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Abstract

The aromatic amines 2-aminofluorene (2AF), 2-acetylaminofluorene, and 2-aminoanthracene, and the heterocyclic amines 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline, and 3-amino-1-methyl-SH-pyrido[4,3-*b*]indole (Trp-P-2) were activated by rat liver cytosolic fractions to form mutagenic metabolites in *Salmonella typhimurium* strains TA98, TA98NR, and TA98/1,8-DNP6. In the case of the Trp-P-2, the cytosolic activation was even more potent than the microsomal activation, which is classically ascribed to *N*-hydroxylation and subsequent esterification. The cytosolic activation was *a*) NADPH-dependent, *b*) induced by pretreatment of rats with 3-methylcholanthrene and especially Aroclor 1254 but not by phenobarbital, and *c*) inhibited by dicoumarol. The hypothesis is that, following a preliminary oxidative step in the cytosol (pure cytosolic activation) or in microsomes via prostaglandin H synthase (mixed microsomal-cytosolic activation), an oxidized intermediate of amino compounds may serve as substrate for DT diaphorase activity and bioelectronically reduced to the corresponding *N*-hydroxyamino derivative. Purified DT diaphorase, in the presence of either NADPH or NADH as electron donor, produced mutagenic derivatives from IQ and Trp-P-2. An NADPH-dependent activation of Trp-P-2 also occurred in the liver cytosol of woodchucks (*Marmota monax*), but was not inhibited by dicoumarol. As previously demonstrated with liver S-12 fractions in both humans and woodchucks, the cytosolic activation of Trp-P-2 was enhanced in animals affected by hepatitis B virus infection. This enhanced metabolism, which persisted even after appearance of primary hepatocellular carcinoma in virus carriers, is likely to be ascribed to mechanisms other than DT diaphorase induction, such as glutathione depletion. -- **Environ Health Perspect** 102(Suppl 6):69-74 (1994)

Key words: aromatic amines, heterocyclic amines, metabolism, cytosol, mutagenicity, dicoumarol, DT diaphorase, viral hepatitis

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Introduction

The first metabolic step in the hepatic activation of mutagenic and carcinogenic aromatic amines (1) and of food-derived heterocyclic amines (2) consists in the microsomal oxidation of the exocyclic amino group, which is primarily catalyzed by cytochromes P4501A1 and especially P4501A2 (3). The *N*-hydroxyamino derivatives are further esterified to form more reactive species, [e.g., via *O*-sulfation (4) or *O*-acylation, which takes place both in

mammalian liver cytosol (5) and bacterial cells (6)]. An alternative pathway, especially in extrahepatic tissues, proceeds via one-electron oxidation catalyzed by microsomal prostaglandin H synthase (PHS) (7,8).

Less attention has been paid to the exclusive activation of these compounds in the liver cytosol, although an activation of aromatic amines to mutagenic metabolites has already been reported to occur in the presence of rat liver cytosolic fractions in bacterial test systems (9-11).

We present herein the results of studies investigating the liver cytosolic metabolism of the aromatic amines 2-aminofluorene (2AF), 2-acetylaminofluorene (2AAF) and 2-aminoanthracene (2AA), and of the heterocyclic amines 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) and 3-amino-1-methyl-SH-pyrido[4,3-*b*]-indole (Trp-P-2). The cytosolic activation of all these compounds was inhibited by dicoumarol, a rather specific inhibitor of DT diaphorase activity (12). Addition of purified DT diaphorase, using either NADH or NADPH as electron donor, produced mutagenic derivatives of heterocyclic amines. In addition, in agreement with a previous study using S-12 fractions (13), the activation of Trp-P-2 by liver S-105 fractions was enhanced in woodchucks naturally infected with hepatitis B virus (WHV).

