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THE REDUCTION OF 6-*N*-HYDROXYLAMINOPURINE TO ADENINE BY XANTHINE OXIDASE

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Abstract—The genotoxic and mutagenic compound 6-*N*-hydroxylaminopurine (HAP) can be detoxified *in vitro* by enzymatic N-reduction to adenine. This reaction is catalysed by both rat and rabbit liver cytosolic fractions. The formation of adenine was monitored using HPLC. Subcellular distribution of the activity, kinetic parameters and the influence of various cofactors and inhibitors were determined. The N-reduction required NADH or hypoxanthine or xanthine and was strongly inhibited by allopurinol. These observations suggested that the *N*-reductase activity is due to xanthine oxidase (EC 1.2.3.2). Moreover, the involvement of xanthine oxidase is supported by the observation that purified cow milk xanthine oxidase also catalysed this reaction. The N-reduction of HAP was inhibited only weakly by oxygen. In addition, the formation of adenine is catalysed by either the oxidase or dehydrogenase form of xanthine oxidase. Thus, this reaction should be significant for the *in vivo* detoxification of HAP.

Reductive metabolic processes seem to have been investigated much less frequently as compared to the oxidative, hydrolytic and conjugating biotransformation routes [1]. Only a few comprehensive review articles dealing with the enzymatic reduction of nitrogen-containing functional groups can be found in the literature [2]. An important aspect of N-reductive processes is the "activation" of nitrogen-containing compounds to produce cytotoxic, mutagenic or antimicrobial metabolites [2]. In this context, the reductions of nitro and azo compounds are of particular importance [2]. The reductions of hydroxylamines [2], hydroxamic acids [3] and nitroso compounds [4], on the other hand, generally lead to an "inactivation" or a detoxification, respectively.

Our interest in N-reductions is based, among others, on the observation that the cytochrome P450 enzyme system is able to N-oxygenate the endogenous substance adenine **1** to the genotoxic and mutagenic compound 6-*N*-hydroxylaminopurine **2** (HAP†) [5] (Fig. 1). The question thus arises as to whether the organism possesses a protective mechanism against this reaction which may certainly be classified as a metabolic imbalance. The simplest possibility would be the retroreaction of HAP (**2**) to adenine (**1**) (Fig. 1).

A whole series of mammalian enzymes have already been described which are able to catalyse the reduction of nitrogen-containing functional groups [2, 6–9]. Many of these reductases, however, exhibit a low substrate specificity as well as a low specificity with regard to the functional group [2, 10, 11]. Moreover, the degree of *in vivo* significance of a reduction that has been demonstrated *in vitro* depends decisively upon its sensitivity to atmospheric oxygen [4]. Thus, N-reductions that are catalysed by xanthine oxidase [12], aldehyde oxidase [7], cytochrome P450 [10, 11] and cytochrome P450 reductases [2] are inhibited by atmospheric oxygen; in other words, the reduction can only be observed under anaerobic conditions.

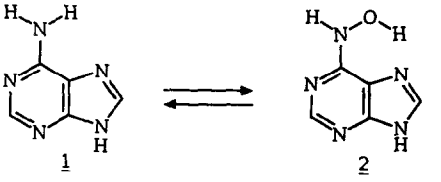

Fig. 1. N-oxygenation of adenine **1** versus N-reduction of HAP **2**.

Thus, the present investigation is concerned with the search for an enzymatic N-reduction of HAP (**2**) which could possibly also take place under physiological, aerobic conditions. For the estimation of the *in vivo* relevance of such an N-reduction, the participating enzymes need to be determined and, additionally, the reaction should be characterized as completely as possible.

MATERIALS AND METHODS

Reagents and biochemicals. Allopurinol, menadione, dicumarol and N^1-methylnicotinamide were purchased from the Sigma Chemical Co. (Deisenhofen, F.R.G.). NADPH (tetra sodium salt), NADH (disodium salt), xanthine, hypoxanthine, sodium dithionite and dithiothreitol were obtained from Merck AG (Darmstadt, F.R.G.), and metyrapone from EGA-Chemie (Steinheim, F.R.G.). 2-Diethylaminoethyl-2, 2-diphenylvaleratehydrochloride (SKF 525A) was kindly supplied by

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† Abbreviations: HAP, 6-*N*-hydroxylaminopurine; SKF-525A, 2-diethylaminoethyl-2,2-diphenylvaleratehydrochloride.

