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METABOLISM OF 2-NITRO-1-PHENYLPROPANE BY RABBIT LIVER MICROSOMES

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Abstract—The *in vitro* metabolism of 2-nitro-1-phenylpropane by rabbit liver microsomes was examined. The metabolites, identified by gas chromatography–mass spectrometry, were benzyl alcohol, benzoic acid, phenylacetone, 1-phenyl-2-propanon-1-ol, and 1-phenyl-1,2-propanediol. The mass spectra of these compounds are discussed in terms of the major diagnostic peaks. The levels of the metabolites formed were determined by quantitative gas chromatography, and the major metabolites were phenylacetone and 1-phenyl-2-propanon-1-ol. The formation of these two compounds appears to be P-450 dependent since the reactions are sensitive to CO and 2,4-dichloro-6-phenylphenolxethylamine (DPEA) and inducible by phenobarbital pretreatment of the rabbits.

Although Scott [1] reported the *in vivo* conversion of an aliphatic nitro compound to a carbonyl derivative in rabbits in 1942, it is only recently that interest in the conversion of nitro alkanes to ketone compounds has intensified.

Kido *et al.* have reported the conversion of 2-nitropropane to acetone by oxygenases isolated from both fungus [2] and yeast [3] cell-free extracts. Recently, Sakurai *et al.* [4] reported the results of the oxidation of several aliphatic nitro compounds by rat hepatic microsomes and indicated that most of the substrates examined formed a cytochrome P-450 complex as the —NO derivative, with the corresponding release of the appropriate aldehyde or ketone that would result from the oxidative cleavage of the C—N bond.

The metabolism of *R*(−)-amphetamine by rabbit liver preparations yields approximately equal amounts of phenylacetone (VII) [5] (or its primary metabolite, 1-phenyl-2-propanol (X) [6]), and *N*-hydroxyamphetamine (II) [7], but the corresponding oxime (III) [8–10] and the nitro metabolite (VIII) have been found as well† (see Fig. 1). Incubation of phenylacetone oxime (III) also produces VIII with either rat [11] or rabbit [12] liver homogenates. Incubation of the nitroso derivative (not shown) [13] or the hydroxylamine metabolite (II) [14] in rabbit liver homogenates also produces VIII. In addition, a nitro derivative has been found as a metabolite of *N*-hydroxyphentermine *in vitro* [15] and as a metabolite of *p*-chlorophentermine *in vivo* [16].

We have demonstrated recently that the hydroxylamine (II) is a precursor of the nitro metabolite (VIII) without prior conversion to the oxime (III) [17], and that this same hydroxylamine is converted to phenylacetone (VII) via VIII. A preliminary report on the metabolism of VIII revealed that phenylacetone (VII) is the major metabolite formed

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† R. C. Kammerer *et al.*, unpublished observations.

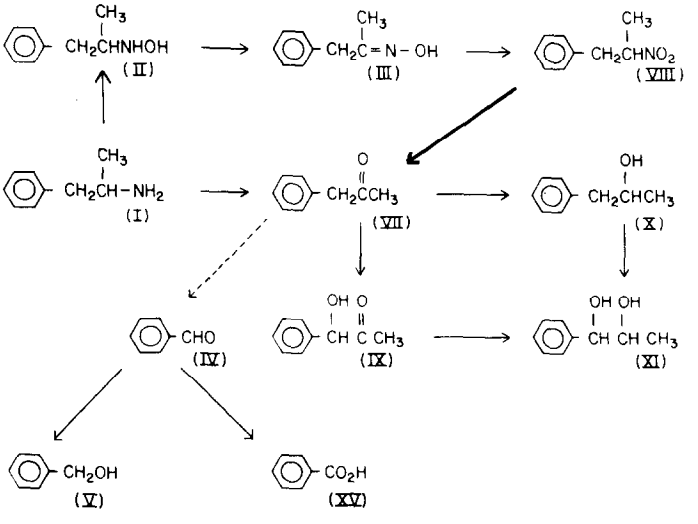

Fig. 1. Metabolic pathways of amphetamine.

from VIII when incubated with rabbit liver microsomes [18]. This study reports additional evidence that the enzymatic denitrification of VIII is cytochrome P-450 mediated and provides identification of other metabolic products of this biotransformation reaction. A preliminary report of this work has appeared [19].