Materials and Methods

Chemicals and Biochemicals

Test mutagens included the heterocyclic amines Trp-P-2, IQ, and MeIQ (gifts of T Sugimura and K Wakabayashi, National Cancer Center Research Institute, Tokyo, Japan), and the aromatic amines 2AF (Ega-Chemie KG, Steinheim/Albuch, Germany), 2AAF and 2AA (both from Sigma Chemical Co., St. Louis, MO). All test mutagens were dissolved and diluted in dimethylsulfoxide (DMSO). Dicoumarol (Sigma) was dissolved in 0.01 N NaOH. DT diaphorase (EC 1.6.99.2), purified from the liver cytosol of 3-methylcholanthrene-treated rats (14), was a gift from L Ernster, C Lind, and J Segura Aguilar (Arrhenius Laboratory, University of Stockholm, Sweden). Its molecular activity, as assayed with menadione as substrate (15), was 72,500 mole/mole FAD/min.

Animals

Four groups (five animals each) of male adult Sprague-Dawley rats (Morini strain) were either untreated or treated with one of the following inducers (all of them diluted in corn oil): phenobarbital (Merck AG, Darmstadt, Germany) 3 ip injections of 60 mg/kg during the 3 days before killing, 3-methylcholanthrene (Fluka AG, Buchs, Switzerland) single ip injection of 80 mg/kg 24 hr before killing, or Aroclor 1254 (Monsanto Co., St. Louis, MO) single ip injection of 500 mg/kg 5 days before killing.

The livers of 17 woodchucks (*Marmota monax*), either uninfected or infected with WHV or additionally carrying primary hepatocellular carcinoma (PHC), were kindly supplied by I Millman (Fox Chase Cancer Center, Institute for Cancer Research, Philadelphia, PA).

Preparation of Liver Subcellular Fractions

Liver preparations were obtained as previously described (16). They included: *a*) whole cell homogenates, obtained by homogenizing, in a Potter-Elvehjem apparatus, minced livers in a 50 mM Tris-0.25 M sucrose solution, pH 7.4 (3 ml/g wet tissue); *b*) S-12 fractions, i.e., supernatants obtained by twice centrifuging cell homogenates for 20 min at 12,000g; *c*) S-105 or cytosolic fractions, i.e., supernatants obtained by centrifuging S-12 fractions for 1 hr at 105,000g; and *d*) microsomal fractions, i.e., the corresponding pellets, washed once and resuspended in a 50 mM Tris 0.1 M EDTA solution, pH 7.4, supplemented with 20% glycerol (0.5 mg/g of original tissue). All the cell preparations were divided into small aliquots and immediately stored at -80°C until use. For use in mutagenicity assays, liver preparations were

thawed and incorporated, in varying amounts, into S-9 mix, i.e., an NADPH-generating system composed of 8 mM MgCl₂, 33 mM KCl, 5 mM G6P, 4 mM NADP⁺ and 100 mM sodium phosphate, pH 7.4; in assays with microsomes this mix was supplemented with yeast G6PD (8 IU/ml).

Mutagenicity Assays

The mutagenicity of test compounds in the presence of the above described metabolic systems, at the doses indicated under Results, was evaluated in the *Salmonella* reversion test, according to the plate incorporation procedure (17), using the *S. typhimurium* strains TA98 (gift of BN Ames, University of California, Berkeley, CA) and its nitroreductase- or *O*-acetyltransferase-deficient derivatives TA98NR and TA98/1,8-DNP6 (gifts of H.S. Rosenkranz, University of Pittsburgh, PA). Briefly, two consecutive preincubation steps were performed, the first (10 min at 37°C) involving the mixture of 500 μ l either of metabolic systems or its control (lacking liver preparations and/or cofactors) with 100 μ l of either dicoumarol or its solvent, the second (30 min at 37°C) involving incubation with 100 μ l of either test mutagens or their controls (DMSO) before plating in top agar with the appropriate *Salmonella* strain. All the assays were performed in triplicate plates.

