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Cytochrome P-450 Isozyme Pattern Is Related to Individual Susceptibility to Diethylnitrosamine-induced Liver Cancer in Rats

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Differences in susceptibility to chemical carcinogenesis between rodent strains and species have been linked to variations in genetically-determined mixed function oxidase activities. In order to verify whether such variations also determine the susceptibility of individual animals of the same strain to a chemical carcinogen, outbred male Wistar rats were administered diethylnitrosamine (DEN) (1, 2, or 3 mg/kg) five times a week for 20 weeks. The relationship was examined between the outcome (i.e., presence or absence of liver tumors, and latency period) and the hepatic activities of mixed function oxidases and conjugating enzymes, as well as of O^6-methylguanine-DNA-methyltransferase, measured before the carcinogen treatment. In addition, the metabolic profiles of two model drugs, antipyrine and disopyramide, in the urine were analyzed and correlated with the carcinogen susceptibility. The length of the latency period of hepatocellular tumors in individual rats was negatively related to the activities of hepatic dimethylnitrosamine *N*-demethylase, aryl hydrocarbon hydroxylase and epoxide hydrolase and positively related to the amount of microsomal protein. Consistent relationships between the other 10 measured parameters and the susceptibility to DEN-induced carcinogenesis were not detected. Long-term treatment with DEN slightly decreased the proportion of metabolism of antipyrine into norantipyrine, and increased the share of 4-hydroxyantipyrine; a decrease in the metabolism of disopyramide to N-deisopropyldisopyramide was also detected. It is concluded that the pattern of cytochrome P-450 isoenzymes is related to differences in individual susceptibility to nitrosamine-induced carcinogenesis. The relationship was most marked at low dose levels, which are the levels at which nitrosamine exposures of humans are known to occur.

Key words: Nitrosamine — Hepatocellular tumor — Cytochrome P-450 — Monooxygenase — Individual susceptibility

The majority of chemical carcinogens — including those which are active in humans — are metabolically activated into ultimate carcinogens in the body. Most often, these activation steps include oxidation by the cytochrome P-450 dependent mixed function oxidase (MFO) system. Genetic and environmental factors that influence the MFO activities are also therefore suspected to affect chemically induced carcinogenesis. Indeed, induction of the MFO by polychlorinated biphenyls (PCB), 3-methylcholanthrene, phenobarbital, or β-naphthoflavone increased the incidence of hyperplastic liver nodules caused by 2-acetylaminofluorene in the rat,[1] while α- and β-naphthoflavone treatments decreased the carcinogenicity of polycyclic aromatic hydrocarbons.[2,3] Tetrachlorodibenzo-*p*-dioxin, the most potent inducer of MFO, decreased the carcinogenic activity of polycyclic aromatic hydrocarbons in mouse skin.[4,5] Inbred mouse strains with different inducibilities of MFO showed different sensitivities to polycyclic aromatic hydrocarbon (PAH)-induced carcinogenicity.[6,7] 1,2 -Dimethylhydrazine-induced colorectal carcinogenesis was related to arylhydrocarbon hydroxylase (AHH)-induction capacity.[8] It has also been reported that differential expression of cytochrome P-450 species may play a role in determining responsiveness to hepatocarcinogenesis-promoting agents, such as 2-acetylaminofluorene.[9] There is circumstantial evidence that genetically determined MFO activity may be related to susceptibility to chemically induced cancer even in humans.[10–13]

Dialkylnitrosamines are carcinogenic in a variety of species and organs,[14,15] and are activated in a monooxygenation reaction to labile α-C-hydroxylated products, which after hydrolysis give rise to active alkylating agents. Changes in MFO activity, caused by inhibition, induction, or genetic regulation, have been shown to lead to altered carcinogenicity of dialkylnitrosamines; for example, β-naphthoflavone, pregnenolone 16-α-carbonitrile, benzo[*a*]pyrene, 3-methylcholanthrene and PCB, as well as piperonyl butoxide, modified the carcinogenic response of dimethylnitrosamine (DMN) in rodents,[16–21] and ethanol and PCB modified the carcinogenicity of diethylnitrosamine (DEN).[22] Induction and inhibition of MFO in hepatic microsomes also altered the alkylation capacity *in vitro* and *in vivo* of DMN[23–25] and DEN,[21,26]

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as well as the generation of mutagenic species from DMN and DEN *in vitro*.[27-34] It is thus evident that marked changes in monooxygenation capacity may lead to variation in susceptibility to chemical carcinogens. However, no data are available to determine whether minor differences, such as those normally encountered between individuals in outbred populations of experimental animals, or in humans, may also contribute to interindividual differences in susceptibility to chemical carcinogens.

