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HEPATIC MICROSOMAL N-HYDROXYLATION OF ADENINE TO 6-*N*-HYDROXYLAMINOPURINE

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Abstract—The enzymatic N-hydroxylation of the purine base adenine to the genotoxic and mutagenic compound 6-*N*-hydroxylaminopurine is reported for the first time. Adenine was N-oxygenated *in vitro* by aerobic incubations with 3-methylcholanthrene or isosafrole induced microsomal fractions of rat liver homogenates and NADPH. The formation of 6-*N*-hydroxylaminopurine in the incubation mixtures under widely differing conditions was assayed using newly-developed, high-performance liquid- and thin-layer chromatographic methods. Optimal reaction conditions and kinetic parameters were determined. Neither superoxide anion nor hydrogen peroxide was directly involved in the N-hydroxylation reaction. Oxidases like xanthine oxidase and peroxidase (in the presence of hydrogen peroxide) did not catalyse this N-hydroxylation. The involvement of cytochrome P-450 isoenzymes in this reaction is supported by the observation that the N-hydroxylation is only observed after pretreatment of the rats with 3-methylcholanthrene or isosafrole. Other inducers (phenobarbital, ethanol, 5-pregnen-3βol-20-one-16α-carbonitrile) were without effect. This is the first example of the microsomal transformation of an endogenous substance to a toxic derivative by usually foreign substances (xenobiotics) metabolizing cytochrome P-450 isoenzymes. The significance for the *in vivo* situation is discussed on the basis of the data obtained in this study.

All species possess numerous cytochrome P-450 isoenzymes [1]. These can be subdivided on the one hand into those which are characterized by a high substrate specificity and participate in the biosynthesis of endogenous substances such as steroids and prostaglandins and, on the other hand, into those which are non-specifically responsible for the metabolism of widely differing exogenous compounds (xenobiotics) and which, in part, are induced by foreign substances [2]. The question now arises whether or not the latter forms, on account of their low substrate specificities, can also attack endogenous substances and, in analogy to some xenobiotics, even convert them—depending on the structural features—into toxic compounds. An example of this type of reaction of an endogenous substrate is described in the present publication, namely the microsomal N-hydroxylation of the nucleic base adenine **1** to 6-*N*-hydroxylaminopurine **2** (HAP†) (Fig. 1). From other investigations it was already known that HAP **2** is genotoxic (to eucaryotes and procaryotes) and carcinogenic, whereby evidence was presented both for a reaction as a base analog [3] as well as for a covalent bonding to DNA [4, 5].

The fact that the N-hydroxylation of adenine **1** was the first endogenous compound investigated in this connection is a direct consequence of our previous biotransformation studies on amidines [6–8] in which their N-oxygenations by probable cytochrome P-450 [7] were demonstrated. In the course of this work,

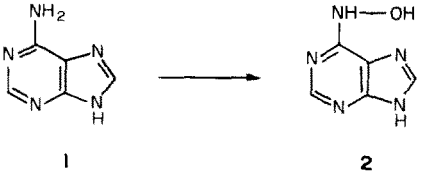

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Fig. 1. N-Hydroxylation of adenine **1** to 6-*N*-hydroxylaminopurine (HAP) **2**.

it was found [6] that an N-oxygenation by cytochrome P-450 was observed when, as a result of the lack of suitable structural elements, a competing N-dealkylation reaction was not possible. These structural prerequisites for an N-dealkylation reaction are also absent in adenine **1**. Although this substrate should actually be considered as an amino-substituted heteroaromatic compound exhibiting only weakly basic properties, the exocyclic amino group bonded to C6 in conjunction with the ring nitrogen atom N1 can also be visualized as the components of an amidine-like structural moiety. At first we were mainly interested in an N-oxygenation of the exocyclic NH_2-group to furnish HAP **2** on account of its significant toxicological relevance [3–5] and have paid less attention to N-oxygenations and C-oxygenations of the purine ring.

The only previous reports about a comparable N-hydroxylation of an exocyclic amino group bonded to a carbon atom which is part of an aromatic system and is bonded to at least one other further ring nitrogen atom were concerned with a group of foreign substances possessing other heterocyclic systems (pyrolysis products from proteins and amino acids) [9].

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† Abbreviations used: HAP, 6-*N*-hydroxylaminopurine; 3-MC, 3-methylcholanthrene; SKF-525A, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride; PCN, 5-pregnen-3-βol-20-one-16α-carbonitrile; HPLC, high-performance liquid chromatography.

