	17.6 ± 1.2[d]	7.11 ± 0.17[f]	44.5 ± 4.1[f]	3.03 ± 0.10

Animals were induced, microsomes prepared and activities assayed as described in the Materials and Methods.
C = control; AAF = 2-acetylaminofluorene; MC = 3-methylcholanthrene.
The values presented are the means ± S.D. for 3–9 animals.
[a] Significantly different from the control value for male rat at the level of $P < 0.05$.
[b] Significantly different from the control value for male rat at the level of $P < 0.01$.
[c] Significantly different from the control value for male rat at the level of $P < 0.001$.
[d] Significantly different from the control value for the same species at the level of $P < 0.05$.
[e] Significantly different from the control value for the same species at the level of $P < 0.01$.
[f] Significantly different from the control value for the same species at the level of $P < 0.001$.

Table 3. The pattern of 2-acetylaminofluorene metabolism by liver microsomes from control, 2-acetylaminofluorene- and 3-methylcholanthrene-treated animals

Animal	Treatment	Metabolites (nmol/min–nmol cytochrome P-450)						
		Total	7-hydroxy	9-hydroxy	5-hydroxy	3-hydroxy	1-hydroxy	*N*-hydroxy
Male rat	C	245 ± 21	143 ± 10	45.0 ± 3.8	2.60 ± 0.71	8.11 ± 0.92	14.2 ± 1.2	32.0 ± 4.6
	AAF	1105 ± 373[e]	472 ± 152[e]	131 ± 14[f]	218 ± 104[e]	175 ± 73[e]	16.8 ± 7.5	92.8 ± 4.4[f]
	MC	3866 ± 790[f]	1470 ± 280[f]	175 ± 51[f]	1220 ± 223[f]	881 ± 174[f]	22.4 ± 7.1	98.1 ± 24.3[e]
Female rat	C	38.3 ± 10.8[c]	15.2 ± 2.10[c]	2.19 ± 0.69[c]	3.91 ± 1.22	4.1 ± 2.29[a]	2.00 ± 1.30[c]	10.9 ± 3.6[b]
	AAF	2456 ± 532[f]	1000 ± 218[f]	120 ± 44[e]	588 ± 125[f]	432 ± 102[f]	38 ± 10[e]	278 ± 42[f]
	MC	10100 ± 692[f]	3760 ± 276[f]	480 ± 123[e]	3230 ± 150[f]	2318 ± 132[f]	58 ± 30[e]	233 ± 73[e]
Hamster	C	2028 ± 486[b]	1052 ± 231[b]	250 ± 49.4[b]	434 ± 97[c]	99.8 ± 13.5[c]	105 ± 71[a]	87 ± 38[a]
	AAF	1920 ± 281	990 ± 310	235 ± 96.8	284 ± 53	95.0 ± 10.8	176 ± 18	140 ± 34
	MC	2830 ± 230	1520 ± 175[d]	159 ± 16.1[d]	278 ± 33	56.8 ± 11.9[e]	245 ± 35[d]	572 ± 34[f]
Guinea pig	C	1930 ± 317[c]	1817 ± 315[c]	<10[c]	37.3 ± 11.8[b]	5.8 ± 2.8	70 ± 16.3[b]	<1[c]
	AAF	2270 ± 240	2070 ± 120	<10	55.8 ± 21.9	6.8 ± 1.2	136 ± 87	<1
	MC	2650 ± 279[d]	1940 ± 128	<10	17.9 ± 3.1	5.8 ± 2.5	686 ± 162[e]	<1
DBA/2 mouse	C	1030 ± 278[b]	698 ± 220[b]	<5[c]	86.5 ± 28.5[b]	17.6 ± 5.7[a]	67 ± 16[b]	161 ± 35[b]
	AAF	1107 ± 40	858 ± 44	<5	66.0 ± 5.9	12.7 ± 2.7	59 ± 22	111 ± 14[d]
	MC	1430 ± 157	738 ± 75	<5	191 ± 26.6[c]	35.6 ± 6.3[e]	123 ± 13[e]	343 ± 38[e]
C57BL/6 mouse	C	1029 ± 79[c]	449 ± 56[c]	34.1 ± 4.6[a]	143 ± 7.7[c]	27.1 ± 9.7[a]	107 ± 9.3[c]	269 ± 21[c]
	AAF	1155 ± 287	595 ± 215	58.6 ± 7.1[e]	117 ± 8.9[e]	31.4 ± 5.0	106 ± 12	247 ± 50
	MC	7889 ± 998[f]	4090 ± 732[f]	141 ± 42[e]	1700 ± 517[e]	560 ± 131[f]	310 ± 60[e]	1088 ± 219[e]