Professor K. Netter (Marburg, F.R.G.). Adenine was purchased from Janssen Chimica (Beerse, Belgium) and 6-chloropurine from the Aldrich Chemical Co. (Steinheim, F.R.G.). Xanthine oxidase (cow milk, 20 U/mL) was obtained from Boehringer Mannheim GmbH (Mannheim, F.R.G). All other chemicals and solvents (GR) were obtained from Merck.

Synthesis. HAP **2** was prepared with slight modifications from hydroxylamine and 6-chloropurine according to the procedure of Ginner-Sorolla and Bendich [13] and checked for purity by standard methods and HPLC.

Preparation of rat 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.). The homogenates were centrifuged at 9000 *g* for 30 min. The supernatant was carefully decanted and used as the enzyme source. 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 the 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/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.* [14].

Preparation of rabbit liver homogenates. The 9000 *g* and 100,000 *g* supernatant fractions and the washed microsomes of rabbit liver were prepared as described previously [15].

Ammonium sulfate precipitation. Ten millilitres of the 100,000 *g* supernatant cooled in an ice bath were treated with 5.0 g (0.038 mol) of ammonium sulfate and stirred carefully until a solution was obtained. The mixture was then centrifuged at 9000 *g* for 10 min and the pellet was suspended in 5 mL of the buffered KCl solution (1.15% + phosphate buffer 10 mM pH 7.4). The clear solution was dialysed three times for 4 hr each time against 500 mL of buffered KCl solution (1.15%, pH 7.4) at 4°. The cut-off limit of the dialysis tube was 1500 Da. The dialysed protein solution thus obtained was used as the enzyme source.

Converstion of liver xanthine oxidase and xanthine dehydrogenase. The 100,000 *g* supernatant was incubated for 30 min at 37° with dithiothreitol (10 mM final concentration) using micro test tubes 1.5 mL (Eppendorf, Hamburg, F.R.G.) to yield the dehydrogenase form [4]. To convert the xanthine dehydrogenase to the oxidase form, the 100,000 *g* supernatant was incubated aerobically for 30 min at 37° [4].

Aerobic incubations. Incubations were carried out in a shaking water bath at 37° in the presence of atmospheric oxygen using micro test tubes 1.5 mL. The standard incubation mixture (usually 0.6 mL) contained the following components: phosphate buffer (pH 7.4; 8.7 mM KH_2PO_4, 30.4 mM Na_2HPO_4), 400 μM HAP, 500 μM NADH and/or 250 μM xanthine and liver fraction corresponding to 0.3–0.9 mg protein/0.6 mL or milk xanthine oxidase (0.03 mg protein/0.6 mL). After preincubation at 37° for 1 min the reactions were started by addition of HAP. A 3–7 min incubation time was routinely employed. Incubations were terminated by adding 0.6 mL ice-cold methanol to the incubation mixture.

Aerobic incubations. Incubations were carried out as described above with the following modifications: all solutions were degassed at 0–4° under reduced pressure and treated with nitrogen. The solutions were pipetted under nitrogen into the micro test tubes which were then stoppered. Incubations were terminated by cooling the reaction vessels in an ice bath, followed by adding 0.6 mL ice-cold methanol to the incubation mixture.