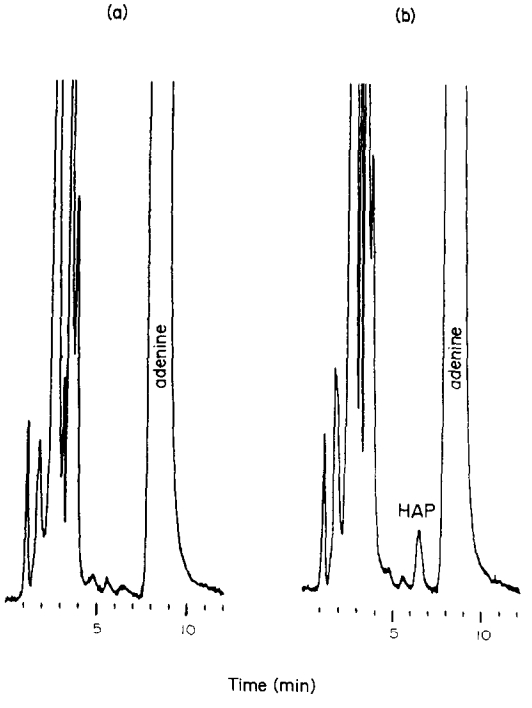

Fig. 2. Representative HPLC chromatogram of adenine metabolism by 9000 *g* supernatant fractions of rat liver homogenates. See Materials and Methods for details of reaction mixture content, incubation, sampling and analysis. Key: (a) omission of NADPH; (b) complete system; HAP, 6-*N*-hydroxylaminopurine.

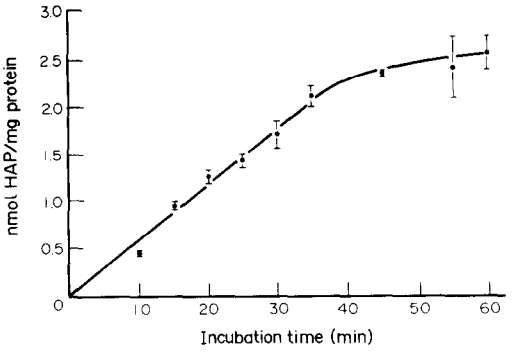

Fig. 3. Formation of 6-*N*-hydroxylaminopurine by 9000 *g* supernatant fractions of rat liver homogenates as a function of time (pretreatment with 3-MC). Each point is the mean of three determinations ±SD. See Materials and Methods for details of reaction mixture content, incubation, sampling, and analysis.

MATERIALS AND METHODS

Reagents and biochemicals

Superoxide dismutase from bovine erythrocytes (3000 units/mg), catalase (bovine liver, 65,000 units/mg), peroxidase (horse radish, 227 units/mg), xanthine oxidase (cow milk, 20 units/mL), and glucose oxidase (Asp. niger, 208 units/mg) were purchased from Boehringer-Mannheim GmbH (Mannheim, F.R.G.). NADPH (tetra sodium salt), NADP (disodium salt), and glucose-6-phosphate dehydrogenase were obtained from Merck (Darmstadt, F.R.G.). Metyrapone and 3-MC were products from EGA-Chemie (Steinheim, F.R.G.). SKF 525A was kindly supplied by Prof. K. Netter (Marburg, F.R.G.). Adenine was purchased from Janssen Chimica (Beerse, Belgium), 6-chloropurine from the Aldrich Chemical Co. (Milwaukee, WI), PCN, and isoguanine (6-amino-2-hydroxypurine, 2-hydroxyadenine) from the Sigma Chemical Co. (St Louis, MO). All other chemicals and solvents (GR) were obtained from Merck.

Synthesis

6-*N*-Hydroxylaminopurine **2** was prepared from hydroxylamine and 6-chloropurine according to the procedure of Giner-Sorolla and Bendich [10]. Adenine 1-*N*-oxide [11], adenine 3-*N*-oxide [12], adenine 7-*N*-oxide [13], and 8-hydroxyadenine(6-amino-6-hydroxypurine) [14] were also synthesized as reported previously. All compounds were checked for purity by standard methods.