Measuring MFO activities in humans *in vivo* is difficult because of limited access to tissue specimens. Therefore, metabolism of model (probe) drugs has been proposed as a replacement for direct *in vitro* enzyme activity determinations, and several different drugs have been explored, eg., warfarin, antipyrine, debrisoquine, and caffeine.[35-38] It is not yet known, however, whether the metabolism of model drugs accurately reflects the activities of various specific mixed function oxidases,[39,40] or whether the variation in metabolism of model drugs reflects sensitivity to chemical carcinogens.

The purpose of the present study was to elucidate the potential of *in vitro* determinations of different carcinogen-metabolizing enzyme activities, and the metabolism of model drugs *in vivo*, as indicators of individual susceptibility to DEN-induced hepatocarcinogenesis in an outbred Wistar rat strain, whereby animals were not pretreated with agents affecting the MFO. At the same time, the relationship between *in vitro* activities of drug-metabolizing enzymes, and the metabolism of model drugs *in vivo* was investigated.

MATERIALS AND METHODS

Animal treatment Seventy-five adult (14 weeks old at the beginning of the experiment) male outbred Wistar rats (433 ± 25 g), were submitted to a two-thirds hepatectomy[41] under light ether anesthesia. During the subsequent 24 h, 20% D-glucose was given as drinking water. After 4–5 weeks, the metabolism of disopyramide was investigated after a single intraperitoneal dose of 75 mg/kg disopyramide, by collecting the urine excreted during 24 h. The following week, the metabolism of antipyrine was studied in a similar way: the animals were given 75 mg/kg antipyrine, and the urine was collected during 24 h. One week later, treatment with DEN was started: rats, divided into three groups of 25 animals each, were given 1, 2, or 3 mg/kg DEN in an aqueous solution (prepared daily) by intragastric gavage 5 days per week, for 20 weeks. At the 6th and 17th weeks, the metabolism of disopyramide was reinvestigated, and at the 7th and 18th week, that of antipyrine was re-examined. The animals were kept for life, or until moribund, and a full autopsy was performed. The relationship between susceptibility to DEN-carcinogenesis (presence/absence of tumors in different organs, and latency) and the various parameters measured during the experiment was analyzed.

Chemicals ^{3}H-Labeled and unlabeled benzo[*a*]pyrene were gifts from Dr. John R. Bend, NIEHS; 3-hydroxyantipyrine, norantipyrine and 4-hydroxyantipyrine from Dr. D. Breimer, Sylvius Laboratories, Leiden; disopyramide phosphate from Leiras Ltd., Finland, and N-deisopropyldisopyramide and *p*-chlorodisopyramide from Roussel Laboratories, UK. 3-OH-Benzo[*a*]pyrene and benzo[*a*]pyrene-4,5-dihydrodiol were obtained from the National Cancer Institute Chemical Repository. Other chemicals were from commercial sources. The solvents used in liquid chromatography were of HPLC grade and were used without further purification. Water used in HPLC was deionized and passed through a microfilter before use. The absence of interfering chemicals in the chloroform used for disopyramide extraction was verified before using a new bottle. Silica thin-layer chromatographic plates (LK6DF), 5×20 cm, with a spotting/concentration zone, were purchased from Whatman.

Enzyme assays The excised portion of the liver was immediately homogenized in 0.125 mol/liter KCl-5 mmol/liter Sörensen phosphate buffer, pH 7.4. Microsomes and cytosolic fractions were prepared by differential centrifugation, as described,[42] and were divided into several 1 ml portions and stored at −70°C until use. In each series of enzyme analyses, one or two control specimens (a batch of pooled control rat liver microsomes/cytosol) were analyzed, and to compensate for daily variations in the assays, the results were corrected using the results of the control specimens.