MATERIALS AND METHODS

Reagents and animals. White New Zealand male rabbits (2.0–2.5 kg) were purchased from the Curd Corp. (Los Angeles, CA). J. T. Baker & Co. (Phillipsburg, NJ) was the source of reagent grade potassium chloride, sodium azide, magnesium chloride, potassium phosphate, sodium cyanide, and benzoic acid. Glucose-6-phosphate dehydrogenase, NADP, glucose-6-phosphate monosodium salt, and catalase were purchased from the Sigma Chemical Co. (St. Louis, MO). Trifluoroacetic anhydride, nitroethane, benzaldehyde, sodium borohydride, phenylacetone, 1-phenyl-2-butanone, benzyl alcohol, and phenylacetic acid were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Diethylether and methylene chloride were purchased from the Burdick & Jackson Co. (Muskegon, MI). Sodium phenobarbital was obtained from the UCLA Pharmacy, School of Medicine (Los Angeles, CA). *N*-Nitrosomethyl urea, used in generating diazomethane, was from ICN (K & K Labs Division) (Plainview, NY). $H_2^{18}O$ was donated by the Los Alamos Scientific Laboratory (Santa Fe, NM). 2,4-Dichloro-6-phenylphenoxyethylamine (DPEA) was a gift from Dr. R. E. McMahon of Lilly Research Laboratories (Indianapolis, IN). Carbon monoxide was purchased from Matheson Gas Products (East Rutherford, NJ) and oxygen was procured from Ohio Medical Products (Madison, WI).

Syntheses. 2-Nitro-1-phenylpropane (VIII) was prepared by condensing benzaldehyde and nitroethane according to the method of Gairaud and Lappin [20]. The resulting 2-nitro-1-phenyl-1-propene was reduced with sodium borohydride according to Shechter *et al* [21] to yield the desired propane, which was then fractionally distilled. The product gave a single peak on the gas chromatograph and a consistent mass spectrum. The syntheses of erythro and threo 1-phenyl-1,2-propanediol (XI) and 1-phenyl-2-propanon-1-ol (IX) have already been reported [6].

Microsomal preparation and incubation. Rabbits were stunned by a blow on the head and exsanguinated. The livers were removed and homogenized in 1.15% KCl solution (10 g liver in 30 ml) with a Teflon homogenizer in a glass mortar and centrifuged at 9000 *g* for 20 min. The supernatant fluid was poured into another centrifuge tube and centrifuged at 100,000 *g* for 60 min. The supernatant fraction was discarded, an equal amount of fresh 1.15% KCl was added, and the microsomal pellet was resuspended by means of a Teflon mortar and spun at 100,000 *g* for 30 min to form a washed microsomal pellet. This pellet was then resuspended via light homogenization in cold fresh 1.15% KCl to prepare the microsomal suspension for incubations.

Microsomes were also prepared by homogenizing the liver in sucrose (0.32 M) and filtering the 9000 *g* supernatant fluid on Sepharose 2B as described by Tangen *et al* [22]. Microsomal incubations were carried out in a 25-ml Erlenmeyer flask placed in a Dubnoff metabolic incubator (37°) with 1 ml of microsomal suspension (3-7 mg/ml), 1 ml of 0.6 M potassium phosphate buffer (pH 7.4), substrate as an aqueous solution, and an NADPH-generating system containing 12 mM glucose-6-phosphate sodium salt, glucose-6-phosphate dehydrogenase (8 units), $NADP^+$ (0.5 mM), and $MgCl_2$ (2.4 mM), in a final volume of 5 ml. No metabolic differences were found in the microsomes prepared by gel filtration when compared to the microsomes prepared by centrifugation.

Extractions, gas–liquid chromatography–mass spectrometry. After incubation for the required time period, the flasks were removed, and the incubation mixtures were poured into 10 ml of cold (0°) dichloromethane containing 0.5 μmole of 1-phenyl-2-butanone as an internal standard, extracted by shaking for 20 min, and then centrifuged at 3000 rpm for 10 min. Benzoic acid was determined by extraction of the microsomes (adjusted to pH 1 by the addition of 0.5 ml of 12 N HCl) with ice-cold ether containing 0.5 μmole phenylacetic acid as an internal standard). Eight milliliters of the dichloromethane (or ether) solution was then pipetted into an evaporation tube and evaporated under a stream of N_2 until about 100 μl remained. For benzoic acid determination, 1 ml of diazomethane solution in ether (generated from *N*-nitroso methyl urea according to Arndt [23]) was added, mixed well, and evaporated to 100 μl final volume which was then assayed for methyl benzoate. For other metabolic samples, 25 μl of trifluoroacetic anhydride was added to the evaporated methylene chloride extract, mixed, and then allowed to stand for 10 min. Aliquots (2 μl) were then injected into a Hewlett–Packard 5830A gas chromatograph equipped with a 6 ft × 2 mm i.d. 3% OV-17 Gas Chrom Q glass column and a flame ionization detector. Conditions of gas chromatography were as follows unless stated otherwise; flame ionization detector temperature 260° and injector temperature 240°; the flow rate of N_2 carrier gas was 40 ml/min; the column temperature was programmed from 100 to 160° at 25°/min starting 4.5 min after injection of the sample.