Animals were induced, microsomes prepared and 2-acetylaminofluorene metabolism analyzed as described in the Materials and Methods. The values presented are the means ± S.D. for 3–6 animals.

C = control; AAF = 2-acetylaminofluorene; MC = 3-methylcholanthrene.

[a] Significantly different from the control value for male rat at the level of $P < 0.05$.

[b] Significantly different from the control value for male rat at the level of $P < 0.01$.

[c] Significantly different from the control value for male rat at the level of $P < 0.001$.

[d] Significantly different from the control value for the same species at the level of $P < 0.05$.

[e] Significantly different from the control value for the same species at the level of $P < 0.01$.

[f] Significantly different from the control value for the same species at the level of $P < 0.001$.

Effects on total microsomal cytochrome P-450

Table 2 presents the control levels of total cytochrome P-450 per mg microsomal protein in the different species, as well as the effects of treatment with 2-acetylaminofluorene or 3-methylcholanthrene on this parameter. Such measurements are of limited value only, since there are many different isozymes of cytochrome P-450 whose levels can vary more-or-less independently under different conditions [7]. Nonetheless, induction of cytochrome P-450-catalyzed activities is usually accompanied by an increase in the total level of this cytochrome.

In no case did the control level of total cytochrome P-450 per mg microsomal protein differ significantly from that of the male rat, with the exception of the female rat, whose liver microsomes contained half as much of this cytochrome per mg protein as the male of this species. Treatment of the animals with 2-acetylaminofluorene significantly increased the specific microsomal content of total cytochrome P-450 only in the case of the male rat and male C57B1/6 mouse. These increases were relatively small and no spectral shifts in the absorption maximum of the difference spectrum between the carbon monooxide-reduced cytochrome complex and reduced cytochrome P-450 were observed. This finding for the male rat agrees with our earlier reports of an approximately 50% induction of total cytochrome P-450 with 2-acetylaminofluorene [7, 16–18].

On the other hand, treatment of the animals with 3-methylcholanthrene induced total cytochrome P-450 in all cases except the DBA/2 mouse, which is known to be relatively non-responsive to this xenobiotic. The magnitudes of these increases varied greatly, being largest for the rat (322% of control) and the responsive C57B1/6 mouse (300%). In all of the liver microsomal preparations from 3-methylcholanthrene-treated animals there was a 1–2 nm blue shift in the absorption maximum of the difference spectrum (i.e. the absorption maximum was at 448–449 nm).

Effects on cytochrome P-450-catalyzed activities

Ideally, in order to compare the levels of different cytochrome P-450s in liver microsomes from animals of different sexes and species, one would like to quantitate the amounts of the different isozymes present using immunochemical methods. However, in most cases the only antibodies presently available are directed towards the rat proteins and it is uncertain to what extent these antibodies crossreact with the corresponding hepatic proteins in the other species employed here. In addition, activity measurements would seem to be more relevant with regard to the functional aspects of drug metabolism.

For these reasons we have measured here a spectrum of commonly studied cytochrome P-450-catalyzed activities, including *N*-demethylation, *O*-deethylation, aromatic hydroxylation and *N*-hydroxylation. In Tables 2 and 3 specific activities per nmol cytochrome P-450 are given in order to facilitate comparisons between the different species and sexes. These values can be converted to total activities using the relevant parameters from Table 1.