The activity of aryl hydrocarbon hydroxylase was determined by a modification of the fluorimetric method of Dehnen *et al.*[43]; hydroxylated benzo[*a*]pyrene derivatives were measured after 20±1 min, quantitation was based on authentic 3-hydroxybenzo[*a*]pyrene, and daily calibration was achieved with quinine sulfate. Ethoxycoumarin deethylation was measured as described.[44] Dimethylnitrosamine *N*-demethylation was determined by following the generation of formaldehyde by means of the Nash reaction.[45] Large amounts of the NADPH regenerating system (1.25 mmol/liter NADP, 12.5 mmol/liter glucose-6-phosphate) were necessary to guarantee the linearity of the reaction with respect to time and amount of tissue. The final concentration of DMN was 4 mmol/liter. Since large amounts of tissue were necessary at low substrate concentrations, for each tissue specimen, control incubations were carried out in the absence of DMN to correct for the turbidity, and possible formation of formaldehyde independently of DMN oxidation.

Quantitation was achieved by comparing the result with a calibration curve obtained with formaldehyde; this was done daily, in order to correct for a slow change in the color of the Nash reagent with time. The activity of epoxide hydroxylase was determined with [^{3}H]-benzo[*a*]-pyrene-4,5-oxide as the substrate, using the thin-layer chromatographic method of Jerina *et al.*[46] The carry-over of radioactivity was corrected for by using zero-time incubations. The activity of glutathione *S*-transferase(s) was determined using benzo[*a*]pyrene-4,5-oxide as the substrate essentially as described,[47,48] at pH 10.0. The results were corrected for non-enzymatic conjugation and nonspecific binding of radioactivity to tissue using zero-time and zero-tissue incubations. Activities of UDP-glucuronosyltransferase and sulfate conjugation were measured using 4-methylumbelliferone as the substrate[49,50]; the microsomal protein content was measured by using Lowry's method,[51] and the protein concentration of the cytosolic fraction by means of the biuret reaction.[52] Extent of alkylation of hemoglobin (ethylcysteine) after treatment of the animals with DEN was determined by the method developed by Farmer[53]; urinary excretion of thioethers was analyzed by the method of van Doorn *et al.*,[54] and O^6-methylguanine-DNA methyltransferase was determined by the method of Pegg *et al.*[55]

Determination of antipyrine, disopyramide and their metabolites in urine Disopyramide and its major metabolite, N-deisopropyldisopyramide (MND), were determined by gas chromatography after extraction into chloroform from alkaline urine, using *p*-chlorodisopyramide as an internal standard, and alkaline flame detection.[38] The urinary concentrations of antipyrine, 3-hydroxyantipyrine, 4-hydroxyantipyrine, and norantipyrine were determined by modifications of the method of Danhoff and coworkers.[56] Antipyrine and 3-hydroxyantipyrine were analyzed in one HPLC run, after alkaline extraction at high salt concentration into dichloromethane, using a 25 cm, 10 μm RP-2 column, with acetic acid-methanol as the eluant. 4-Hydroxyantipyrine and norantipyrine were analyzed in another run, after enzymatic hydrolysis and extraction with dichloromethane-pentane (30:70), using a 10 cm ODS RAC column with 22% tetrahydrofuran in 1% acetic acid as the eluant. For both analyses of antipyrine metabolites, phenacetin was used as the internal standard, and the quantitation was achieved by using a 5-point standard curve based on ratios of peak areas to that of the internal standard.

Statistical analysis Correlations between latency and levels of the measured metabolic parameters were estimated by using Pearson's product moment correlation coefficient. Multiple correlation coefficients were also calculated in order to adjust for the dose level of DEN. Tests of significance were based on normal approximations. Differences in survival rates between treatment groups were tested using the general Wilcoxon statistic. Pair-wise *t* tests were used to test the significance of the differences in metabolite patterns of antipyrine and disopyramide before and after DEN administration.

RESULTS

The survival of the rats was decreased with increasing dose of DEN ($P<0.01$); the median survival times were 468, 268, and 186 days in the animals that received 1, 2 and 3 mg/kg body wt./day DEN, which correspond to total doses of 100, 200 and 300 mg/kg body wt., respectively. With the exception of four animals which died early, all rats had tumors on autopsy (Table I). The most frequently observed tumors were hepatocellular carcinomas (HCC), which were seen in 13/24 animals in the lowest dose group, 23/25 in the middle dose group, and 19/22 animals in the high dose group. In addition, 7 animals which did not develop HCC had either liver neoplastic nodules, altered foci, or hepatic sarcomas. Esophagus, kidney, lung and nasal cavity were the other target organs for DEN carcinogenicity. The spectrum of tumors varied with dosage. In the highest dose group, only esophageal papillomas and carcinomas appeared often, in addition to HCC, while in the middle dose group, kidney adenomas and adenocarcinomas, esophageal papillomas and carcinomas, pulmonary adenomas and carcinomas, and nasal cavity carcinomas were also frequent. In the low dose group, a large number of different tumors was seen, often with several different primary tumors in the same animal. Since the survival also varied with the dosage, it is not clear whether the variation in the tumor spectrum is linked to survival and/or to dosage. In the intermediate dose group, tumors other than hepatocellular carcinomas were more frequent among animals who survived longer: in animals who survived less than 265 days, only 2 had tumors other than HCC (and 9/11 had HCC); all 14 animals with longer survival developed other tumors (and HCC).

