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MINIREVIEW

PATHWAYS OF METABOLISM OF AMPHETAMINE AND RELATED COMPOUNDS

Arthur K. Cho and Jeremy Wright

Department of Pharmacology, School of Medicine
Center for the Health Sciences, University of California
Los Angeles, California 90024

The metabolism of compounds related to amphetamine has been extensively examined because of experimental and clinical interest in the compounds (1,2) and the possible involvement of metabolites in their pharmacology. However, the metabolic pathways for these compounds may be of more general interest because amphetamine has structural elements common to many other classes of drugs. This review will describe studies investigating the oxidative pathways of amphetamine and phentermine metabolism with primary focus on in vitro studies. In vivo data is reported where relevant to relate the in vitro data to whole animal work.

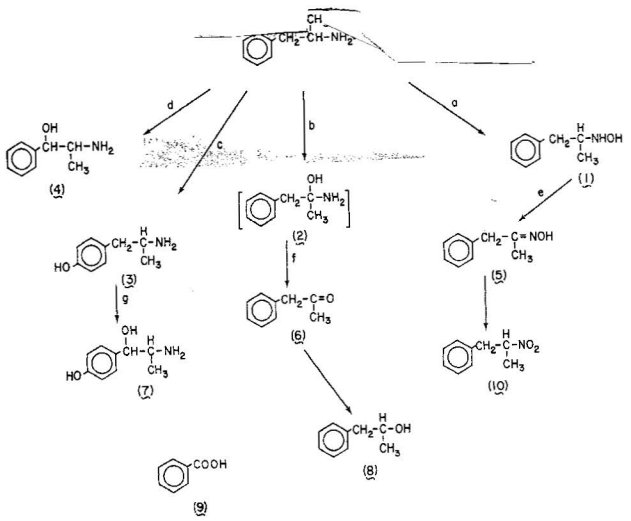
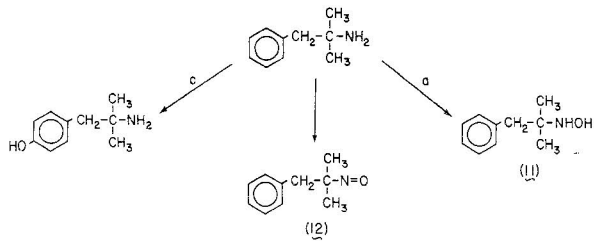


FIG. 1b



The metabolic pathways of amphetamine that have been reported are summarized in Figure 1a. The primary pathways of metabolism result from hydroxylation of (a) the nitrogen, (b) the α-carbon, (c) the aromatic 4-carbon and (d) the β-carbon. These metabolites then undergo further metabolism to form the secondary metabolites, phenylacetone oxime (5), phenylacetone (6), p-hydroxynorephedrine (7), 1-phenyl-2-propanol (8) and benzoic acid (9). The enzymes linking the primary and secondary metabolites have not all been demonstrated. Phentermine (Figure 1b) lacks the α-hydrogen so that pathways b and e are not possible and the secondary metabolic pathways differ. Conjugation reactions will not be discussed here.

Pathway (a) N-Hydroxylation

N-Hydroxyphentermine (11) has been found as an *in vivo* metabolite (6), but N-hydroxyamphetamine (1) has not. The N-hydroxylation reaction was demonstrated in liver preparations from rabbit with amphetamine and phentermine as substrates (7,8,9,10), but the reaction in rat liver was demonstrated only with phentermine (12). Data collected in studies with rabbit liver microsomes using phentermine as substrate indicate that the reaction is mediated by an oxidase which is sensitive to CO and SKF525A and which is NADPH dependent (7). The kinetics of the reaction in rabbit liver preparations are complex and do not give linear reciprocal plots (7). Part of this may be due to catalysis by hemoglobin (12).