HPLC. The terminated incubation mixtures were centrifuged at 6000 *g* for 5 min. Aliquots (5 or 10 μL) of the resulting supernatant were determined directly using HPLC (655 A-11, Merck-Hitachi, Darmstadt, F.R.G.) equipped with a variable wavelength UV monitor (655 A-22, Merck-Hitachi) set at 260 nm and an autosampler (WISP 170a, Waters Associates, Eschborn, F.R.G.). The areas under the peaks were integrated with a chromato-integrator (D-2000, Merck-Hitachi). Separation and quantification of adenine were performed at room temperature on a prepacked cationic exchange column (200 mm length × 4 mm i.d., partical size 5 μm; Nucleosil® 5 SA, Macherey-Nagel GmbH, Düren, F.R.G.). The mobile phase was methanol–ammonium phosphate buffer 0.15 M pH 3.0 (45:55, v/v). This mobile phase flowed through the column at a 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 or sonicated. Standard curves (peak area) at the levels of 1, 2, 4, 8, 14, 20, 30 and 50 μM adenine were constructed by introducing known amounts of adenine into the usual incubation mixtures (omission of substrate HAP); the mixtures were then incubated and treated in the same way as the experimental sample. Standard curves were linear over this range with correlation coefficients of 0.998 or greater. The levels of adenine in unknown incubation mixtures were determined directly from these standard curves which were run in parallel with the experimental samples. The recovery of adenine from incubation mixtures fortified with adenine was 96.1 ± 2.9% (N = 48) of that obtained using samples which contained the same amount of adenine dissolved in methanol–water (45:55, v/v). The detection limit of adenine in one incubation mixture was 0.25 μM which corresponds to a rate of N-reduction of 0.1 nmol adenine/min/mg protein. The retention times were 9.3 min for adenine and 6.3 min for HAP. The determination of HAP was performed with the

same method. Standard curves were linear over a range of 1–500 μM with correlation coefficients of 0.997 or greater. The recovery of HAP was 97.2 ± 3.8% (N = 48).

UV spectrum of the metabolite adenine. After incubation at 37° for 15 min the reaction mixture (usual concentrations, vol. 3 mL) was freeze-dried. Two millilitres of methanol were added to the resulting lyophilisate and centrifuged at 6000 *g* for 3 min. The clear methanolic solution was concentrated by evaporation under reduced pressure to a volume of approximately 100 μL. This solution was again centrifuged (6000 *g*, 3 min) and aliquots (15 μL) of the supernatant were analysed using HPLC (Waters 600 multisolvent delivery system, Waters Associates) linked to a diode array detector (Waters 990). The UV spectrum of the metabolite with a retention time of 9.3 min was identical to a spectrum of adenine recorded by the diode array detector under the same conditions.

Xanthine oxidase and dehydrogenase assays. Xanthine oxidase and dehydrogenase activities were determined spectrophotometrically by following the formation of uric acid without NAD or in the presence of added NAD as described previously [4].

RESULTS

Qualitative and quantitative HPLC analysis of adenine formed by enzymatic N-reduction of 6-N-*hydroxylaminopurine*

The qualitative and quantitative determination of adenine could be performed with slight modifications by the method developed previously [5]. In the course of biotransformation studies, it was possible for the first time to detect adenine which had been formed from HAP by the action of cytosolic enzymes under aerobic conditions.

Throughout the HPLC analyses and even with differing solvent systems, one signal always appeared with a retention time in agreement with that of a 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 identical conditions (UV maxima at 208 and 260 nm).

The HPLC method enabled the simultaneous quantification of adenine and the substrate HAP, so that the determination of the *N*-reductase activity could be controlled by either formation of adenine or disappearance of HAP. No differences between these determinations of the *N*-reductase activity could be observed, so that it is concluded that the reduction of HAP yields exclusively adenine.

Subcellular distribution of the N-*reductase activity, cofactor requirement and effect of inhibitors*

Of all the fractions of rat and rabbit liver homogenates tested, only the 9000 *g* and the 100,000 *g* supernatant (cytosol) showed a measurable activity, whereby the formation of adenine was detectable without addition of cofactors (Table 1). Only NADH and not NADPH increased significantly the *N*-reductase activity (Table 1).