Preparation of liver homogenates

Wistar rats of either sex, approximately 200–250 g in weight, were used and fed on a standard diet ("Altromin", supplied by Lage, F.R.G.). They were allowed drinking water *ad lib*. Rats were decapitated and the livers removed immediately. Usually 5–10 livers were pooled. All subsequent operations were carried out at 0–4°. The livers were washed three times with phosphate buffer (pH 7.4; 8.7 mM KH_2PO_4, 30.4 mM Na_2HPO_4), blotted dry, and weighed. The minced livers were homogenized with 4 vol. of 1.15% (w/v) KCl solution (0.154 M) using a motorized Teflon pestle glass tube homogenizer (Potter S, for 30 mL, B. Braun Melsungen AG, F.R.G.) or alternatively with an Ultra-Turrax homogenizer (3 × 15 sec, interrupted by cooling in an ice bath for 1 min). Both procedures gave tissue fractions with similar specific activities. The homogenates were transferred to plastic tubes and centrifuged at 9000 *g* for 30 min. The supernatant was carefully decanted and used as the enzyme source. The preparations were stored at −80° and could be used for 3 months without a significant loss of activity. The microsomal pellet and the 100,000 *g* supernatant were obtained by further centrifugation at 100,000 *g* for 90 min. The pellet of microsomes was resuspended in phosphate buffer (pH 7.4; 8.7 mM KH_2PO_4, 30.4 mM Na_2HPO_4) with a Potter homogenizer and again centrifuged as above. The washed microsomes were finally suspended in isotonic KCl solution, usually at a concentration of 20 mg protein per mL. The microsomes were stored at −80° and could be used for 1 month without a significant loss of activity. Protein concentrations were measured using the method of Gornall *et al.* [15]. The content of cytochrome P-450 in the microsomes was determined as described by Omura and Sato [16].

Xenobiotic treatment

Phenobarbital. Wistar rats of either sex, approximately 200–250 g in weight, were used. Drinking

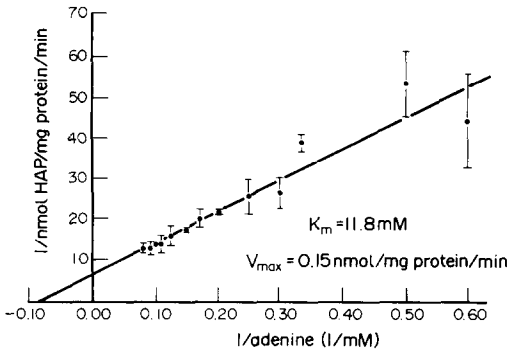

Fig. 4. Lineweaver–Burk plot of the N-hydroxylation of adenine measured by 6-*N*-hydroxylaminopurine formation in the incubation mixture. Each point is the mean of three experiments ±SD (three different enzyme preparations). Incubations were carried out as described in Materials and Methods.

water was replaced for 5 days by a solution containing 0.1% sodium phenobarbital. Control animals received only drinking water.

3-Methylcholanthrene. Male or female Wistar rats were injected subcutaneously with a corn oil solution of 25 mg 3-MC/kg body weight once daily for 2 days and twice on the third day. Control animals received only corn oil.

PCN and isosafrole. Male Wistar rats were injected intraperitoneally with a corn oil solution of, respectively, 50 mg PCN/kg body weight and 150 mg isosafrole/kg body weight once daily for 3 days. Control animals received only corn oil.

In all protocols the animals were fasted for 12 hr before they were killed. The microsomal fractions were prepared as described above.

Incubations

Incubations were carried out in a shaking water bath at 37° in the presence of atmospheric oxygen using 1.5 mL microtubes (Eppendorf, Hamburg, F.R.G.). The standard incubation mixture (usually 0.15 mL) contained the following components: phosphate buffer (pH 7.4; 8.7 mM KH_2PO_4, 30.4 mM Na_2HPO_4), 3.4 mM $MgCl_2$, 0.4 mM NADPH, 9000 *g* supernatant or microsomes corresponding to 0.5 mg protein/0.15 mL. Since preliminary experiments had shown that incubations of microsomes either in the presence of NADPH or of an NADPH-generating system gave similar results, NADPH was used in all of the experiments reported. NADPH (0.4 mM) was found to be the optimum amount for the reactions at the selected concentrations as an increase of the amount of NADPH did not result in an increase in the reaction rate. After preincubation at 37° for 1 min the reactions were initiated by addition of the cofactor solution. A 30-min incubation time was routinely employed. Incubations were terminated by freeze-drying.