In a recent report, Beckett and Belanger (8) described the oxidation of phentermine to the hydroxylamine and the nitroso compound in rabbit liver preparations. They found that the hydroxylamine was stable under incubation conditions that catalyzed the formation of the nitroso metabolite and concluded that the latter is not formed from the hydroxylamine but arises by a separate path. In these studies, hydroxylamine formation increased in preparations from phenobarbital pretreated rabbits, while nitroso compound formation decreased. The insensitivity of the N-hydroxylation reaction to cyanide led these workers to propose catalysis by a flavin dependent system rather than a P_{450} dependent oxidase. A flavin dependent, P_{450} independent N-oxidase has been isolated from pig liver which oxidizes a number of tertiary, secondary, as well as primary amines (13), but not amphetamine (14). However, cyanide is not a useful reagent for testing P_{450} involvement in a reaction because it is a selective inhibitor of the P_{450} system. For exam-

ple, Comai and Gaylor (15) have reported three forms of P_{450} characterized by widely differing affinities for cyanide (k = 0.5 mM to 5.0 mM). Curiously, in two studies quoted by Beckett and Belanger to support their use of cyanide, it was described as being a poor inhibitor (10% inhibition at 1 mM) of aromatic ring hydroxylation in one (16) and was not used in the other (17). Cyanide does inhibit one component of a P_{450} dependent demethylase system with N,N-dimethylamphetamine as the substrate (18).

Phentermine N-hydroxylation activity is also present in guinea pig and rat liver preparations, but levels are about 1/2 those for the rabbit (7). In the rat, it is induced with phenobarbital and not 3-methylcholanthrene and is inhibited by SKF525A and DPEA (12). As reported for the rabbit, part of the N-hydroxylation activity in rat liver microsome preparations is lost in washing. This loss in activity was noted only for N-hydroxylation and not for dimethylaniline demethylation and aniline-4-hydroxylation which were examined for comparison (12). Preliminary evidence indicates that this washable factor is hemoglobin present in low levels in the microsomal preparation (12). Hemoglobin has been demonstrated to effect other P_{450}-type oxidations, but high levels are required (19) in contrast to the results obtained for N-oxidation.

Amphetamine exhibits substantial species difference with respect to N-hydroxylation. Rabbit liver preparations readily N-hydroxylate amphetamine (9,10,11), but the metabolite could not be demonstrated in rat liver preparations (Sum, unpublished). The α-hydrogen in amphetamine makes analysis of its metabolism more complex than phentermine, since N-hydroxyamphetamine can be converted to the oxime (5) chemically and enzymatically. N-Hydroxyamphetamine decomposes in aqueous solution (20,9) and under certain gas chromatographic conditions (11). This instability has led to some confusion in the literature regarding the identification of N-hydroxyamphetamine and phenylacetone oxime as metabolites of amphetamine (11). The majority of the evidence indicates that both compounds are metabolites with the N-hydroxy compound being the major one. R(-) Amphetamine appears to have a lower affinity for the N-hydroxylation system but has a higher rate than the S(+) isomer so that when incubated separately the R(-) isomer forms the hydroxylamine faster than the S(+). When a racemic mixture is incubated, the S(+) isomer with the higher affinity is preferentially metabolized but at a slower overall rate than the R(-) alone (21).

The oxidation of nitrogen may be involved in other interactions of amphetamine and microsomes. Amphetamine forms a complex with rat and rabbit liver microsomes that is characterized by an absorption maximum at 455 nmeters (22) and which is time and substrate dependent. The complex is also O_2/NADPH dependent, SKF525A inhibited and inducible with phenobarbital (23). It is formed more rapidly and in greater amounts when N-hydroxyamphetamine is used as the ligand instead of amphetamine. These observations suggest that some metabolic reaction is occurring in the formation of the complex and that N-oxidation is involved. Ligand specificity is also evident and N-methylamphetamine forms the complex (22), but phentermine (22,24) does not. The chemical basis for the interaction has been proposed to involve the oxidation of the nitrogen (24,25) and Mansay et al. (25) suggest that the nitroso derivative is the chemical species.