Since such a large majority of the animals had hepatocellular cancer, and since the survival time was similar in rats that died of HCC or of other tumors, the parameter considered to be most useful to evaluate the individual susceptibility to DEN hepatocarcinogenesis, was time to tumor, i.e., latency period. Because true latency could not be accurately measured, time to death was used as the surrogate. Among the 14 parameters measured (O^6-methylguanine-DNA methyltransferase, and parameters reflecting activities of different hepatic drug-metabolizing enzyme activities, see "Materials and Methods"), only the activities of DMN-*N*-demethylase (Fig. 1), aryl hydrocarbon hydroxylase (Fig. 2), and

Table I. Latency Period (Time from the Beginning of Treatment to Death) and Tumors Detected in Rats Treated with 1, 2, or 3 mg/kg Diethylnitrosamine Orally 5d/w for 20 Weeks

Latency days	Tumor sites and types
A. Dose 1 mg/kg/d	
130	None
191	None
374	Liver neoplastic nodules
377	Liver altered foci, esophageal papilloma, nasal cavity tumor
380	Hepatocellular carcinoma with pulmonary metastasis
398	HCC, kidney adenoma and adenocarcinoma, colonic adenocarcinoma
398	HCC
398	Pulmonary and renal adenocarcinoma, pituitary adenoma
419	Liver AF, adrenal cortical adenoma, subcutaneous neurinoma
431	Liver haemangiosarcoma, tracheal papilloma
443	HCC, renal adenoma
468	Renal adenoma, esophageal papilloma
468	HCC, renal adenoma, pulmonary adenocarcinoma
468	HCC, pulmonary adenoma, tracheal papilloma
503	HCC, adrenal cortical adenoma
510	HCC, renal adenoma, pulmonary adenocarcinoma, meningeal sarcoma
531	Liver AF, NN, pulmonary adenocarcinoma
531	HCC, esophageal papilloma, subcutaneous fibrosarcoma
541	HCC with pulmonary metastasis, esophageal papilloma
541	Liver NN, renal adenoma, tracheal papilloma, meningeal sarcoma
562	HCC, pulmonary adenocarcinoma, testicular mesothelioma
586	HCC, pulmonary carcinoma, esophageal papilloma, nasal cavity tumor
607	Pulmonary adenocarcinoma
607	HCC, pulmonary adenocarcinoma, renal adenoma
B. Dose 2 mg/kg/d	
131	None
179	Esophageal papilloma, nasal cavity tumor
191	HCC with pulmonary metastasis
239	HCC with pulmonary metastasis
242	HCC with pulmonary metastasis
242	HCC with pulmonary metastasis
250	HCC, esophageal papilloma
252	HCC
261	HCC with pulmonary metastasis
263	HCC
268	HCC with pulmonary metastasis, esophageal papilloma
268	HCC with pulmonary metastasis, carcinoma of the tongue
268	HCC, nasal cavity tumor, renal adenoma, esophageal papilloma and carcinoma
272	HCC, esophageal carcinoma, lower jaw carcinoma
272	HCC, esophageal papilloma, renal adenoma, nasal cavity tumor
279	HCC, esophageal papilloma and carcinoma, nasal cavity tumor
292	HCC, esophageal papilloma, renal adenoma, pulmonary adenocarcinoma, nasal cavity tumor
305	HCC, renal adenoma, nasal cavity tumor
306	HCC with pulmonary metastasis, esophageal papilloma, renal adenoma, nasal cavity tumor
320	HCC, pulmonary adenocarcinoma, esophageal papilloma and carcinoma, nasal cavity tumor
324	HCC, esophageal carcinoma, pulmonary adenoma, renal adenoma
332	HCC, esophageal papilloma, renal adenocarcinoma, nasal cavity tumor
353	HCC, renal adenocarcinoma, nasal cavity tumor
332	HCC, esophageal carcinoma, nasal cavity tumor
C. Dose 3 mg/kg/d	
52	None
165	HCC with pulmonary metastasis, esophageal papilloma
167	HCC
169	HCC
169	Liver altered foci
170	HCC with pulmonary metastasis
176	HCC, esophageal papilloma
177	HCC
179	HCC
179	HCC with pulmonary metastasis
184	HCC
186	HCC
188	HCC with pulmonary metastasis
189	HCC, esophageal papilloma
191	HCC with pulmonary metastasis
194	HCC, esophageal papilloma
200	HCC, renal adenoma
202	Testicular mesothelioma, nasal cavity tumor
205	HCC with pulmonary metastasis, nasal cavity tumor
208	HCC
209	HCC
213	HCC with pulmonary metastasis, esophageal papilloma, renal adenoma, nasal cavity tumor