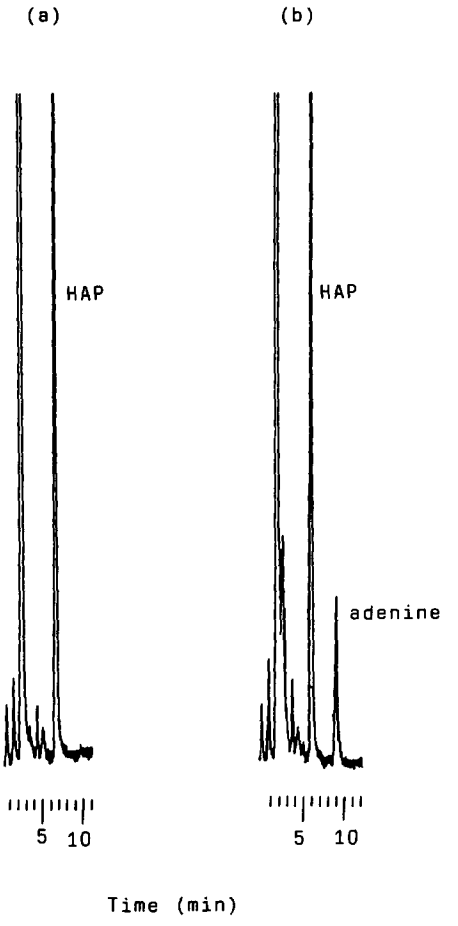

Fig. 2. Representative HPLC chromatogram of HAP metabolism by 100,000 *g* supernatant fractions of rat and rabbit liver homogenates. See Materials and Methods for details of reaction mixture content, incubation, sampling and analysis. (a) Omission of enzyme preparation; (b) complete system.

In order to be able to investigate the influence of cofactors specifically, the reasons for the pronounced activity without the addition of cofactors must first be determined.

In principle, two possibilities are available for discussion: either sufficient cofactors are present in the cytosolic fractions or HAP is not only reduced but also oxidized and can thus serve as an electron donor. Therefore, the 100,000 *g* supernatant was separated from components with low M_r by protein precipitation with ammonium sulfate [16] and subsequent dialysis. Formation of adenine would then be possible only when the HAP is concomitantly reduced and oxidized. The enzyme solution thus obtained is referred to hereafter as the "ammonium sulfate fraction".

It was found that the natural content of cofactors in the 100,000 *g* supernatant is indeed responsible for the activity observed in the control incubations (Table 2). The low residual activity (approx. 10% of the maximum activity) of the ammonium sulfate

Table 1. Subcellular distribution of the N-reduction of HAP and influence of NADPH and NADH

Species	Enzyme reaction	Cofactor	Adenine (nmol/min/mg protein)
Rat	9000 *g*	—	1.9 ± 0.34
	supernatant	NADPH	2.4 ± 0.15
		NADH	4.2 ± 0.21*
	100,000 *g*	—	3.5 ± 0.19
	supernatant	NADPH	3.5 ± 0.41
		NADH	4.9 ± 0.24*
	Microsomes	—	<0.1
		NADPH	<0.1
		NADH	<0.1
Rabbit	9000 *g*	—	0.7 ± 0.16
	supernatant	NADPH	0.7 ± 0.21
		NADH	1.4 ± 0.19*
	Microsomes	—	<0.1
		NADPH	<0.1
		NADH	<0.1

* Values are presented as the means of three determinations with the respective enzyme preparation (five rat livers or 10 rabbit livers were pooled) ± SD.
Reaction mixtures were composed as described in Materials and Methods.
* Statistically different from control (incubations without addition of cofactor), $P < 0.001$ (Student's *t*-test).

Table 2. Effects of cofactors on the N-reduction of HAP to adenine

Cofactor	Adenine (nmol/min/mg protein) 100,000 *g* supernatant	Ammonium sulfate fraction
—	2.6 ± 0.19	0.3 ± 0.03
Xanthine (250 μM)	2.5 ± 0.52	2.0 ± 0.06
Hypoxanthine (250 μM)	2.9 ± 0.35	2.1 ± 0.11
Sodium dithionite (170 μM)	<0.1	—
N^1-Methylnicotinamide (250 μM)	0.6 ± 0.12	0.2 ± 0.05
NADH (0.5 mM)	3.9 ± 0.34	1.3 ± 0.11
NADH (0.5 mM) + xanthine (250 μM)	6.19 ± 0.18	3.28 ± 0.19

Values are presented as the means of three determinations from one enzyme preparation (100,000 *g* supernatant and ammonium sulfate fraction from 10 pooled rat livers).
Reaction mixtures were composed as described in Materials and Methods.

fraction without the addition of cofactors can be explained in terms of the oxidation of HAP. The activity was increased in all enzyme preparations by the addition of xanthine or hypoxanthine. The maximum activity was exhibited by the combination of NADPH with xanthine (Table 2). Sodium dithionite and N^1-methylnicotinamide do not represent electron donors for the N-reduction (Table 2).