N-Hydroxyphentermine (11) can be oxidized chemically to the nitroso compound (12) (20), but the nitroso compound also forms enzymatically in rabbit liver preparations (8). The hydroxylamines are also reduced *in vivo*, and N-hydroxyphentermine (26) and N-hydroxy-p-chloroamphetamine (27) were rapidly reduced to the parent amine after injection. Reduction of N-hy-

droxyphentermine also occurs in microsomes from several species (28,8) and properties of the enzyme system have been described for the rat (28). The reductase activity in rat liver microsomes is inducible with phenobarbital and sensitive to SKF525A, carbon monoxide, as well as oxygen. These observations suggest that P_{450} may be responsible for the reduction, and if it is, it would indicate that P_{450} effects both oxidative and reductive reactions depending on the availability of oxygen. Thus, the rapid *in vivo* reduction of the hydroxylamines described above may reflect the oxygen tension in the liver cell.

Phenylacetone oxime (5) is metabolized in rabbit liver preparations to 2-nitrophenylpropane (10) (10,29) while with rat liver preparations phenylacetone and benzylalcohol were isolated. The oxime appears to be chemically stable in the absence of NADPH, but under vigorous conditions such as strong acid it can be hydrolyzed to the ketone. The oxime was proposed by Hucker to be the major *in vitro* metabolite of amphetamine and has been isolated by several different groups (30,10,11). However, it is not a major metabolite at the early time points, and previous work describing its formation (30) is difficult to interpret because of the ready decomposition of the hydroxylamine to the oxime during its direct gas chromatography. The hydroxylamine is gas chromatographically stable when converted to its trifluoroacetamide (11).

Pathway (b) α-Carbon Hydroxylation

This pathway was proposed by Brodie et al. (31) to account for the formation of phenylacetone in rabbit liver microsome preparations (32). The reaction, called deamination by Axelrod, is equivalent to other N-dealkylation reactions in that a carbonyl compound and an amine are the products. Phenylacetone and its metabolites, phenyl-2-propanol (8) and benzoic acid (9) have also been found *in vivo* as metabolites of amphetamine in rabbits (33).

In his *in vitro* studies, Axelrod (32) identified and estimated the phenylacetone formed as its 2,4-dinitrophenylhydrazone. When he measured both the phenylacetone formed and amphetamine consumed the phenylacetone represented only about 50% of the total amphetamine metabolized and most of this could be accounted for by the subsequent metabolism of phenylacetone to a then unidentified metabolite. More recent experiments in this laboratory indicate that phenylacetone is reduced to phenylisopropanol by a highly active NADPH dependent reductase (11) which, although soluble, has enough residual activity in washed microsomes to reduce substantial quantities of phenylacetone (unpublished observations). N-Hydroxyamphetamine and phenylacetone oxime were not noted by Axelrod, and the reason for this may be the vigorous (1.0 N NaOH, 2.0 N HCl) extraction conditions used. Most of his experiments were conducted with rabbit liver preparations, and he found very little activity in microsomes from dog, guinea pig or rat. Rat liver microsomes inhibited amphetamine metabolism by rabbit tissue in proportion to the quantity added, suggesting the presence of an inhibitor. This inhibition was eliminated by heating in boiling water and the boiled preparation caused an increase in deamination when added to rabbit microsomes. This inhibition by rat liver has been noted for other microsomal oxidations (16).

The stereochemical nature of the deamination reaction is similar to that for N-hydroxylation and although the R(-) isomer is more extensively metabolized during incubations of one or more hours, the S(+) isomer appears to control metabolism when the racemic mixture is presented to the enzyme (21).

In studies with amphetamine-α-^{2}H, in which the α-hydrogen was substituted with deuterium, a substantial isotope effect was observed for ketone and oxime formation, but N-hydroxylation exhibited a reverse effect, i.e. a higher rate with deuterium substitution (10). A similar increase with deuterium enrichment was noted in P_{450} complex formation (24).