Results

The mutagenicity assay of the heterocyclic amines Trp-P-2, IQ, and MeIQ in strain TA98 of *S. typhimurium*, in the presence of varying amounts of liver subcellular fractions from Aroclor-treated rats (Figure 1), showed that the activation by reconstituted cytosolic plus microsomal fractions is even greater than that produced by the original postmitochondrial fractions. In addition, cytosolic fractions were as active (IQ and MeIQ) or even more active (Trp-P-2) than the corresponding microsomal fractions, at equivalent weight of liver, in activating these heterocyclic amines. The addition of dicoumarol resulted in an evident decrease of the mutagenicity of all three compounds, and especially of Trp-P-2, in the presence of S-12, S-105, or S-105 plus microsomal fractions, but not in the presence of pure microsomal fractions (Figure 1). As assessed with Trp-P-2, the activation by either S-12 or S-105 fractions was interchangeably NADPH- or NADH-dependent, the mutagenic response being poor when no pyridine nucleotide was included in the composition of S-9 mix (data not shown).

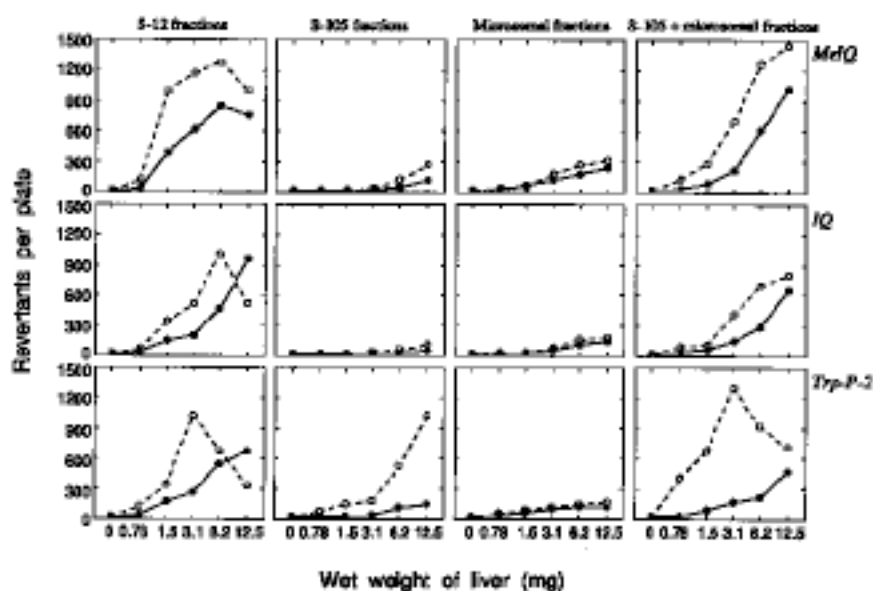


Figure 1. Metabolic activation of Trp-P-2 (20 ng/plate), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) (8 ng/plate) and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) (2 ng/plate) to mutagenic metabolites in strain TA98 of *S. typhimurium*, in the presence of varying amounts of liver post-mitochondrial (S-12), cytosolic (S-105), microsomal, or reconstituted cytosolic plus microsomal fractions from Aroclor-treated rats. The assays were

carried out either in the presence or in the absence of 50 μ M dicoumarol.

As shown in the experiment reported in Figure 2, the cytosolic activation of Trp-P-2, IQ, and MeIQ to mutagenic metabolites was poor when liver preparations from either untreated or phenobarbital-treated rats were used; whereas, metabolism was stimulated by pretreatment of rats with 3-methylcholanthrene and even more with Aroclor 1254. Again, addition of dicoumarol inhibited the cytosolic activation of these heterocyclic amines, to a marked extent in the case of Trp-P-2. Inhibition by dicoumarol was dose-dependent (Figure 3).