In order to obtain information about the participating enzymes, the effects of specific inhibitors on the N-reduction of HAP were investigated. In addition to the inhibitors of microsomal enzymes such as SKF 525A and metyrapone [17], menadione as an inhibitor of aldehyde oxidase [18], dicumarol as an inhibitor of DT diaphorase [19], and allopurinol as an inhibitor of xanthine oxidase [20] were also employed (Table 3). With the exception of allopurinol, none of the other inhibitors exerted any significant influence on the *N*-reductase activity.

For the investigation of the effect of oxygen on the rate of formation of adenine, purified xanthine oxidase (cow milk) and 100,000 *g* supernatants (rat liver) were employed as enzyme sources (Table 4). NADPH and xanthine served as the cofactors. The

Table 3. Effect of inhibitors on the N-reduction of HAP to adenine

Inhibitor	Final concn	Adenine (nmol/min/mg protein)
—		5.2 ± 0.24
Metyrapone	0.1 mM	5.4 ± 0.06
SKF 525A	5 mM	5.9 ± 0.45
Menadione	1 μM	5.5 ± 0.31
	100 μM	5.9 ± 0.38
Dicumarol	1 μM	4.9 ± 0.32
	100 μM	5.0 ± 0.63
Allopurinol	5 μM	0.8 ± 0.13*
	100 μM	<0.10*

Values are presented as the means of three determinations with one enzyme preparation (100,000 *g* supernatant; 10 livers were pooled).

Reaction mixtures were composed as described in Materials and Methods.

* Statistically different from control (complete incubation mixture, 500 μM NADH) with $P < 0.001$ (Student's *t*-test).

Table 4. Effect of oxygen on the N-reduction of HAP to adenine

	Aerobic incubation		Anaerobic incubation	
Cofactor	100,000 *g* supernatant	Cow milk xanthine oxidase	100,000 *g* supernatant	Cow milk xanthine oxidase
NADH (500 μM)	4.9 ± 0.24	46.4 ± 4.0	5.4 ± 0.23	59.7 ± 4.36
Xanthine (250 μM)	2.5 ± 0.20	56.3 ± 7.3	2.5 ± 0.17	86.1 ± 2.0
NADH (500 μM) + xanthine (250 μM)	6.2 ± 0.18	83.2 ± 2.8	7.1 ± 0.28	154.8 ± 2.8

N-reductase activity: nmol adenine/mg/mg protein.

Values are presented as the means of three determinations with one enzyme preparation each.

See Materials and Methods for details of reaction mixture content, incubation, sampling and analysis.

Table 5. Determination of rat liver xanthine oxidase or xanthine dehydrogenase activity according to Kutcher and McCalla [4], and *N*-reductase activity (formation rate of adenine)

	Pretreatment of the 100,000 *g* supernatants		
	Without pretreatment	+ Dithiothreitol	Aerobic incubation
Oxidase activity*	3.0 ± 0.39	1.0 ± 0.19	2.4 ± 0.18
Dehydrogenase activity*	<0.1	1.9 ± 0.24	<0.1
N-reductase activity†			
NADH (500 μM)	3.6 ± 0.21	5.2 ± 0.67	3.3 ± 0.31
Xanthine (250 μM)	2.4 ± 0.21	3.6 ± 0.40	1.9 ± 0.26
NADH (500 μM) + xanthine (250 μM)	5.4 ± 0.42	6.7 ± 1.10	5.7 ± 0.22

* nmol uric acid/min/mg protein; † nmol adenine/min/mg protein.

Values are presented as means of three determinations ± SD.

Pretreatment of the enzyme source was carried out as described in Materials and Methods.

relative inhibition by atmospheric oxygen amounted to between 0 and 13% in the incubations with 100,000 *g* supernatant and between 22.3 and 46% in those with cow milk xanthine oxidase.