Abbreviations: HCC=hepatocellular carcinoma, NN=liver neoplastic nodules, AF=liver altered foci.

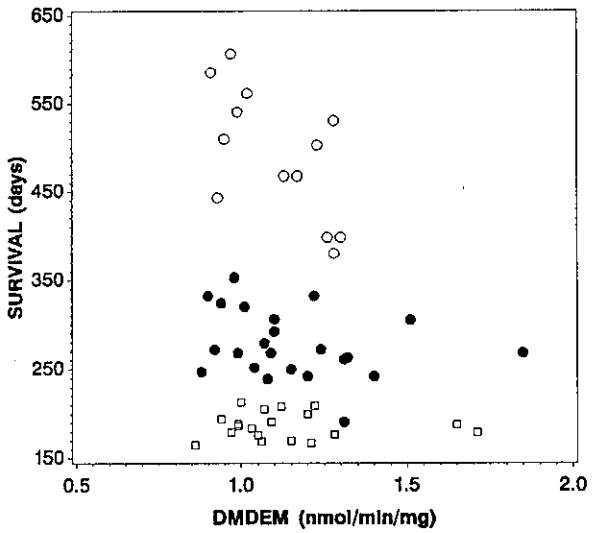

Fig. 1. Survival of rats that died with hepatocellular carcinoma after being treated with 1 (○), 2 (●) or 3 (□) mg/kg diethylnitrosamine 5 days/week for 20 weeks as a function of the activity of dimethylnitrosamine demethylase (nmol of formaldehyde formed/min/mg microsomal protein), measured in microsomes from a portion of liver excised in partial hepatectomy 8–10 weeks before starting the diethylnitrosamine treatment.

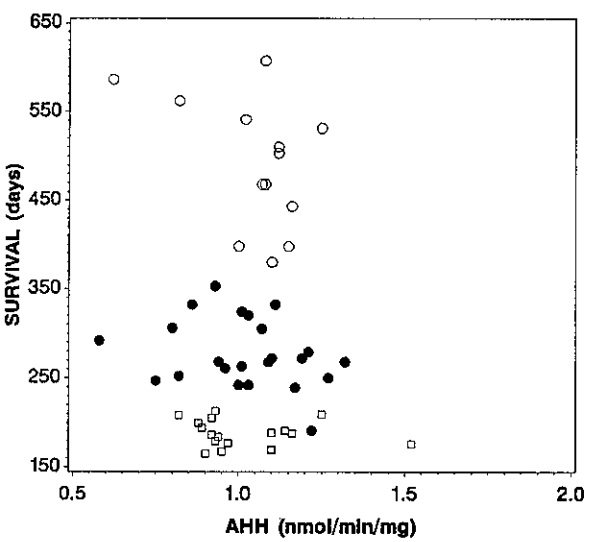

Fig. 2. Survival of rats that died with hepatocellular carcinoma after being treated with 1 (○), 2 (●) or 3 (□) mg/kg diethylnitrosamine 5 days/week for 20 weeks as a function of the activity of aryl hydrocarbon hydroxylase (nmol of 3-OH benzo[*a*]pyrene formed/min/mg microsomal protein), measured in microsomes from a portion of liver excised in partial hepatectomy 8–10 weeks before starting the diethylnitrosamine treatment.