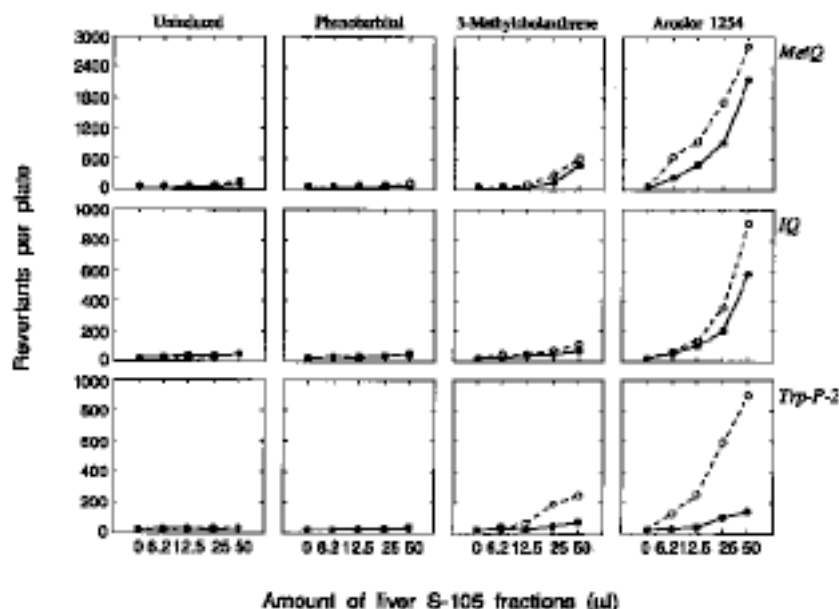


Figure 2. Metabolic activation of Trp-P-2 (20 ng/plate), 2-amino-3-methylimidazo[4,5-*f*]quinoline(IQ) (8 ng/plate) and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline(MeIQ) (2 ng/plate) to mutagenic metabolites in strain TA98 of *S. typhimurium*, in the presence of varying amounts of liver cytosolic fractions from either uninduced, phenobarbital-, 3-methylcholanthrene- or Aroclor 1254-induced rats. The assays were carried out either in the presence or in the absence of 50 μ M dicoumarol.

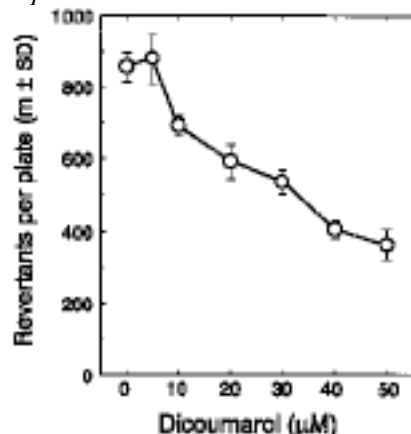


Figure 3. Decrease of Trp-P-2 (20 ng/plate) mutagenicity in strain TA98 of *S. typhimurium*, in the presence of liver cytosolic fractions (50 μ l/plate) from Aroclor-treated rats, as related to the amounts of dicoumarol (5-50 μ M).

The mutagenicity of Trp-P-2 in the presence of rat liver S-12 or S-105 fractions was similar in the *S. typhimurium* strains TA98 and TA98NR; whereas, it was decreased but not abolished (about half in the experiment shown in Figure 4) in TA98/1,8-DNP6. Inhibition by dicoumarol was evident and significant ($p < 0.001$, as assessed by Student's *t*-test) in all three strains (Figure 4).

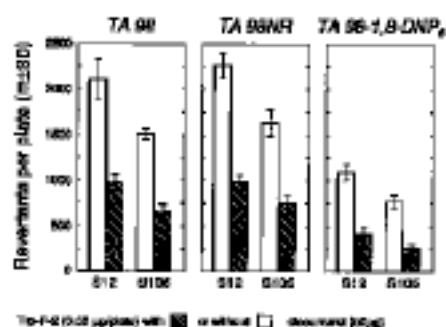


Figure 4. Mutagenicity of Trp-P-2 (50 ng/plate) in strains TA98, TA98NR, and TA98-1,8-DNP6 of *S. typhimurium* following activation by either S-12 (2.5 μ l/plate) or S-105 (12.5 μ l/plate) fractions from Aroclor-treated rats, either in the presence (dashed columns) or in the absence (open columns) of 50 μ M dicoumarol.