Participation of xanthine oxidase or xanthine dehydrogenase in the N-reduction of 6-N-hydroxylaminopurine to adenine

The dehydrogenase form is considered to be the

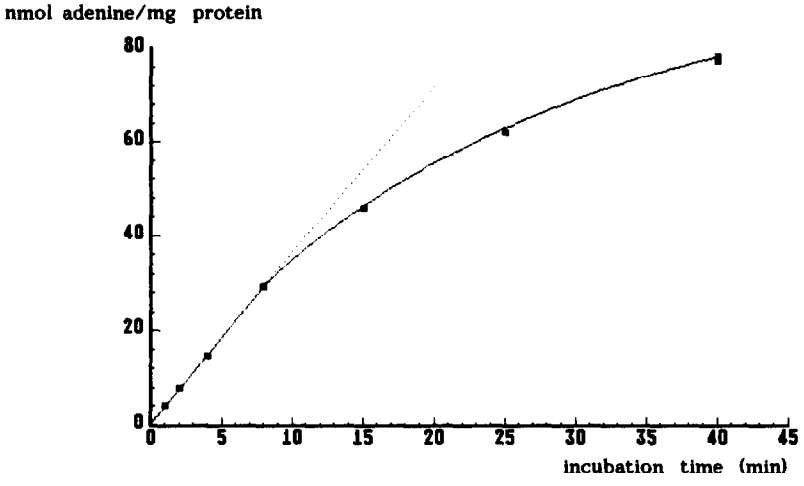

Fig. 3. Formation of adenine by 100,000 *g* supernatant fractions of rat liver homogenates as a function of time. Each point is the mean of three determinations. Similar results were obtained with 100,000 *g* supernatant fractions of rabbit liver homogenates. See Materials and Methods for details of reaction mixture content, incubation, sampling and analysis.

native form of xanthine oxidase [21, 22]. The conversion of xanthine dehydrogenase to the oxidase form can be achieved by oxidation of thiol groups of the enzyme (reversible) and by calcium-induced, proteolytic cleavage of the enzyme (irreversible) [22, 23]. Conversion of the oxidase form to the dehydrogenase form can be realized by incubation with dithiols. On the other hand, aerobic incubation of xanthine dehydrogenase results in its transformation to the oxidase form [22, 16].

Pretreatment of the 100,000 *g* supernatants of rat liver homogenates with dithiothreitol resulted in a ratio of dehydrogenase to oxidase activity of 1.9:1 (Table 5). Dehydrogenase activity cannot be detected after aerobic incubation of the enzyme source. This complete transformation of the native dehydrogenase form into the oxidase form can also be observed in the untreated 100,000 *g* supernatant (Table 5). Both the 100,000 *g* supernatant pretreated with dithiothreitol as well as that pretreated by aerobic incubation exhibit high *N*-reductase activities (Table 5). A comparison of the rates of formation of adenine revealed a 23–45% higher activity for the 100,000 *g* supernatant pretreated with dithiothreitol as compared to the enzyme source pretreated by aerobic incubation (Table 5).

Kinetics of the N-reduction of 6-N-*hydroxylaminopurine*

The reaction rate of the enzymatic formation of adenine was approximately linear over a period of 7 min at a substrate concentration of 200 μM (Fig. 3). This linearity was controlled by determination of the residual substrate HAP, which had to be more than 90%. An incubation time of 5 min was selected in order to obtain a sufficient amount of the metabolite adenine for its quantitative determination.

The N-reduction of HAP during incubation with 100,000 *g* supernatant of rat liver homogenates and NADH (500 μM) followed Michaelis–Menten kinetics. The apparent K_m value calculated from the Lineweaver–Burk plot (Fig. 4) was 0.42 ± 0.06 mM, while the apparent V_{max} value was found to be 21.3 ± 1.4 nmol adenine produced/min/mg of protein (values obtained with two different experiments, two different enzyme preparations, 5–10 livers pooled in each experiment).

Influence of pH value and buffer

When the 100,000 *g* supernatant from rat liver homogenates as well as the purified xanthine oxidase from cow milk was used, a pH optimum for phosphate buffer (50 mM) and one for Tris–HCl buffer (50 mM) were observed in each case (Table 6). This effect was also found in the case of anaerobic incubation (Table 6).

DISCUSSION

With the help of the newly developed HPLC analyses, it was possible to identify adenine as an *in vitro* metabolite of HAP. The enzymatic N-reduction of HAP was localized in the 100,000 *g* supernatant of rat and rabbit liver homogenates (Table 1).