High-performance liquid chromatography

Methanol (250 μL) was added to the freeze-dried incubation mixture and centrifuged at 6000 *g* for 3 min. Aliquots (5–10 μL) of the supernatant were analysed using a high pressure liquid chromatography (655 A-11, Merck-Hitachi, Darmstadt, F.R.G.) equipped with a variable wavelength UV monitor (655 A-22, Merck-Hitachi) set at 270 nm. The areas under the peaks were integrated with a chromato-integrator (D-2000, Merck-Hitachi). Separation and quantification of 6-*N*-hydroxylaminopurine were performed at room temperature on a prepacked cationic exchange column (200 mm length × 4 mm i.d., particle size 5 μm; Nucleosil® 5 SA, Macherey-Nagel GmbH, Düren, F.R.G.). The mobile phase was methanol/ammonium formate buffer 0.1 M pH 3.0 (67:33, v/v). This mobile phase flowed through the column at the rate of 0.8 mL/min. Solvents used in the analysis were filtered through a membranous filter CRC-255 (Schleicher and Schuell, F.R.G.), degassed by bubbling with helium and sonicated. Standard curves (peak area) at the level of 0.5, 1.0, 1.5, 2.5, 4.0, 5.0, 6.0, 10.0, 15.0, 20.0, 50.0, and 200 μM 6-*N*-hydroxylaminopurine were constructed by introducing known amounts of 6-*N*-hydroxylaminopurine into the usual incubation mixtures (omission of NADPH), the mixtures were then incubated and treated in the same way as the experimental samples. Standard curves were linear over this range with correlation coefficients of 0.998 or greater. The levels of 6-*N*-hydroxylaminopurine in unknown incubation mixtures were determined directly from these standard curves which were run in parallel with the experimental samples. The recovery of 6-*N*-hydroxylaminopurine from incubation mixtures fortified with 6-*N*-hydroxylaminopurine was 97.2 ± 1.2 (N = 48) of that obtained using samples which contained the same amount of 6-*N*-hydroxylaminopurine dissolved in methanol. The detection limit of 6-*N*-hydroxylaminopurine in one incubation mixture is 0.4 μM which corresponds to a rate of N-hydroxylation of 0.004 nmol 6-*N*-hydroxylaminopurine/min/mg protein. The retention times were 6.4 min for 6-*N*-hydroxylaminopurine, and 8.3 min for adenine.

UV spectrum of the metabolite 6-N-*hydroxylaminopurine*

The methanolic solution of a freeze-dried incubation mixture (usual concentrations, 6 mL) obtained as described above was processed as follows: the solution was concentrated by evaporation under reduced pressure to a volume of 3 mL. The solid (predominant adenine), which had been formed after standing for 2 hr at −30°, was precipitated by centrifugation at 3000 *g*. Aliquots of the supernatant were analysed using a high pressure liquid chromatograph (Waters 600 multisolvent delivery system, Waters Associates, Eschborn, F.R.G.) linked to a diode array-detector (Waters 990). The UV spectrum of the metabolite with a retention time of 6.4 min was identical with a spectrum of synthetic 6-*N*-hydroxylaminopurine recorded by the diode array-detector under the same conditions.

Thin-layer chromatography

The methanolic solution of a freeze-dried incubation mixture (usual concentrations, 6 mL) was concentrated as described above (see UV spectrum of the metabolite 6-*N*-hydroxylaminopurine) to a volume of *ca.* 0.5 mL. To the so formed suspension

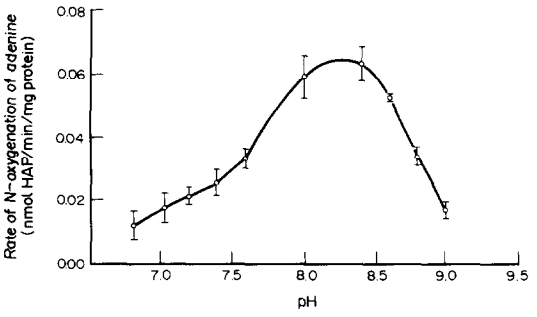

Fig. 5. N-Oxygenation of adenine activity as a function of pH. Incubations were carried out with Tris–HCl buffer (50 mM) as described in Materials and Methods.

0.5 mL formic acid was added and the clear solution was used for the TLC separation. Chromatograms were obtained using 20 × 20 cm precoated glass plates RP-8 F_{254S} (Merck), 0.25 mm thickness. The solvent system used was methanol/water/1-octanesulfonic acid (60:40:0.0018 M, v/v/M), adjusted to pH 3.0 with phosphoric acid. Spots were visualized under UV light (254 nm). The R_f values were 0.60 for 6-*N*-hydroxylaminopurine, 0.35 for adenine, 0.40 for adenine-1-*N*-oxide, 0.51 for adenine-3-*N*-oxide (tailing), 0.47 for adenine-7-*N*-oxide (tailing), 0.38 for 8-hydroxyadenine and 0.66 for isoguanine (2-hydroxyadenine).

Carbon monoxide inhibition

Experiments were performed as described previously for the microsomal N-demethylation of *N*-methylbenzamidine [8] using the usual concentrations of components (see above) in a total volume of 10 mL. Alternatively experiments were performed according to the method reported by Lotlikar and Zaleski [17] using the same incubation mixtures as above.