Four separate experiments with IQ and one experiment with Trp-P-2 indicated that addition of purified DT diaphorase, in the presence of either NADH or NADPH as electron donor, resulted in a poor (from 1.9- to 4.6-fold) yet reproducible and dose-dependent enhancement of mutagenicity, which was inhibited by dicoumarol (Table 1).

Table 1. Mutagenicity in strain TA98 of *S. typhimurium* of heterocyclic amines in the presence of either NADH or NADPH and purified DT diaphorase, and its inhibition by dicoumarol.^a

Exp. no.	Compound, 10 μ g/plate	Electron donor	Purified DT diaphorase	Revertants per plate	
				NaOH	50 μ M dicoumarol
1	IQ	NADH(4mM)	—	120 $\pm$ 3	141 $\pm$ 23
			+ (1U)	312 [x2.6]	139 [x1.0]
2	IQ	NADH (4mM)	—	44 $\pm$ 11	47 $\pm$ 8
			+ (2U)	203 [x4.6]	NT
			+ (1U)	128 [x2.9]	NT
			+ (0.5U)	105 [x2.4]	NT
3	IQ	NADPH (4 mM)	—	62 $\pm$ 14	59 $\pm$ 11
			+ (2U)	203 [x3.3]	74 [x1.2]
		NADH (4 mM)	—	73 $\pm$ 8	66 $\pm$ 19
			+ (2U)	171 [x2.3]	88 [x1.3]
4	IQ	NADPH (4mM)	—	85 $\pm$ 22	93 $\pm$ 15
			+ (1U)	173 [x2.0]	97 [x1.0]
		NADH (4mM)	—	67 $\pm$ 10	64 $\pm$ 9
			+ (1U)	126 [x1.9]	71 [x1.1]
5	Trp-P-2	NADPH (4mM)	—	44 $\pm$ 8	51 $\pm$ 7
			+ (1U)	91 [x2.1]	54 [x1.1]

^a Results are expressed as mean SD of triplicate plates in the absence of DT diaphorase and of individual results in its presence. Values between brackets indicate the relative increase of revertants produced by the pure enzyme.

Similar indications were provided by experiments with the aromatic amines 2AF, 2AAF, and 2AA, shown in Figure 5. All three compounds were activated not only by S-12 but also by rat liver S-105 fractions, and dicoumarol produced a significant decrease of mutagenicity.

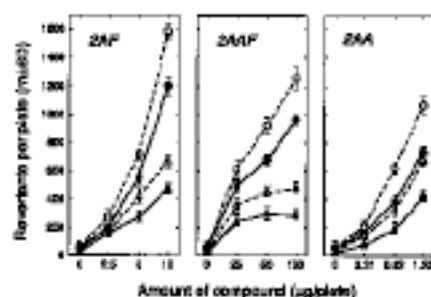


Figure 5. Mutagenicity of varying amounts of the aromatic amines 2-aminofluorene, 2-acetylaminofluorene, and 2-aminoanthracene in strain TA98 of *S. typhimurium* after activation by either S-12 (s,d) or S-105 (n,m) fractions from Aroclor-treated rats, either in the absence (s,n) or presence (d,m) of 50 μ M dicoumarol. Based on preliminary dose-response curves, 10 μ l S-12 and 20 μ l S-105 were used for 2AF and 2AA activation, whereas 40 μ l S-12 and 80 μ l S-105 were used for 2AAF activation.