Worthy of particular note are, above all, the following four cytosolic liver enzymes which reduce nitrogen-containing functional groups in a relatively unspecific manner [2]: the flavoenzyme DT diaphorase (EC 1.6.99.2), the molybdenum-containing flavoenzymes xanthine oxidase (EC 1.2.3.2) and aldehyde oxidase (EC 1.2.3.1), as well as the zinc-containing alcohol dehydrogenase (EC 1.1.1.1) [2].

Of the reduced dinucleotides NADH and NADPH employed, only NADH effected an increase in the reduction rate of the N-reduction of HAP. Of the additionally tested cofactors, only xanthine and hypoxanthine brought about an increase in the activity (Table 2). These cofactors are substrates of

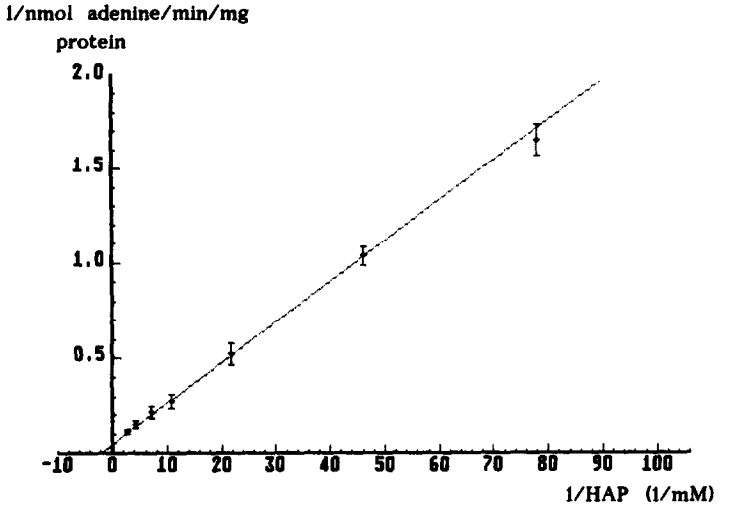

Fig. 4. Lineweaver–Burk plot of the N-reduction of HAP measured by adenine formation in the incubation mixture. Each point is the mean of six determinations ± SD (two different enzyme preparations, three determinations each). Aerobic incubations were carried out as described in Materials and Methods.

Table 6. Effect of pH and buffer on the N-reduction of HAP

Enzyme source	pH optima	
	Phosphate buffer	Tris–HCl buffer
Cow milk xanthine oxidase (aerobic)	7.2	7.8
100,000 *g* supernatant (aerobic)	6.9	7.9
100,000 *g* supernatant (anaerobic)	7.7	8.3

Incubations were carried out as described in Materials and Methods. Cofactor was NADH (500 μM).

xanthine oxidase and serve as electron donors for the N-reductions of nitro compounds [4, 12] and of purine *N*-oxides [24], for example.

N^1-Methylnicotinamide, as a typical cosubstrate of N-reductions of aldehyde oxidase [3, 7], did not cause a rate increase in the reduction of HAP. The relatively high rate of formation of adenine without the addition of cofactors to the 100,000 *g* supernatants used as enzyme source (Table 1) is reduced to $\frac{1}{10}$ of the original value by removal of the components with low M_r (Table 2). The remaining residual activity can be explained in terms of an oxidation of HAP. With regard to the N-reduction of adenine-1-*N*-oxide catalysed by xanthine oxidase, an analogous oxidation of this purine *N*-oxide has been described [24].

These results are indicative of participation of xanthine oxidase in the N-reduction of HAP. For further substantiation of this assumption, the effects of inhibitors of cytosolic and microsomal enzymes were investigated. The specific inhibitors menadione (aldehyde oxidase [18, 25]) and dicumarol (DT diaphorase [19]), as well as metyrapone and SKF 525A (cytochrome P450 [17]) did not have any effect on the rate of N-reduction (Table 3). On the other hand, the addition of allopurinol to the incubation mixtures brought about a highly significant inhibition (Table 3). Allopurinol selectively inhibits oxidations [20, 26] and reductions [4, 27] catalysed by xanthine oxidase. Furthermore, the N-reduction of HAP is catalysed by purified cow milk xanthine oxidase. In summary, it can be assumed that the sole participation of liver xanthine oxidase in the N-reduction of HAP to adenine is very probable. Catalysis by other enzymes appears to occur to a very low extent (<0.1 nmol/min/mg protein), if at all.