Statistical analysis was performed using Student's *t*-test for measuring the significance of the difference between the means of independent groups.

RESULTS

Qualitative and quantitative analysis of 6-N-*hydroxylaminopurine formed by microsomal N-hydroxylation of adenine*

The qualitative determination of 6-*N*-hydroxylaminopurine in the presence of adenine from *in vitro* biotransformation reactions is possible by freeze-drying the incubation mixture, dissolving the residue in methanol, and subsequently analysing the sample by TLC or HPLC methods. These newly developed analytical methods also enable the detection of small amounts of 6-*N*-hydroxylaminopurine in the presence of a large excess of adenine. Another procedure for the work-up and a comparable HPLC system for the detection of 6-*N*-hydroxylaminopurine has been described by Lam *et al.* [18].

In the course of biotransformation studies, it was possible for the first time to detect 6-*N*-hydroxylaminopurine which had been formed from adenine by the action of microsomal enzymes. The results has been confirmed several times. Throughout the HPLC analysis and even with differing solvent systems, one analyte always appeared with a retention time in agreement with that of a synthetic reference sample. A typical chromatogram from such an HPLC analysis is shown in Fig. 2. With the aid of a diode array-detector, the complete UV spectrum of the metabolite was recorded. It was identical to the spectrum of the reference compound under analogous conditions (UV maximum at 270 nm).

In order to exclude the possibility of the presence of a ring-oxygenated metabolite of adenine fortuitously having an identical chromatographic behaviour, adenine 1-*N*-oxide [11], adenine 3-*N*-oxide [12], adenine 7-*N*-oxide [13], and 8-hydroxyadenine [14] were prepared. 2-Hydroxyadenine (6-amino-2-hydroxypurine, isoguanine) is commercially available (Sigma). All of these substances exhibit differing retention times or R_f values when analysed under the chosen chromatographic conditions; however, since the signals or spots of these substances, with the exception of adenine 3-*N*-oxide, are overlapped by those of adenine or of components of the enzyme source, it is not possible to draw any conclusions concerning their possible microsomal formation. Adenine 3-*N*-oxide could not be detected.

It was not necessary to prepare adenine 9-*N*-oxide (or 9-*N*-hydroxyadenine) for comparative purposes since, in acidic media, this substance gives rise to clearly different UV spectra (two maxima at 245 nm and 259 nm) [19].

On TLC analysis of the freeze-dried biotransformation mixtures, a substance with the same R_f value as 6-*N*-hydroxylaminopurine was also found.

The exact quantitative characterization of this newly discovered N-hydroxylation of adenine was possible by means of the developed HPLC analytical method throughout the entire concentration range of interest.

Microsomal enzymes, cofactor requirement, and effect of inhibitors

Of all the fractions of rat liver homogenates tested, only the 9000 *g* supernatant and the microsomes showed a measurable activity (Table 1). It was also found that the reaction was proportional to protein concentration up to 3.5 mg microsomal protein per mL incubation mixture (Table 2).

The reactions required the presence of NADPH in addition to oxygen. Addition of magnesium chloride resulted in a higher activity (not significant). Some of the inhibitors of the cytochrome P-450 system [20] SKF 525A, metyrapone and 1-octylamine inhibited the microsomal formation of 6-*N*-hydroxylaminopurine, while potassium cyanide did not have a significant influence on the metabolic rate (Table 1).

In addition, it was investigated whether the rate of metabolism is dependent on the sex of the animals. This could be unequivocally excluded (Table 1).

The influence of carbon monoxide was more thoroughly studied. The proportions of CO to O_2 were varied and the respective inhibition in comparison to a control determined. It was ensured that the respective oxygen concentration (also in the control experiment) remained constant. Thus, it can be excluded that a supposed inhibition is only a result of a decreased amount of available oxygen [21].

Table 1. Effect of cofactors and of inhibitors of cytochrome P-450 on the rate of adenine N-oxygenation by rat liver microsomal enzymes and sex differences

Enzyme (pretreatment with 3-MC)	Incubation mixture	nmol HAP/min/mg protein
Supernatant (9000 *g*)	Complete (female)	0.051 ± 0.008
	Complete (male)	0.052 ± 0.007
	−NADPH	<0.004*
	$-Mg^{2+}$	0.049 ± 0.006
	$-O_2$	<0.004*
	+SKF 525A (5 mM)	0.021 ± 0.0022*
	+KCN (1 mM)	0.046 ± 0.014
	+Metyrapone (0.1 mM)	<0.004*
	+1-Octylamine (0.07 mM)	0.026 ± 0.0072*
Microsomes	Complete (female)	0.037 ± 0.009+
	Complete (male)	0.036 ± 0.011+
Supernatant (100,000 *g*)	Complete	<0.004

Values are presented as the means of three determinations with one enzyme preparation (10 livers were pooled) ±SD; reaction mixtures contained the components described in Materials and Methods, except for the omission of cofactors or the addition of inhibitors; +0.033 ± 0.008 nmol HAP/min/nmol cytochrome P-450; +0.032 ± 0.01 nmol HAP/min/nmol cytochrome P-450.