The activation of Trp-P-2 to mutagenic metabolites was also obtained in the presence of woodchuck liver S-105 fractions (Figure 6). The results of these experiments are expressed as relative metabolic efficiency (RME), i.e., the ratio of mean revertants induced by Trp-P-2 in the presence of each liver preparation to mean revertants induced by Trp-P-2 in the presence of S-105 buffer. In spite of a considerable interindividual variability, activation by liver S-105 fractions from WHV-infected animals (mean $\pm$ SD = 6.8 ± 3.83) was significantly greater than that from uninfected animals (2.7 ± 0.55) ($p < 0.05$, as assessed by Student's *t*-test). The former value did not significantly differ from that produced by the nontumorous tissue preparations of WHV carriers bearing PHC (4.6 ± 1.57). Within this group of animals, the liver cytosol from the cancer tissue was significantly less efficient in activating Trp-P-2 than the surrounding nontumorous tissue ($p < 0.05$, as assessed by Student's *t*-test for paired data). Activation by woodchuck liver cytosol was almost exclusively NADPH dependent, but was not affected by addition of dicoumarol (data not shown). There was a high ($r = 0.88$) and significant ($p < 0.001$) correlation between activation by woodchuck liver S-105 fractions, as reported in the present study, and activation by the corresponding S-12 fractions, as it had been reported in a previous study (13) (Figure 7).

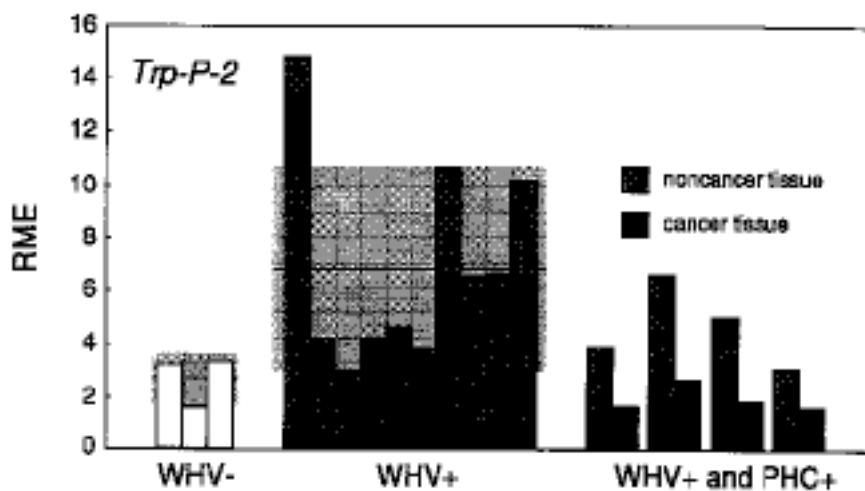


Figure 6. Metabolic activation of Trp-P-2 (1 μ g/plate) in strain TA98 of *S. typhimurium* by liver S-105 fractions (amounts corresponding to 2 mg S-105 protein/plate) from captive woodchuck either uninfected with hepatitis B virus (WHV-) or infected with hepatitis B virus (WHV+) or additionally bearing primary hepatocellular carcinoma (PHC+).

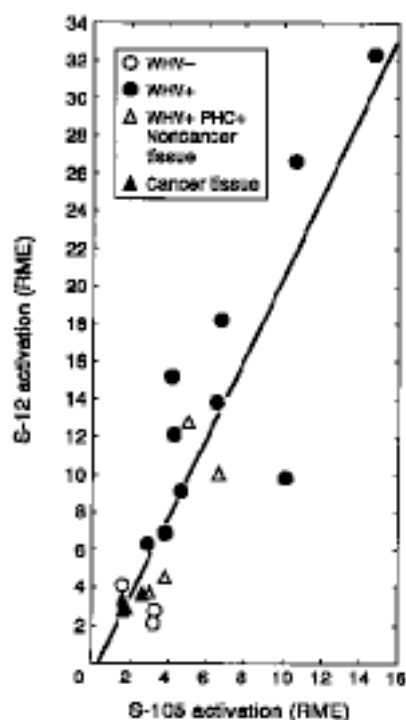


Figure 7. Correlation between metabolic activation by woodchuck liver S-105 fractions (Figure 6) and metabolic activation by the corresponding S-12 fractions (13).