With regard to control activities in liver microsomes from untreated animals both male and female rats demonstrated lower aminopyrine *N*-demethylase, benzo(*a*)pyrene monooxygenase, ethoxyresorufin *O*-deethylase, ethoxycoumarin *O*-deethylase and total 2-acetylaminofluorene metabolism than did the other species examined (Tables 2 and 3). Male and female rats had approximately the same aminopyrine *N*-demethylase and benzo(*a*)pyrene monooxygenase activities; males demonstrated a 6-fold faster total metabolism of 2-acetylaminofluorene; whereas the female activities of ethoxyresorufin and ethoxycoumarin *O*-deethylase were 1.6- and 2.65-fold higher, respectively, than the corresponding values for liver microsomes from untreated male rats.

There was also some variation in the control levels of these activities in liver microsomes from the other species examined. For instance, guinea pigs had approximately half the aminopyrine demethylase and twice the benzo(*a*)pyrene monooxygenase activities of the other animals. Control ethoxycoumarin *O*-deethylase activity was particularly high in the hamster and the total metabolism of 2-acetylaminofluorene in liver microsomes from untreated hamsters and guinea pigs was about twice that observed in the two strains of mice used. These other species demonstrated aminopyrine *N*-demethylase, benzo(*a*)pyrene monooxygenase, ethoxyresorufin and ethoxycoumarin *O*-deethylase and total 2-acetylaminofluorene metabolism which were 2–4-fold, 5–40-fold, 3–5-fold, 2–10-fold and 4–50-fold, respectively, higher than those observed in liver microsomes from untreated rats.

Treatment of the animals with 2-acetylaminofluorene has a number of different effects (Tables 2 and 3). Ethoxyresorufin *O*-deethylase, ethoxycoumarin *O*-deethylase and total 2-acetylaminofluorene metabolism were induced by this treatment only in rats, demonstrating respective increases of 613%, 173% and 451% for males, and 1200%, 108% and 6310% for females. Benzo(*a*)pyrene monooxygenase activity was increased in male (to 178% of the control value) and female (1460%) rats and slightly in both mouse strains (148% in DBA/2 and 156% in C57B1/6). Aminopyrine *N*-demethylase activities were unaffected by treatment with 2-acetylaminofluorene, except for significant decreases for the male rat and hamster. Ethoxycoumarin *O*-deethylase activity in hamster liver microsomes was also decreased. This decrease in the case of the rat probably reflects a selective increase in isozymes of cytochrome P-450 which are inefficient in the *N*-demethylation of aminopyrine. However, since the total level of cytochrome P-450 per mg microsomal protein was unaffected by treatment of hamsters with 3-methylcholanthrene, the decreases in this case presumably reflect both a selective increase in certain isozymes of cytochrome P-450 and a concomitant, compensatory decrease in other isozymes.

Treatment of these animals with 3-methylcholanthrene also caused a number of changes in the cytochrome P-450-catalyzed activities measured here (Tables 2 and 3). The most dramatic effects in this case were, as expected, on benzo(*a*)pyrene monooxygenase activities, which increased 1180% for the male rat, 6800% for the female rat and 401% for the

responsive C57B1/6 mouse. Predictably, this activity in the non-responsive DBA/2 mouse was unaffected by 3-methylcholanthrene, which was also true for the guinea pig. Despite a relatively large increase in microsomal cytochrome P-450 upon treatment of hamsters with 3-methylcholanthrene, the microsomal benzo(*a*)pyrene monooxygenase activity per nmol cytochrome P-450 was slightly, but significantly decreased, indicating selective induction of forms of cytochrome P-450 inefficient in the metabolism of benzo(*a*)pyrene.