N-Hydroxylation v.s. C-Hydroxylation as the Pathway for Phenylacetone Formation

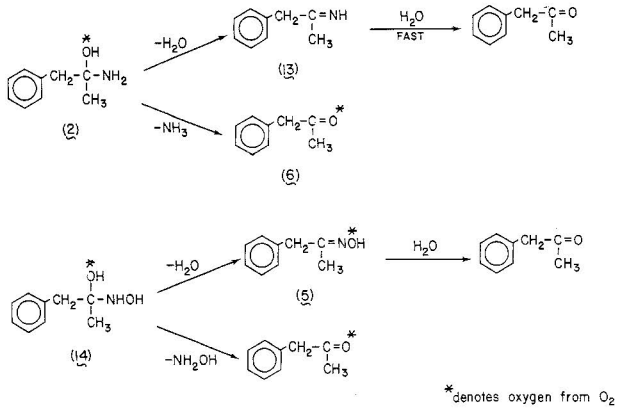
There has been considerable discussion in the literature regarding the role of Pathway (a) v.s. Pathway (b) as the mechanism for ketone formation (9,34,35) from amphetamine (Figure 1a). In Pathway (a) the hydroxylamine is dehydrogenated to oxime which can be hydrolyzed chemically and enzymatically to ketone. Pathway (b) was proposed by Brodie et al. as a general mechanism for all dealkylations, but the carbinol amine (2) has never been isolated. Beckett and Al-Sarraj (9) considered the conversion of hydroxylamine (1) to oxime (5) to be a chemical reaction, but the experiments involved were done at high pH for long periods of time and not under biochemical incubation conditions. Additional support for the hydroxylamine pathway comes from the work of Hucker and co-workers (30) who isolated the oxime as the major metabolite from rabbit liver preparations. They also reported experiments which indicated that the oxime was quantitatively and enzymatically converted to phenylacetone. In studies with $^{18}O_2$ Parli et al. (35) examined the source of oxygen in phenylacetone. As shown in Figure 2, the ketone formed from the carbinol amine (2) by elimination of NH_3 should contain only ^{18}O while that formed by hydrolysis of the oxime (5) should contain ^{16}O from the water. Their results showed that the oxime was 95% ^{18}O enriched while the ketone was only ∿30% ^{18}O enriched suggesting that the majority of the phenylacetone arose from a hydrolytic pathway. As indicated above, these workers also reported a substantial deuterium isotope effect for both ketone and oxime formation (10) while the formation of hydroxylamine was increased with α-carbon deuterium substitution. These latter observations would argue against the N-hydroxylation pathway. In incubations of amphetamine with rabbit liver preparations, Wright et al. (11) found that ketone or its equivalent, the alcohol, was formed more rapidly than the oxime. They also found that N-hydroxyamphetamine itself is oxidized to the oxime as the major metabolite with ketone forming much more slowly. In subsequent isotope dilution experiments in which deuterium labelled amphetamine (α-methyl-$^{2}H_3$) was incubated with N-hydroxyamphetamine, little isotopic mixing in deamination product occurred, indicating that the ketone is formed essentially independently from hydroxylamine (36).

Thus, while there is evidence to suggest that a hydrolytic step such as oxime hydrolysis is involved in ketone formation, the isotope effects of the reaction and isotope dilution studies suggest that most of the ketone arises from a pathway distinct from N-hydroxylation. One possible explanation could be the involvement of α-hydroxylation in both oxime and ketone formation. This is shown by Figure 2 which compares the potential reactions of α-hydroxylated amphetamine (2) and α-hydroxylated N-hydroxyamphetamine (14). If both amphetamine and N-hydroxyamphetamine are α-hydroxylated, the resulting carbinol amines could decompose by loss of water or amine. When amphetamine is the substrate ketone or immine (13) is the product, and when N-hydroxyamphetamine is the substrate ketone or oxime is the product.

2-Imino-1-phenylpropane (13) which is the dehydration product of amphetamine carbinol amine would be immediately hydrolyzed to the ketone. On the other hand, the carbinol amine of N-hydroxyamphetamine would lose water to

form the oxime which is more stable than the immine. The $^{18}O_2$ data of Parli et al. (35) indicated that ketone is formed by hydrolysis rather than by aminolysis. The oxygen of the oxime results from the atmosphere via the N-hydroxylation reaction and therefore has high enrichment.