Discussion

The results of the present study provide evidence that liver cytosol can contribute to the activation not only of aromatic amines, thus confirming the findings reported by other laboratories (9-11), but also of heterocyclic amines. The production of mutagenic metabolites by microsomal and cytosolic fractions was of the same order of magnitude for the two imidazoquinolines; whereas, cytosolic fractions were even more effective than microsomal fractions in activating the tryptophan pyrolysis product.

In any case, as assessed with all three heterocyclic amines, the mutagenic response obtained in the presence of cytosolic and microsomal fractions was less than additive, as compared to the effect of the original S-12 fractions. At least in the case of Trp-P-2, activation was even better by reconstituting cytosolic and microsomal fractions. These results suggest that, besides an exclusive cytosolic metabolism, an interaction between microsomal and cytosolic pathways can also occur, such interaction being better expressed when the two subfractions are reconstituted in the experimental system used. The potentiating effect of the cytosol towards microsomal activation had been previously reported with aromatic and heterocyclic amines, such as 3-amino-5H-pyrido[4,3-*b*]indole (18), 2AF (19), and IQ (20). However, in the last two studies the cytosol alone failed to activate 2AF and IQ. In another laboratory, where 2AF, 2AAF, and 2AA had been reported to be activated by the cytosol alone (9), the cytosol was also found to enhance the S-9 activation of the same compounds (21).

The cytosolic activation of IQ, MeIQ, Trp-P-2, 2AF, 2AAF, and 2AA, as assessed in the present study, was *a*) NADPH dependent, *b*) uninduced by phenobarbital but induced by 3-methylcholanthrene and, even more, by Aroclor 1254, and *c*) inhibited by dicoumarol, with top efficiency in the case of Trp-P-2. Dicoumarol decreased the mutagenic response induced in the presence of either S-12 or S-105 fractions but not in the presence of microsomes. In preliminary assays carried out in our laboratory, the S-12-mediated mutagenicity of MeIQ and Trp-P-2 and, in addition, of a cigarette smoke condensate [which among other compounds contains heterocyclic amines (22) and various quinones (23)] had been found to be decreased by dicoumarol (24). Inhibition by dicoumarol of S-12 and S-105 activation of Trp-P-2

occurred in all tester strains, irrespective of their sensitivity to this compound, which was similar in TA98 and its nitroreductase-deficient derivative TA98NR, and lower (yet still appreciable) in the *O*-acetyltransferase-deficient derivative TA98/1,8-DNP6. This suggests that the results obtained in the presence of liver subfractions are not affected by further metabolism in bacterial cells.

All these patterns converge in suggesting a possible role of DT diaphorase in the cytosolic activation of aromatic and heterocyclic amines. In fact, this FAD-containing flavoprotein is mostly localized in the cell cytosol, where it utilizes both reduced pyridine nucleotides as electron donors, dicoumarol being the most potent inhibitor (12). Moreover, DT diaphorase is induced in rat liver cytosol by 3-methylcholanthrene and even more by Aroclor 1254, but not by phenobarbital (25,26). However, an involvement of DT diaphorase in the metabolism of amino compounds is difficult to be interpreted, because it is obvious that these molecules cannot per se accept electrons. We raise the hypothesis that the amino group may undergo a preliminary oxidation to form an intermediate, acting as a substrate for DT diaphorase. For instance, as shown with pyrolysis products of tryptophan and glutamic acid, heterocyclic amines can be oxidized in the cytosol by hydrogen peroxide plus various peroxidases or catalase (27,28). Alternatively, as reported in the Introduction, amines can undergo a one-electron oxidation catalyzed by microsomal PHS (7,8), which can explain a combined microsomal-cytosolic activation of these compounds. It has been reported that PHS can oxidize 2AF to 2-nitrofluorene (29), and possibly IQ to nitro-IQ (30).