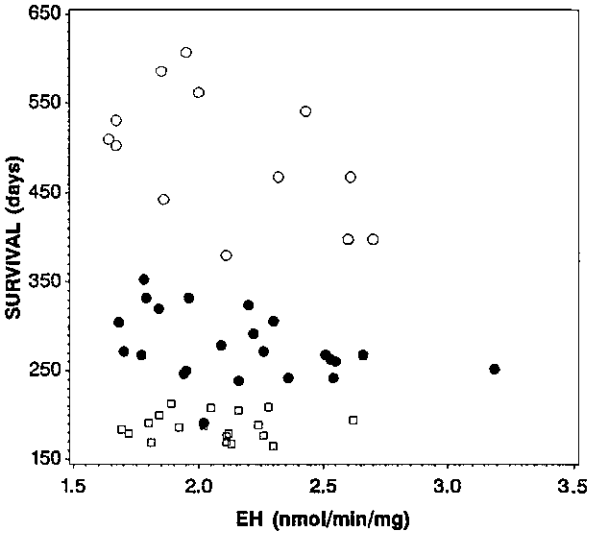

Fig. 3. Survival of rats that died with hepatocellular carcinoma after being treated with 1 (○), 2 (●) or 3 (□) mg/kg diethylnitrosamine 5 days/week for 20 weeks as a function of the activity of epoxide hydrolase activity (nmol/min/mg microsomal protein), measured in microsomes from a portion of liver excised in partial hepatectomy 8–10 weeks before starting the diethylnitrosamine treatment.

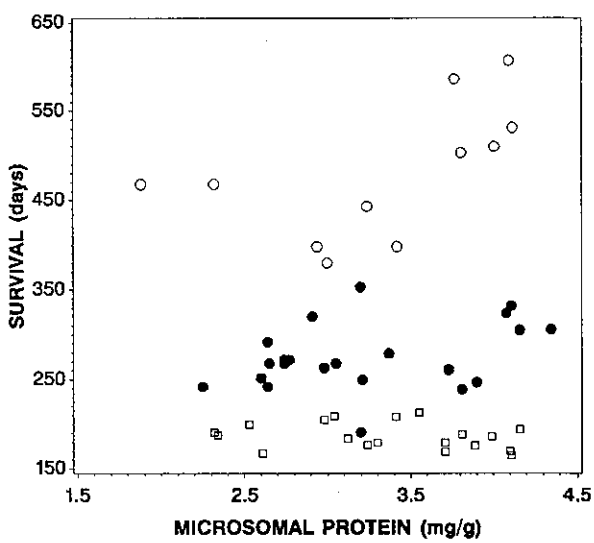

Fig. 4. Survival of rats that died with hepatocellular carcinoma after being treated with 1 (○), 2 (●) or 3 (□) mg/kg diethylnitrosamine 5 days/week for 20 weeks as a function of microsomal protein content (mg/gram liver wet weight), measured in microsomes from a portion of liver excised in partial hepatectomy 8–10 weeks before starting the diethylnitrosamine treatment.

Table II. Correlation Coefficients (r) between the Specific Activities (nmol/min/mg protein) of Different Drug-metabolizing Enzyme Activities in Rat Liver

	AHH	ECDE	DMNDM	UDPGT	EH	GST
ECDE	0.82*					
DMNDM	0.29	0.40*				
UDPGT	0.08	0.13	0.12			
EH	−0.08	−0.19	0.01	0.50*		
GST	0.31	0.29	0.16	0.29	0.13	
SULF	−0.27	−0.20	−0.22	−0.02	−0.12	−0.11

Abbreviations: AHH=arylhydrocarbon hydroxylase, ECDE=ethoxycoumarin deethylase, DMNDM=dimethylnitrosamine demethylase, UDPGT=Uridinediphosphateglucuronyltransferase, EH=epoxide hydratase, GST=glutathione *S*-transferase, SULF=sulfotransferase.
* Denotes statistical significance, $P<0.001$.

epoxide hydrolase, and the microsomal protein content (Fig. 3) were consistently related to the latency period. The latency was shorter in animals with a high activity of DMN *N*-demethylase ($\chi^2=4.78$, $P<0.05$ for all treated animal groups together); this relationship was most marked in the low dose group ($\chi^2=7.8$, $P<0.01$). The latency also tended to be shorter in animals with high liver aryl hydrocarbon hydroxylase activity ($\chi^2=4.32$, $P<0.05$ for all animals) or high epoxide hydrolase activity ($\chi^2=8.33$, $P<0.01$ for all animals). These differences were not significant in any of the dose groups analyzed separately. On the other hand, the latency period was longer in animals with a higher content of total microsomal protein ($\chi^2=10.17$, $P<0.01$); this relationship was restricted to the two lower dose groups (Fig. 4).