For quantitation, standard curves were prepared by adding 0.01 to 5.0 μmoles of benzyl alcohol (V), benzoic acid (XV), 2-nitro-1-phenylpropane (VIII), phenylacetone (VII), 1-phenyl-1,2-propanediol (XI), and 1-phenyl-2-propanon-1-ol (IX) to incubation mixtures and extracting them in the presence of the internal standard. A plot of peak height ratio (product/internal standard) versus μmoles of product added was made, and amounts of products were determined by comparison with these standard curves.

For mass spectrometric studies, the same g.l.c. column was connected to a Hewlett–Packard 5981A GCMS system controlled by a microprocessor system, operated at an ionizing voltage of 70 eV.

RESULTS

Identification of metabolites. The methylene chlor-

Table 1. Identification of the *in vitro* metabolites of 2-nitro-1-phenylpropane

	Gas chromatography retention time (min)	Parent ion [m/e(%)]	Mass spectrometry (70 eV) Relative abundance of prominent fragment ions [m/e(%)]					
Benzyl alcohol*, †	1.36		107(9)	91(100)	77(13)	65(19)		
Benzaldehyde	1.79	106(10)	105(100)	77(75)	65(15)			
1-Phenyl-2-propanol (X)*, †	2.53		119(19)	118(90)	117(35)	91(100)	69(10)	65(13)
1-Phenyl-1,2-propanediol (XI)*, †, ‡	2.79		230(30)	203(100)	175(30)	109(19)	105(42)	77(12) 69(57)
1-Phenyl-1,2-propanediol (XI)*, †, §	3.57		230(32)	203(100)	175(27)	109(22)	105(34)	77(10) 69(57)
Phenylacetone (VII)*	4.82	134(22)	91(93)	65(34)	43(100)			
1-Phenyl-2-propanon-1-ol (IX)*, †	5.74	246(3)	203(19)	175(8)	105(99)	77(100)	69(19)	43(75)
2-Nitro-1-phenylpropane (VIII)*	7.83		119(12)	118(67)	117(22)	91(100)	77(9)	65(7)
Methyl benzoate (XV)‖	1.28	136(21)	121(7.5)	105(100)	77(25)	59(19)		

* Gas–liquid chromatography conditions. temperature program: temp 1, 100°; temp 2, 160°. Heating began at 4.5 min at 25°/min; flow, 40 ml/min; detector, 260°; injector, 240°. Internal standard = 1-phenyl-2-butanone with a retention time of 6.43 min.

† Trifluoroacetyl derivative.

‡ Erythro isomer.

§ Threo isomer.

‖ GLC conditions. Temp, 130°C; flow, 40 ml/min; detector, 260°C; injector, 240°C; internal standard = methylphenyl acetate, with a retention time under these conditions of 1.95 min.

ide extracts of the incubation mixtures were evaporated to 100 μl, derivatized, and injected into the GCMS system. The retention times and mass spectral fragmentation patterns of the metabolites were identical to those of authentic samples (Table 1).

The metabolites that contained a carbonyl function generally gave a parent peak on mass fragmentation. This included benzaldehyde (IV), phenylacetone (VII), 1-phenyl-2-propanon-1-ol (IX), and methyl benzoate (XV). Metabolites in which the benzylic carbon is oxidized, such as IX, XI, benzaldehyde (IV), and methyl benzoate (XV), all yielded diagnostic peaks at *m/e* 105 (ϕ—C≡O^+). All of the hydroxyl containing metabolites were derivatized by trifluoroacetic anhydride to yield a trifluoroacetyl derivative, which yielded an *m/e* peak 69(CF_3^+) upon mass fragmentation. The β-hydroxy metabolites, IX and XI, and benzyl alcohol (V), all yielded an *m/e* 203 fragment which was due to the ϕ-$CHOCOCF_3$ moiety, and they were thus of diagnostic value, since the intensity of the ion was usually quite high.

Requirements of the denitrification reaction. A summary of the effects of various substances on the microsomal denitrification of VIII is presented in Table 2. Elimination of the protein completely stopped the reaction, whereas deletion of the NADPH-generating system yielded only 4 per cent of the control metabolism. These data are consistent with a protein-dependent reaction, where the protein contains a small amount of endogenous NADPH. Decreasing the protein decreased the rate of reaction, and replacement of the incubation air with nitrogen or carbon monoxide also decreased the reaction, indicating the involvement of the cytochrome P-450-dependent mono-oxygenase.