* Statistically different from control (9000 *g* supernatant, complete male) with $P < 0.001$ (Student's *t*-test).

Table 2. Effects of various amounts of microsomal protein on the liver microsomal N-hydroxylation of adenine to 6-*N*-hydroxylaminopurine

Microsomal protein (mg)	nmol 6-*N*-hydroxylaminopurine/min/mg protein
0	<0.01
0.35	0.10 ± 0.026
1.05	0.07 ± 0.014
1.75	0.06 ± 0.009
2.45	0.05 ± 0.004
3.50	0.045 ± 0.006
7.00	0.02 ± 0.002

Values are presented as the means of three determinations from one enzyme preparation (five livers were pooled, 3-MC pretreatment) ±SD; incubations were carried out as described in Materials and Methods.

Since the inhibition of cytochrome P-450-dependent reactions by carbon monoxide is, on the one hand, dependent on the experimental conditions [17, 22] and, on the other hand, also dependent on the substrate [20], various methods have been employed (see Materials and Methods). However, a significant inhibition could not be observed under any conditions or at any ratios of CO to O_2. Instead, practically identical reaction rates were determined (data not shown).

Kinetics of the N-oxygenation of adenine

The reaction rate of the enzymatic formation of 6-*N*-hydroxylaminopurine was linear over a period of 35 min (Fig. 3).

An incubation time of 30–35 min was selected in order to obtain a sufficient amount of the metabolite 6-*N*-hydroxylaminopurine for its quantitative determination.

The N-hydroxylation of adenine during the incubation with the 9000 *g* supernatant of rat liver homogenates (rats pretreated with 3-MC) and NADPH followed Michaelis–Menten kinetics. The apparent K_m value, calculated form the Lineweaver–Burk plot (Fig. 4) was 11.8 ± 1.2 mM, while the apparent V_{max} value was found to be 0.15 ± 0.05 nmol 6-*N*-hydroxylaminopurine produced/min/mg of protein (values obtained with three different experiments, three different enzyme preparations, 5–10 livers were pooled in each experiment).

Influence of pH and buffer

The effect of pH values between 6.8 and 9.0 on the microsomal *N*-oxygenation activity is shown in Fig. 5. The optimum pH value was 8.3 ± 0.15.

The incubations were performed in Tris–HCl buffer (0.05 M) and phosphate buffer (see Materials and Methods). Since phosphate buffer possesses a sufficient buffer capacity only up to pH 8.0 and since no differences in the rates of metabolism could be detected, the results obtained with phosphate buffer are not presented here.

Effects of induction on N-oxygenation

The rate of N-oxygenation of adenine to 6-*N*-hydroxylaminopurine was only detected after pretreatment of rats with 3-MC or isosafrole (Table 3). Untreated rats or rats pretreated with PCN, phenobarbital or ethanol showed no measurable activity (Table 3).

The specific rates of metabolism in reactions with the microsomes obtained after pretreatment with 3-MC or isosafrole were almost identical whereas marked differences were observed with the 9000 *g* supernatant (Table 3).

Table 3. Effect of administration of 3-MC, isosafrole or PCN on the N-hydroxylation of adenine by rat liver microsomal fractions

Treatment	nmol HAP/min/nmol cytochrome P-450	nmol HAP/min/mg protein (microsomes)	nmol HAP/min/mg protein (9000 *g* supernatant)
Control	<0.004	<0.004	<0.004
3-MC	0.032 ± 0.01	0.036 ± 0.011	0.052 ± 0.007
Isosafrole	0.026 ± 0.009	0.027 ± 0.009	0.017 ± 0.004
PCN	—	—	<0.004
Phenobarbital	—	—	<0.004
Ethanol	—	—	<0.004

Values are presented as the means of three determinations from one enzyme preparation (5–10 livers were pooled) ±SD; xenobiotic treatment and incubations were carried out as described in Materials and Methods.