No ethylation of cysteine in hemoglobin was detected. No increase in the urinary excretion of thioethers was seen in DEN-treated rats.

Long-term treatment with DEN caused minor changes in the metabolic profiles of antipyrine and disopyramide excreted in the urine. In the highest dose group, the proportion of 4-hydroxyantipyrine increased from 25.1% to 30.1% ($P<0.001$), and there was a concomitant decrease in the proportion of norantipyrine in the urine (20.3 to 16.8%, $P<0.01$). The proportion of unchanged antipyrine increased from 4.7% to 5.5% (not significant). The changes in other dose groups were smaller. N-Deisopropyldisopyramide represented 14.4% of the total disopyramide derivatives in the urine; its proportion decreased ($P<0.001$) to 13.5, 11.4 or 11.0% in the animals given 1, 2, or 3 mg/kg DEN, respectively.

The relationships between different drug-metabolizing enzyme activities in the liver are summarized in Table II. The specific activities of different monooxygenases were interrelated: notably AHH with ethoxycoumarin deethylase ($r=0.82$ $P<0.001$) and DMN demethylation with ECDE ($r=0.40$, $P<0.001$). The conjugating enzyme activities were not related to MFO activities. The relationship between the metabolism of antipyrine and disopyramide *in vivo* and drug-metabolizing enzyme activities *in vitro* was studied on the basis of both total and specific enzyme activities. The excretion of metabolites was also calculated both as the proportion of the dose given and as the proportion of total drug-related chemical (parent compound plus all metabolites) excreted in the urine. None of the enzyme activities showed any relationship to metabolite excretion.

DISCUSSION

The microsomal metabolism of DEN yields acetaldehyde, ethanol,[57, 58] ethylene,[62] and nitrite and ethylamine.[59–62] All these are generated by the activity of cytochrome P-450-dependent monooxygenases; the generation of nitrite and ethylamine is considered to be a detoxification pathway.[59, 61] The other metabolites are products of an α-hydroxylation pathway that gives rise to an ethylcarbonium cation that can ethylate DNA and proteins. They thus indicate metabolic activation of the nitrosamine[57, 63] (although formation of ethanol and ethylene leads to detoxification of the carbonium ion.[62] Because of these competing metabolic pathways, both catalyzed by cytochrome P-450-dependent enzymes, it is not surprising that different results have been obtained with regard to the effect of MFO induction on nitrosamine (e.g. DMN)-induced carcinogenicity.[17, 28, 29, 64, 65] In the present study, DMN *N*-demethylase activity was used to estimate the activation of DEN *in vivo* because of the higher sensitivity of this assay and because *N*-deethylation of DEN, and *N*-demethylation of DMN are both catalyzed by the ethanol-inducible cytochrome P450IIE1.[32, 62, 66–68] We have determined the activity of DMN *N*-demethylation at a substrate concentration of 4

mmol/liter; thus, the results should reflect the activity of the isozyme with a high affinity toward nitrosamines, and not of the polycyclic hydrocarbon-inducible cytochrome P450IA2, or of the phenobarbital-inducible forms IIB1, IIB7, *h* or *k*, which have a high K_m (or low activity) for nitrosamine metabolism.[32, 62, 67]

An important finding of the present study was that the activity of DMN *N*-demethylation in liver microsomes was positively related to the susceptibility of an individual animal to DEN-induced hepatocellular carcinogenesis, an effect which was most pronounced in the lowest dose group. At high dose levels the relationship was not apparent, probably because of involvement, at high substrate concentrations, of isozymes other than P-450IIE1, in DEN demethylation. This is supported by the finding that the denitrosation of DMN is catalyzed by a cytochrome distinctly different from that involved in demethylation.[69] Our results suggest that none of the probe drugs that we studied measured solely the activity of this cytochrome, which catalyzes the competing, detoxifying denitrosation reaction of dialkylnitrosamines. There was also a negative, albeit weak relationship between aryl hydrocarbon hydroxylase activity and the latency period. It has also been shown that the cytochrome P-450IA2 metabolizes DMN(DEN),[62] producing acetaldehyde and ethylene. No relationship was observed between DEN susceptibility and the activity of ethoxycoumarin deethylation, which is catalyzed by both cytochromes P-450IA2 and IIB.[70] Induction of the IIB superfamily by phenobarbital was ineffective in increasing the microsomal metabolism of DMN *in vitro*, and also in transforming it to a mutagenic species.[32] The correlation of epoxide hydrolase activity with susceptibility to DEN-carcinogenesis is not easily explained since this enzyme has no apparent role in the metabolism of N-nitrosamines. In this study the epoxide hydrolase activity, measured *in vitro*, was not related to mixed function oxidase activities.