Furthermore, treatment with 3-methylcholanthrene also induced ethoxyresorufin *O*-deethylase activity (known to be associated in the rat with cytochrome P-450c) [7] in all the animals tested, with the exception of the non-responsive DBA/2 mouse—from 5-fold in the hamster to 84-fold in the male rat. Ethoxycoumarin *O*-deethylase activity was increased only in rats (4-fold in males and 3-fold in females) and was at the same time significantly decreased in hamsters and guinea pigs, presumably again reflecting the "dilution effect" of a selective induction of forms of cytochrome P-450 inefficient in the catalysis of this reaction. The same effect was seen in decreased microsomal aminopyrine *O*-demethylase activities in the male rat, hamster and C57B1/6 mouse; whereas this same activity was unaffected upon treatment of guinea pigs and DBA/2 mouse with 3-methylcholanthrene and actually induced slightly in the female rat. Finally, 3-methycholanthrene was seen to induce total 2-acetylaminofluorene metabolism markedly only in male (16-fold) and female (260-fold) rats and responsive C57B1/6 mice (7.7-fold), although a slight, but significant increase was also found in the case of guinea pigs.

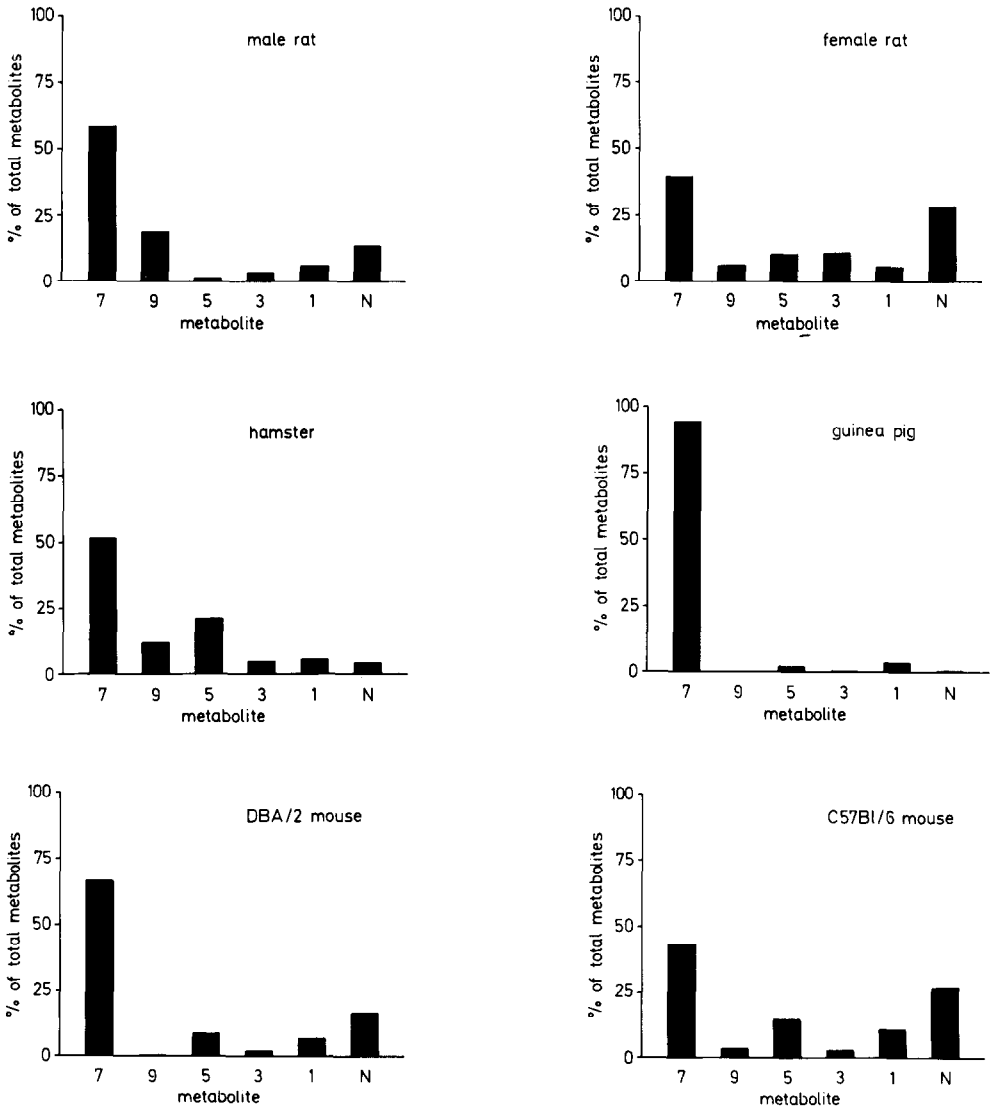

Fig. 1. Pattern of 2-acetylaminofluorene metabolism by liver microsomes from untreated rodents of different species. These patterns were calculated from the data presented in Table 3. 7, 9, 5, 3, 1 and N = 7-hydroxy- 9-hydroxy-, 5-hydroxy-, 3-hydroxy-, 1-hydroxy and *N*-hydroxy-2-acetylaminofluorene, respectively.

Effects on the pattern of 2-acetylaminofluorene metabolism

It was of interest to determine the effects of treating these different animals with 2-acetylaminofluorene or 3-methylcholanthrene on the pattern of 2-acetylaminofluorene metabolism both with regards to the species differences in the carcinogenicity of this compound (see the Discussion) and with regards to the possibility of using this metabolic pattern as a "fingerprint" for the characterization of a certain isozyme(s) of cytochrome P-450 [28].