Table 4. Effects of addition of superoxide dismutase, catalase, and H_2O_2 on the rate of adenine N-oxygenation by 9000 *g* supernatant fractions of rat liver homogenates

Addition	nmol HAP/min/mg protein	% of control activity
None	0.049 ± 0.009	100
Catalase*	0.041 ± 0.014	83
Superoxide dismutase*	0.047 ± 0.01	96
H_2O_2*	<0.004	0

Values are presented as the means of six determinations from two enzyme preparations (five livers were pooled in each experiment, pretreated with 3-MC) ±SD.

* Superoxide dismutase (1000 units/mL final concentration) or catalase (1000 units/mL final concentration) was added to the reaction mixture containing the components described in Materials and Methods; $^+H_2O_2$ (100 mM final concentration) was added to the reaction mixture containing the components described in Materials and Methods but without NADPH.

Influence of catalase and superoxide dismutase

The extent of any role played by hydrogen peroxide or superoxide radicals on the N-oxygenation was investigated by adding catalase or superoxide dismutase or hydrogen peroxide instead of NADPH to the usual incubation mixtures. It was found that neither catalase nor superoxide dismutase had any significant effect on the reaction (Table 4).

NADPH could not be replaced by hydrogen peroxide (Table 4).

Incubations of adenine with H_2O_2, Fenton's reagent, and oxido-reductases

Neither H_2O_2 alone, Fenton's reagent, H_2O_2-forming systems (xanthine oxidase + xanthine, glucose oxidase + glucose), nor combinations of H_2O_2 or of the H_2O_2-forming systems with catalase or superoxide dismutase or peroxidase are able to transform adenine into 6-*N*-hydroxylaminopurine (Table 5).

DISCUSSION

With the help of the newly developed HPLC and TLC analyses, it was possible to identify 6-*N*-hydroxylaminopurine as an *in vitro* metabolite of adenine. The identity of the metabolite was confirmed in several ways and its structure was not identical to that of a ring-oxygenated compound.

The HPLC analysis served for a more detailed characterization of the reaction.

The N-hydroxylation of adenine exhibited the typical properties of a microsomal monooxygenase which requires the presence of NADPH and O_2 in addition to microsomal proteins (Tables 1 and 2) [23]. The N-oxygenation can only be detected when enzyme sources that were obtained from 3-MC or isosafrole pretreated rats were employed. This inducibility permits the immediate assumption of a participation of the cytochrome P-450 enzyme system [21]. It is known that the cytochrome P-450 isoenzymes c and d in the rat are induced by compounds such as 3-MC and isosafrole [24–26]. Comparable N-hydroxylations of similar structural elements (pyrolysis products from proteins and amino acids) are also induced by methylcholanthrene [9].

N-Oxygenations can also be catalysed by the flavine-containing monooxygenase (FMO) [27]. However, an inducibility of this enzyme by 3-MC or isosafrole has never been reported [27]. Furthermore, 1-octylamine would then rather function as an activator than as an inhibitor when this FMO were participating (Table 1) [28].

Table 5. Incubation of adenine with H_2O_2, Fenton's reagent, and several oxidoreductases

Incubation mixture	nmol HAP/min/mL incubation mixture
H_2O_2 (0.1 mM)	<0.013
H_2O_2 (1.0 mM)	<0.013
H_2O_2 (10.0 mM)	<0.013
H_2O_2 (100.0 mM) + Fe(II)SO_4 (7.4 mM)	<0.013
XOD (0.018 units/mL) + xanthine (0.25 mM)	<0.013
GOD (1.3 μg/mL) + glucose (2 mM)	<0.013
H_2O_2 (1 mM) + catalase (100 μg/mL)	<0.013
XOD (0.018 units/mL) + xanthine (0.25 mM) + catalase (100 μg/mL)	<0.013
GOD (1.3 μg/mL) + glucose (2 mM) + catalase (100 μg/mL)	<0.013
XOD (0.018 units/mL) + xanthine 0.25 mM + superoxide dismutase (20 μg/mL)	<0.013
H_2O_2 (1 mM) + POD (250 μg/mL)	<0.013
GOD (1.3 μg/mL) + glucose (2 mM) + POD (250 μg/mL)	<0.013

Incubations (30 min) were carried out in unstoppered 25-mL Erlenmeyer flasks at 37° in a shaking water-bath. The incubation mixtures (total volume 3.0 mL) contained adenine (10 mM final concentration) and phosphate buffer (pH 7.4; 50 mM final concentration). Incubations were terminated by freeze-drying and analysed as described in Materials and Methods.

XOD, xanthine oxidase; GOD, glucose oxidase; POD, peroxidase.