Reductions of nitrogen-containing functional groups under catalysis by xanthine oxidase are frequently carried out under anaerobic conditions since atmospheric oxygen is known to be a powerful inhibitor of these reactions [12, 24, 28]. Kutcher and McCalla [4] showed for the first time that the reduction of nitro compounds by xanthine dehydrogenase from rat liver was inhibited by oxygen to a considerably lesser degree than the reduction catalysed by xanthine oxidase.

In contrast to the previously described N-reductions, the formation of adenine from HAP, which is catalysed by xanthine oxidase both from rat liver and from cow milk, is only inhibited to a small degree by atmospheric oxygen (Table 4). The transformation of the oxidase form to the dehydrogenase form also has only a slight influence on the enzymatic activity (Table 5).

Kinetic studies with 100,000 *g* supernatants of rat liver homogenates have shown that HAP is a very good substrate for xanthine oxidase. The K_m value (0.42 ± 0.06 mM), the maximum rate ($V_{max} = 21.3 \pm 1.4$ nmol/min/mg protein) and the ratio $V_{max}:K_m$ (5.07×10^{-5} L/min/mg protein) are comparable with those of physiological substrates of xanthine oxidase [25, 29]. However, N-reductions of nitro compounds exhibiting similar conversion rates have also been reported [4, 26]. The pH value and the buffer used influenced both the 100,000 *g* supernatant and the purified xanthine oxidase from cow milk: each showed a pH optimum for phosphate buffer and one for Tris–HCl buffer. One reason for this observation could be the differing degrees of ionization of the buffers at the same pH value. The pH optima are in the range of pH 6.9–8.3 and are thus comparable with those of other N-reductions catalysed by xanthine oxidase [6, 24].

The N-reduction of the genotoxic and mutagenic compound HAP by xanthine oxidase from liver and cow milk described in this work unequivocally represents a detoxification reaction. Thus, an *in vivo* relevance is highly probable because of the high activity measured *in vitro* and the fact that the influence of atmospheric oxygen is very low. This should also be valid for xanthine oxidase in the human liver since the substrate specificities in comparison to those of xanthine oxidase from cow milk or rat liver are very similar [16].

The starting point for the present investigations was the question of the extent to which the N-oxygenation of adenine to HAP by cytochrome P450 is involved in carcinogenesis. This reaction was observed after incubation of adenine at high concentrations with microsomes from rats which had been pretreated with 3-methylcholanthrene [5]. A comparison of the $V_{max}:K_m$ value of the N-oxygenation of adenine to HAP with that of the retro-reaction reveals that the value for the N-reduction is four orders of magnitude larger than that for the N-oxygenation. Thus, any HAP formed metabolically in the liver should be immediately reduced again as long as the xanthine oxidase possesses sufficient activity.

Very little information has been reported to date about the influence of inducers and other exogenous factors on the content of xanthine oxidase [27, 29]. The inhibition of xanthine oxidase by allopurinol should have a considerably larger influence on the detoxification of HAP [29]. Since allopurinol has found a wide application as a uricostatic agent [30], this substance could inhibit the reduction of any HAP formed and thus constitute a hazard for the patient.

On surveying the literature on the carcinogenic and mutagenic actions of HAP, it becomes apparent that a possible reduction of HAP has not been taken into consideration. Thus, McCartney *et al.* [31] employed the modified Ames test (addition of 9000 *g* supernatants of rat liver homogenates) [32] for an investigation of the mutagenicity of HAP. The use of 9000 *g* supernatants should drastically reduce the concentration of HAP even under aerobic conditions since the N-reduction by xanthine oxidase can occur under these conditions. In comparison to the results of other authors [33–36], who used the test systems without the addition of 9000 *g* supernatants, the mutagenic activity observed by McCartney *et al.* [31] was markedly less pronounced; this further substantiates the hypothesis presented above. The N-reduction may also be the reason for the relatively low oncological action of HAP, as has been observed in *in vivo* investigations in the rat [37].

In conclusion, reductive processes must, as a general rule, be taken more frequently into consideration since both a detoxification, as presented in this paper, as well as a toxification such as, for example, the reduction of nitro compounds [4] can take place.

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