As can be seen in Table 3, there were quite large

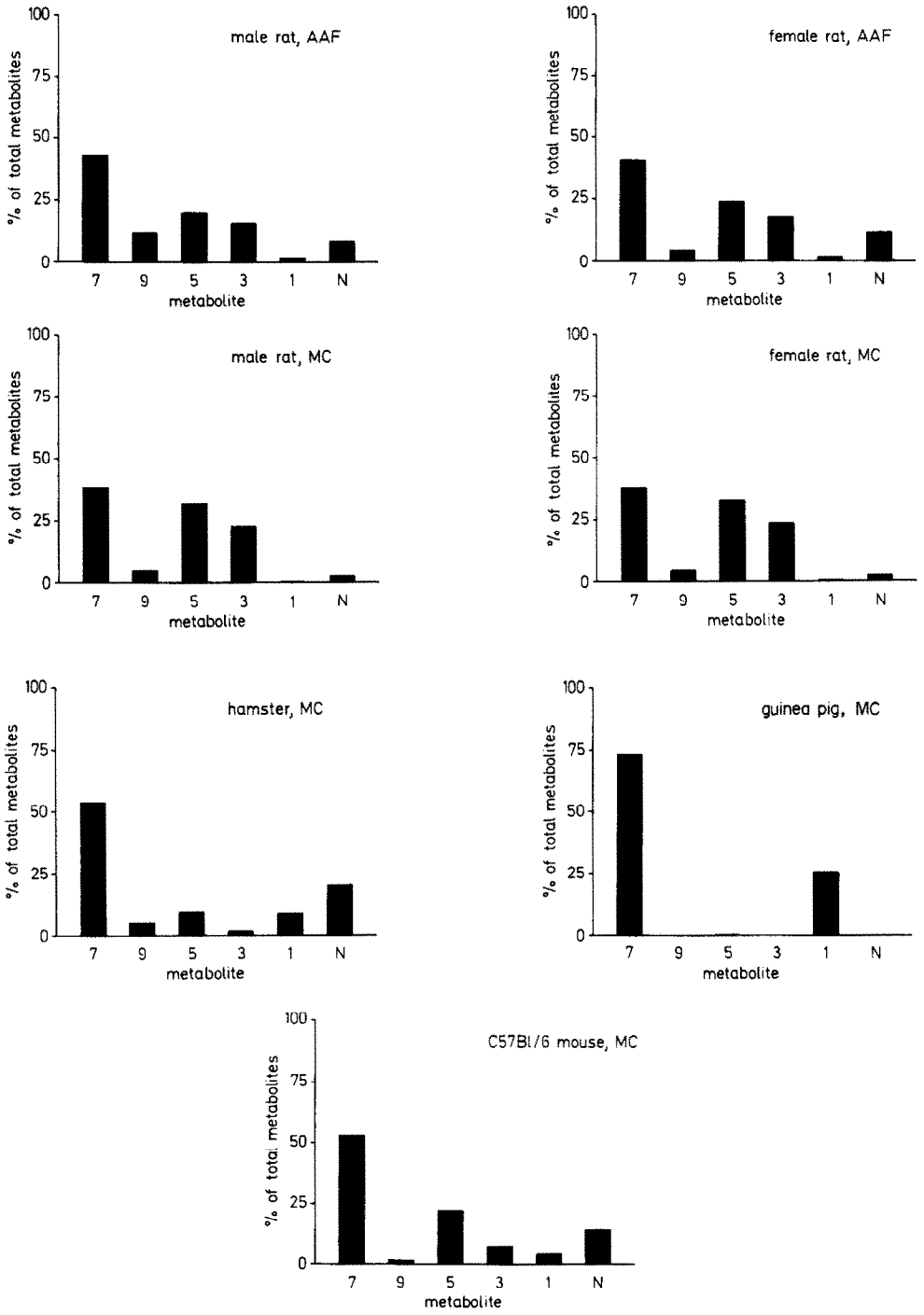

Fig. 2. Pattern of 2-acetylaminofluorene metabolism by liver microsomes from induced rodents of different species. AAF = 2-acetylaminofluorene and MC = 3-methylcholanthrene. For further details, see the legend to Fig. 1.

differences in the patterns of 2-acetylaminofluorene metabolism by liver microsomes from untreated animals. As expected from the total activities (see above), the female rat produced relatively low amounts of all the different metabolites, whereas the hamster yielded relatively large amounts of all metabolites except *N*-hydroxy-2-acetylaminofluorene. The metabolite produced in the largest amount for all the animals investigated was 7-hydroxy-2-acetylaminofluorene. Indeed, in the case of the guinea pig, 95% of the total metabolites produced was the 7-hydroxy derivative. Both the guinea pig and DBA/2 mouse yielded very low levels of 9-hydroxy-2-acetylaminofluorene. And finally, mouse liver microsomes gave rise to relatively high levels of *N*-hydroxy-2-acetylaminofluorene, whereas virtually none of this product was detected with liver microsomes from untreated guinea pigs.

Treatment of the animals with 2-acetylaminofluorene had little effect in any case except for the rats (Table 3). This xenobiotic induced the production of all metabolites in the female rat and all except the 1-hydroxy metabolite in the male. These inductions were much more pronounced for the female rat. Especially large effects were seen for the 5-hydroxy derivative (84-fold induction for the male rat and 150-fold for the female).

Treatment with 3-methylcholanthrene also gave largest effects on the pattern of 2-acetylaminofluorene metabolism in rats (Table 3). Again, only production of the 1-hydroxy metabolite in male rats was unaffected and the largest increases were seen with the 5-hydroxy metabolite (470-fold induction in the male and 826-fold induction in the female). 3-Methylcholanthrene also increased the rate of production of all 2-acetylaminofluorene metabolites by liver microsomes from responsive C57BL/6 mice and, more surprisingly, of the 5-, 3-, 1- and *N*-hydroxy metabolites in non-responsive DBA/2 mice as well, although to a lesser extent. Formation of the 7-, 1- and *N*-hydroxy derivatives was induced by 3-methylcholanthrene in the hamster, whereas only formation of the 1-hydroxy metabolite was increased in the guinea pig.