It is likely that *N*-hydroxy compounds are common metabolites to heterocyclic amines and their nitroderivatives, via monooxygenases and nitroreductases, respectively (31). In this study, however, bacterial nitroreductases did not affect the cytosolic activation of Trp-P-2. Therefore, we propose that, following a preliminary oxidation to the nitroso- or the nitro-derivative, either in the cytosol or in the endoplasmic reticulum, DT diaphorase may catalyze one or two consecutive two-electron reductions, as it is typical for this enzyme activity (at least with quinones) (32). The weak yet consistent generation of mutagenic derivatives following addition of purified DT diaphorase to IQ or Trp-P-2, in the presence of either NADH or NADPH, may possibly be ascribed to traces of oxidized amines in the reaction mixture. It is noteworthy that DT diaphorase has already been shown to metabolize nitrocompounds, such as 4-nitroquinoline 1-oxide (24,33) and dinitropyrenes (DNP) (34). In the case of DNP isomers, activation by liver cytosol contrasts with detoxification by microsomal or S-12 fractions (34,35). Extensive studies now in progress in our laboratory show that the mutagenicity of 1,3-DNP, 1,6-DNP, and 1,8-DNP is inhibited by rat liver S-12 or microsomal fractions, irrespective of Aroclor induction, as well as by cytosolic fractions from Aroclor-treated rats. The mutagenicity of 1,3-DNP is considerably enhanced only by using the cytosol from uninduced rats, with an NADPH-dependent and dicoumarol-inhibitable mechanism. The mutagenicity of all these compounds is also enhanced by reduced glutathione (unpublished data).

Trp-P-2 was also activated by the liver cytosol of woodchucks, and cytosolic activation correlated with S-12 activation, which had been investigated in a previous study using the same liver specimens (13). The cytosolic activation was NADPH dependent but was not inhibited by dicoumarol, which rules out any involvement of DT diaphorase in this rodent species. Therefore, different mechanisms appear to be involved in the cytosolic activation of Trp-P-2 in rat and woodchuck liver.

Previous studies using liver postmitochondrial fractions from patients affected by chronic active hepatitis (36), wild-caught woodchucks affected by chronic active hepatitis (37), and the specimens of captive woodchucks analyzed in this study (13), had demonstrated that infection with the specific hepadnaviruses, i.e., hepatitis B virus (HBV) in humans and WHV in woodchucks, results in an enhanced activation of Trp-P-2 to mutagenic metabolites. This inducing effect has been confirmed now by using woodchuck liver cytosolic fractions. Moreover, in agreement with the data obtained by testing the corresponding S-12 fractions, stimulation of the cytosolic activation of Trp-P-2 in WHV-infected animals persisted also after PHC formation. The cancer tissue itself, in accordance with the resistant hepatocyte

model (38), exhibited a reduced Trp-P-2-activating ability, as compared to the surrounding noncarcinogenic tissue. Since all these effects were not affected by dicoumarol, and DT diaphorase activity was not altered by WHV infection (13), other cytosolic mechanisms, such as the marked depletion of hepatocellular glutathione produced by WHV (13), are likely to account for the modulation of the cytosolic metabolism of procarcinogens in viral hepatitis B.

Heterocyclic amines produce higher levels of DNA adducts in the liver than in other organs, which correlates with their hepatocarcinogenicity (39). Taking into account that humans are exposed ubiquitously to these compounds through the ingestion of cooked foods, an enhancement of their metabolic activation in the liver of hepadnavirus carriers, both in the cytosol and the endoplasmic reticulum, may bear relevance in the etiopathogenesis of the PHC forms associated with HBV infection.

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