Hence, it was especially surprising that the typical cytochrome P-450 inhibitor carbon monoxide [21] did not exhibit a significant influence on the rate of the metabolism. However, the absence of inhibition by CO of other unambiguously cytochrome P-450-dependent reactions (e.g. the N-hydroxylation of 2-acetamidofluorene) has also been reported [22]. The inhibition appears to depend on the experimental conditions chosen. Thus, experiments with CO alone do not allow any final conclusions about the participation of cytochrome P-450 to be drawn [20].

Other inhibitors of cytochrome P-450 [20] such as SKF 525A, metyrapone, and 1-octylamine inhibit the microsomal N-hydroxylation of adenine (Table 1).

After pretreatment of the animals with inducers of other cytochrome P-450 isoenzymes such as phenobarbital, ethanol, and PCN [29], and similar to the case with untreated animals, the metabolite HAP could not be detected (Table 3). In addition to other substrates, adenine was studied by Lam *et al.* [18] with regard to a microsomal N-hydroxylation. The N-hydroxylation described here was not detected by these authors and this could be attributable to the absence of 3-MC or isosafrole pretreatment of the animals used by Lam *et al.* [18].

The cytochrome P-450 enzyme system is also able to reduce oxygen to H_2O_2 in the presence of NADPH [30]. The formation of H_2O_2 could proceed by way of superoxide radicals [31]. Other enzymes are also able to form active oxygen species in the presence of NADPH [32]. However, these active oxygen species do not play a part in this newly discovered N-hydroxylation of adenine since the addition of superoxide dismutase or catalase to the system did not have any effect on the N-hydroxylation of adenine (Table 4).

In order to exclude a possible participation of other enzymes of the liver preparations, incubations with adenine and xanthine oxidase, peroxidase, and catalase were also performed in the presence of H_2O_2 or H_2O_2-forming systems. All of those systems were unable to N-hydroxylate adenine (Table 5). A purely chemical transformation of adenine into 6-*N*-hydroxylaminopurine by H_2O_2 can also be discounted (Table 5).

The pH optimum determined (8.3, Fig. 5) is markedly higher than that of other cytochrome P-450 dependent N-oxygenations [33].

The question of the *in vivo* relevance of the microsomal N-hydroxylation of adenine to the mutagenic and carcinogenic compound 6-*N*-hydroxylaminopurine arises. The determinations of the apparent K_m (11.8 ± 1.2 mM), V_{max} (0.15 ± 0.05 nmol/mg protein/min) and V_{max}/K_m (0.13×10^{-7} l/min/mg) indicate that adenine is only a very poor substrate for the participating enzymes and that only very little adenine is transformed in the *in vitro* studies. However, the V_{max}/K_m values for reactions of unspecific cytochrome P-450 isoenzymes are generally very low [34, 35]. Furthermore, it must be taken into account that only about 0.5 g of uric acid is excreted daily by a human although up to 5 g of free purines are formed each day [36]. Thus, the larger proportion of the purines such as adenine is not degraded but rather retained or reused [37]. A comparison of the V_{max}/K_m value for adenine N-hydroxylation with the V_{max}/K_m values for competing reactions is important for the *in vivo* significance. Adenine phosphoribosyltransferase may be primarily responsible for keeping concentrations of free adenine very low in the mammalian tissues [37]. The V_{max}/K_m value for this enzyme is clearly higher [38] than the experimentally determined V_{max}/K_m value for the N-hydroxylation. In contrast to exogenous substances, however, adenine is continuously available as a substrate for the respective enzymes so that the transformation, albeit to a very small extent, can take place continuously. Hence, 6-*N*-hydroxylaminopurine could play a part in cancerogenesis.

In this process, the formation of HAP would of course be dependent on the amount of cytochrome P-450 isoenzymes belonging to the methylcholanthrene-inducible gene family [24–26] that are

present. It is known that corresponding forms of this gene family exist not only in the rat (isoenzymes c and d), in the mouse (isoenzymes P_1 and P_3), and in rabbits (isoenzymes LM6 and LM4) but also in humans, although significant differences in amounts occur in different individuals [24–26]. Thus, depending on the enzyme status (e.g. depending on smoking habits [25]), differing risk rates must be taken into consideration. One of the most powerful inducers of the discussed isoenzymes is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) even though the metabolites of this compound are not responsible for its toxicity [39]. On the basis of experimental results, it is assumed for TCDD that the compound itself is not carcinogenic but rather that it exerts tumor-promoting properties [40]. Could these not be due to an increased transformation of endogenous compounds such as adenine into genotoxic substances such as HAP as a result of the induction of the corresponding cytochrome P-450 isoenzymes?

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