In terms of carcinogenic potency not only is the absolute rate of formation of different 2-acetylaminofluorene metabolites of interest, but even the relative rates of formation of ring hydroxylated and the *N*-hydroxylated product are of interest (see the Discussion). For this reason Figs 1 and 2 were constructed to illustrate the percentage distributions of the different metabolites using liver microsomes from untreated animals and after induction (in those cases where induction had a marked effect), respectively. The most interesting observations to be made here are as follows: treatment of rats with either 2-acetylaminofluorene or 3-methylcholanthrene caused a decrease in the percentage of the total metabolites accounted for by the *N*-hydroxy derivative, this effect being more pronounced after induction with 3-methylcholanthrene. 3-Methylcholanthrene also decreased the percentage of *N*-hydroxy-2-acetylaminofluorene formed by liver microsomes from the responsive C57BL/6 mouse, while dramatically increasing this value in the case of the hamster. Finally, treatment with 2-acetylaminofluorene resulted in a large increase in the relative formation of 5-hydroxy-2-acetylaminofluorene in the male rat and treatment with 3-methylcholanthrene dramatically increased the relative production of the 1-hydroxy metabolite by the guinea pig.

DISCUSSION

The general observation to be made from the present study—namely, that there are large differences in the control levels of different cytochrome P-450-catalyzed activities and in the inducibility of these activities in the livers of different rodent species and sexes—is in agreement with numerous other reports in the literature (for reviews, see [13–15]). More specifically, four patterns seen here are worth noting:

(1) For all the activities measured, i.e. aminopyrine *N*-demethylase, benzo(*a*)pyrene monooxygenase, ethoxyresorufin *O*-deethylase, ethoxycoumarin *O*-deethylase and total 2-acetylaminofluorene metabolism, the lowest levels were observed in untreated rats. Again, this is in general agreement with the literature (e.g. [29–31]).

(2) With a few minor exceptions, the only species tested in which cytochrome P-450-catalyzed activities were induced by treatment with 2-acetylaminofluorene was the rat. A number of other reports, both from our own laboratory [16, 17] and others (e.g. [32–34]), have also demonstrated that 2-acetylaminofluorene induces its own metabolism through the cytochrome P-450 system in rat liver.

(3) Although a larger number of the species tested were susceptible to induction by 3-methylcholanthrene, this xenobiotic proved also to be in general most potent as an inducer in the rat. Numerous other studies have also demonstrated that 3-methylcholanthrene induces cytochrome P-450 and associated activities in the rat (e.g. [30, 34–37]), hamster (e.g. [30, 35, 37, 38]), guinea pig (e.g. [30, 39]) and mouse (e.g. [30, 40, 41]). Noteworthy in this respect is the finding that ethoxyresorufin *O*-deethylase activity, known to be highly correlated to the induction of cytochrome P-450c in the rat [7], was also highly induced in the other species tested, including the guinea pig, which is known to be relatively refractory to the induction of several cytochrome P-450-catalyzed activities by 3-methylcholanthrene [30, 39].

(4) There are relatively large differences between the male and female rat both in terms of control cytochrome P-450-catalyzed activities and in the inducibility of these activities by 2-acetylaminofluorene and 3-methylcholanthrene. In general, both of these xenobiotics proved to be more potent inducers in the female than in the male.

Thus, it is quite clear that in quantitative terms the hepatic microsomal cytochrome P-450-catalyzed activities and their inducibility by 2-acetylaminofluorene or 3-methylcholanthrene in the male Sprague–Dawley rat are not representative for other rodent species or even for the female of the same species.

Several other interesting observations can be made from the present data:

It would appear from these studies in the rat that

2-acetylaminofluorene is a weak 3-methylcholanthrene-type inducer, i.e. gives qualitatively the same sorts of changes, but to a smaller extent. This impression was at least partially confirmed by a recent study in which we investigated the different isozymes of cytochrome P-450 induced in the liver of the male Sprague–Dawley rat by 2-acetylaminofluorene [7]. The major isozyme induced is, indeed, immunochemically identical to the major isozyme induced by 3-methylcholanthrene, i.e. form c. However, 2-acetylaminofluorene also induces forms b + e, the major forms induced by phenobarbital, as well as form d. In addition, when effects on phase II enzymes are also examined [7, 16], 2-acetylaminofluorene is seen to give an overall pattern of induction which is distinct from that obtained with 3-methylcholanthrene.