FIG. 2



Pathway (c) 4-Hydroxylation

p-Hydroxyamphetamine (3) and its glucuronide conjugate have been identified as urinary metabolites of amphetamine in a number of different species including man, dog, rabbit, rat and mouse but not guinea pig (33). Of all the species examined, the rat appears to be the most active in forming this metabolite (33) and 50-60% of the administered amphetamine dose is excreted as p-hydroxyamphetamine. Similar data were found for phentermine in this species (37).

p-Hydroxyamphetamine is a tyramine-like indirectly acting sympathomimetic amine (38) and thus contributes to the pharmacology of the parent drug. Extensive in vivo studies on its formation and subsequent β-hydroxylation to p-hydroxynorepinephrine (7) have been conducted (39,4,5,40,41) as a possible explanation for the tolerance as well as the catecholamine depleting actions of amphetamine. The in vivo formation of p-hydroxyamphetamine is inhibited by a number of P_{450}-mixed function oxidase inhibitors or substrates including SKF525A (40), iprindol (41) and desmethylimipramine (DMI) (42). Conversion is increased in phenobarbital pretreated animals but only after prolonged treatment (43,44).

Early attempts to investigate this reaction in vitro met with limited success (45,46) because of the low yields of product (<0.5%) obtained. However, more recent studies indicate that substrate inhibition occurs and substrate levels less than 0.5 mM need to be used (47,48). The reaction has been studied at these concentrations with hepatocytes and perfused liver (49, 50) as well as microsomes. These experiments demonstrated inhibition by

SKF525A, DPEA and iprindol (50), DMI, fenfluramine and CO (49) and indicated that hydroxylase activity was not inducible with phenobarbital pretreatment for either 3 or 5 days. Similarly, phentermine 4-hydroxylation activity was not inducible with phenobarbital or 3-methylcholanthrene and was inhibited by SKF525A and iprindol at μM concentrations (51).

Pathway (d) β-Hydroxylation

(±) Amphetamine (52) and S(+)-p-hydroxyamphetamine (53) are β-hydroxylated in vivo to form norephedrine and p-hydroxynorephedrine. Dopamine-β-hydroxylase (DBH), one of the norepinephrine biosynthetic enzymes, has been shown to effect this transformation with S(+)-p-hydroxyamphetamine but not the R(-) enantiomer (54) and this stereospecificity is consistant with in vivo observations. The p-hydroxynorephedrine is of considerable pharmacological interest as it has the properties of a "false transmitter" (55) and has been implicated in the chronic effects of amphetamine (3,4). Little has been published on the enzymatic source of norephedrine. In preliminary experiments in this laboratory, we have found that p-hydroxyphentermine is not β-hydroxylated by DBH, which might be expected if one considers that phentermine has an α-methyl group in both S and R configurations.

One metabolite that deserves more attention is benzoic acid. Phenylacetone or its equivalent is thought to be the precursor, but such compounds are not excreted by the rat or mouse (31) which do excrete benzoic acid as an amphetamine metabolite. One possible pathway is through norephedrine, since ephedrine is oxidized readily to benzoic acid by several species including rat and mouse (56).

Conclusions

The metabolism of amphetamine involves reactions common to other drug substrates, but the enzyme systems involved appear to have different properties. For example, while both amphetamine and aniline are ring hydroxylated by a carbon monoxide sensitive system, only aniline hydroxylase is inducible. The ring hydroxylation of imipramine (57) is also not increased in liver preparations from phenobarbital treated animals (54) so that this difference is not unique to phenylethylamine derivatives. The N-hydroxylation reaction also exhibits different induction properties; phenobarbital pretreatment increases microsomal N-hydroxylation of phentermine but not phenacetin (12). If this selective induction reflects different enzymes or enzyme subunits, then the same reaction is being catalyzed by different units and the so-called nonspecific mixed function oxidase does indeed display substantial substrate selectivity.

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