The amount of microsomal protein was positively correlated with the length of the latency of hepatocellular tumors. This observation may indicate that other microsomal enzymes, the amounts of which are related to the amount of total endoplasmic reticulum and thus microsomal protein — perhaps cytochromes other than IIE1, IA2, and IIB — may play a protective role in DEN carcinogenesis.

Activity associated with cytochrome P450IIE1 has been detected in the nasal mucosa and kidney in the rabbit[62, 71] and kidney of the rat.[72] DEN *N*-deethylation activity was also reported in the nasal mucosa of the rat.[73] The nasal cavity and kidney were target organs for DEN carcinogenesis in this study. However, no detectable cytochrome P450IIE1 activity has been reported in lungs, adrenals, brain or testis,[71, 72] which were also target organs. DEN carcinogenesis in the respiratory tract of hamsters could be prevented by administration of piperonyl butoxide, an inhibitor of cytochrome P-450.[21] These facts are consistent with catalysis of DEN activation by other cytochromes in addition to the form IIE1; ethylene and acetaldehyde were generated from DEN by cytochromes IIE1, IIB, and IA2 in reconstituted systems from rabbit liver.[62] While 80–90% of rat hepatic microsomal demethylation of DMN was inhibited by antibodies toward cytochrome P450IIE1,[72] DMN was also metabolized at low rates by cytochromes *d*, *f*, *h*, and *k* from rat liver.[67]

Glutathione is a scavenger for a large number of electrophilic reactants in the cell; the reaction is catalyzed by several glutathione *S*-transferases. In the present study, glutathione *S*-transferase activity, measured with benzo[*a*]pyrene 4,5-oxide as the substrate, had no apparent relationship with DEN-induced carcinogenesis, in keeping with the observation that neither glutathione, nor glutathione *S*-transferases decreased the binding of DMN to DNA *in vitro*.[74]

No relationship was detected between the susceptibility to DEN-induced carcinogenesis and metabolism of the two model drugs used in the present study. Previous data have suggested that the 3- and 4-hydroxylation and demethylation of antipyrine are preferentially catalyzed by different forms of cytochrome P-450.[75–77] In the present study, however, no positive correlations between the metabolism of antipyrine at different carbons and different cytochrome activities, measured with ethoxycoumarin, benzo[*a*]pyrene, and DMN as the substrates, could be detected. Our negative findings suggest that antipyrine is either metabolized preferentially by cytochromes other than those measured by the probe substrates or that at this low level of dosage — as indicated by a metabolism of approx. 95% of the dose — factors other than hydroxylation are rate-limiting.

Treatment with DEN caused a decrease in the proportion of norantipyrine excreted in the urine, with a concomitant increase in the amount of 4-hydroxyantipyrine. Simultaneously, there was a minor increase in the urinary excretion of unchanged antipyrine. Similarly, the excretion of nonmetabolized disopyramide increased. All these changes were dose-dependent and were accentuated by prolonged treatment with DEN. Thus the changes are inducible by treatment and cannot be attributed only to ageing, which has been shown to change drug-metabolizing enzyme activities in senescent rats.[78–80] Chronic treatment with DEN[81] or DMN[82] has been reported to decrease the activities of aniline hydroxylase and DMN demethylase; even a single high dose of DMN decreased the amount of cytochrome P-450 and the activity of aminopyrine demethylase, measured two months after dosing.[83]

Based on our results, the development and application of non-invasive assays to measure P450 IIE1-associated mixed function oxidases in human liver seems warranted; populations that are (putatively) exposed to nitrosamines have been identified.[84-86] Also of interest are the recent findings in humans that support the relevance of our results in rodents: pulmonary AHH- and epoxide hydrolase activities were negatively correlated with the survival of patients who had undergone thoracic surgery for tobacco-related lung cancer.[87]

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