Also noteworthy is the finding that 3-methylcholanthrene induces somewhat the rate of formation of 5-hydroxy-, 3-hydroxy-, 1-hydroxy- and *N*-hydroxy-2-acetylaminofluorene in the non-responsive DBA/2 mouse, whereas all other activities examined were, as expected, unaffected. This finding may simply reflect the fact that the non-responsiveness of this strain is a relative, rather than an absolute characteristic [8, 9].

Finally, treatment of male hamsters with 3-methylcholanthrene causes a rather pronounced (2.5-fold) increase in the hepatic microsomal cytochrome P-450 content, but brings about corresponding increases only in ethoxyresorufin *O*-deethylase and the rate of production of 7-hydroxy-, 1-hydroxy- and *N*-hydroxy-2-acetylaminofluorene, of the activities tested. This finding suggests that 3-methylcholanthrene may be a rather specific inducer of a certain isozyme(s) of cytochrome P-450 in the hamster liver.

The question remains as to why there are such large differences in control cytochrome P-450-catalyzed activities and in their inducibility in different rodent species and sexes. Functionally, such differences may reflect differences in the metabolism of endogenous substances such as steroids by the same isozymes of cytochrome P-450 and/or differences in the pattern of exposure to environmental xenobiotics.

Differences in inducibility may be explained mechanistically by differences in the cellular components involved in the induction process and/or differences in the metabolism of the inducer. Thus, the liver of the non-responsive DBA/2 mouse contains fewer receptors with a lower affinity for 3-methylcholanthrene and TCCD than does the liver of the responsive mouse C57BL/6 [8, 9], which explains why 3-methylcholanthrene is generally ineffective as an inducer of the cytochrome P-450 system in the former animal. In addition, we have recently reported evidence that although treatment of rats with 2-acetylaminofluorene induces cytochromes P-450c, b + e and d in the liver, it is the metabolite 2-aminofluorene which actually brings about induction of cytochrome P-450c, possibly through binding to the same receptor which binds 3-methylcholanthrene [7]. Consequently, lack of induction of cytochrome P-450c (or an analogous form) in an animal by 2-acetylaminofluorene may not reflect a lack of receptors, but rather a metabolic pattern which does not result in sufficient accumulation of 2-aminofluorene.

Finally, we would like to comment upon the pattern of 2-acetylaminofluorene metabolism and its changes upon induction in relationship to the susceptibilility of the different rodent species and sexes to the hepatocarcinogenicity of this compound. There are large species and sex differences in this susceptibility: the male Sprague–Dawley rat is most susceptible to the hepatocarcinogenicity of 2-acetylaminofluorene; the female Sprague–Dawley rat, hamster and mouse are approximately equally susceptible and much less susceptible than the male rat; and the guinea pig is virtually resistant [42].

N-Hydroxylation is thought to be an obligatory step in the formation of the carcinogenic metabolite from 2-acetylaminofluorene and there is thought to be a general correlation between the rate and/or percentage of *N*-hydroxylation in different animals and their susceptibility to the hepatocarcinogenicity of this compound [42]. The data presented here are only partially in accord with hypothesis. Indeed, the resistant guinea pig produces much less *N*-hydroxy-2-acetylaminofluorene than the other animals examined, but the percentages of the *N*-hydroxy metabolite formed in male and female rats and in both mouse strains is about the same and the absolute rate of formation of this metabolite is considerably higher in the mice.

In addition, treatment of male rats with 3-methylcholanthrene, which reduces their susceptibility to the hepatocarcinogenicity of 2-acetylaminofluorene [42], also reduces the percentage formation of the *N*-hydroxy metabolite, as the hypothesis would predict and as has been pointed out earlier [42]. However, treatment with 3-methylcholanthrene also dramatically increases both the percentage and rate of formation of *N*-hydroxy-2-acetylaminofluorene in the hamster liver without apparently increasing the susceptibility of this animal to the hepatocarcinogenicity [42]. Obviously, this question is a complicated one and future investigations must take more interest in the further metabolism of *N*-hydroxy-2-acetylaminofluorene, among other factors.

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