Cyanide had no effect on the reaction, but cyanide is selective in its binding to cytochrome P-450 enzymes [24]. Catalase, and azide, which could inhibit the endogenous catalase present in the micro-

Table 2. Effects of various substances on the microsomal denitrification of 2-nitro-1-phenylpropane

Substances added	Enzyme activity (% of control)*
Complete	100 ± 8
-Microsomes	0
-NADPH-generating system	4
N_2	17
NaCN (1 mM)	92
CO/O_2 (4:1)	5
DPEA† (0.10 μM)	96
DPEA (0.50 μM)	80
DPEA (1.00 μM)	33
DPEA (5.00 μM)	6
Catalase (0.2 mg/ml)	94
SOD‡ (0.04 mg/ml)	88
Phenobarbital (4 × 60 mg/kg, i.p.)	286 ± 25
NaN_3 (1 mM)	92

* Control = 30 nmoles phenylacetone (VII) plus 1-phenyl-2-propanon-1-ol (IX) formed per mg protein per 20 min. Substrate concentration = 0.5 mM. Expressed as ± S.E. The data are the means of at least three separate experiments.

† DPEA = 2,4-dichloro-6-phenylphenoxyethylamine.

‡ SOD = superoxide dismutase.

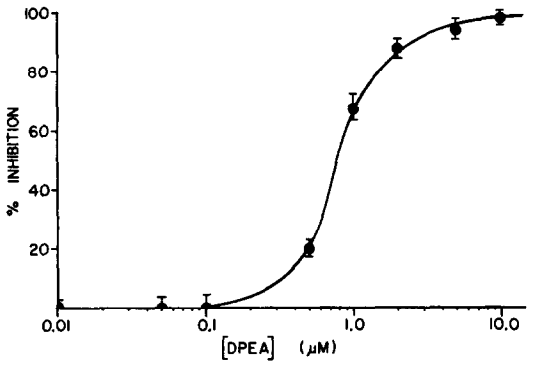

Fig. 2. Concentration–response curve of the denitrification of VIII to DPEA.

somes [25], had no effect on the reaction, so that H_2O_2 does not appear to be involved.

The denitrification reaction was increased markedly (three times) by phenobarbital induction and inhibited by DPEA with an EC_{50}* of less than 1 μM. A log concentration–response curve was obtained (Fig. 2) when the concentration of DPEA was plotted against the percent inhibition of the denitrification reaction of VIII. DPEA was first shown to be a competitive inhibitor of the microsomal demethylation of butynamine [26], and it has since been shown to bind to both microsomes and soluble cytochrome P-450, and to greatly inhibit the interactions of substrates with cytochrome P-450 [27].

Metabolism. Amounts of the various metabolites of 2-nitro-1-phenylpropane (VIII) formed are presented in Table 3. Very small amounts of benzyl alcohol (V), benzoic acid (XV), and glycols (XI), were formed. These amounts were metabolically formed, however, since their production is time-, protein- and NADPH-dependent. Variable, but trace, amounts of benzaldehyde (IV), and 1-phenyl-2-propanol (X), were also formed but are not shown in the table. The mass balance is not complete, however, since the chromatographs of extracts of longer incubations (30 min and longer) indicate unknown peaks appearing with long retention times. These new metabolites have not yet been identified. The levels of phenylacetone (VII), and ketol (IX), are presented together because for a given substrate concentration and incubation time, the relative amounts of VII and IX varied tremendously from experiment to experiment until it was noted that starving the animal on the day before killing it greatly induced the formation of IX (at the expense of VII), but did not change the total amount of VII and IX produced from VIII under that set of conditions. The formation of VII and IX from VIII plotted against protein was linear over a range of 0.4 mg/ml to 16 mg/ml protein.

We reported earlier [18] that the major product after a 45-min incubation of VIII was IX, arising from the benzylic oxidation of phenylacetone (VII), which was produced at earlier time points. Ketol (IX), has been shown to be a metabolite of VII *in vitro* [6]. We also reported that this incubation, when carried out in $H_2^{18}O$ enriched media, produced IX which contained ^{18}O in the keto function of the molecule but not in the benzylic hydroxyl group. Incubation of phenylacetone (VII), in buffer containing $H_2^{18}O$ (47%) for 20 min gave 20 per cent incorporation of ^{18}O into the keto oxygen. Incubation of VII in the presence of microsomes and $H_2^{18}O$ enriched (47%) buffer gave only 25 per cent incorporation of ^{18}O as the carbonyl oxygen, indicating that most, if not all, of the ^{18}O incorporation could be explained by nonenzymatic exchange with water. Incorporation of ^{18}O as the carbonyl oxygen of ketol, in $H_2^{18}O$ enriched (47%) buffer, was considerably faster, perhaps due to the participation of the neighboring hydroxyl function in the exchange. Indeed, microsomal hydroxylation of the benzylic position, in 1-benzyl-4-cyano-4-phenylpiperidine, yielded the dealkylation product, benzaldehyde, which in initial studies, utilizing $^{18}O_2$ in the incubation air, produced only a low (28 per cent) incorporation of ^{18}O into the product benzaldehyde [28]. When the same hydroxylation reaction was reinvestigated with a large excess of NADH (the cofactor necessary for the aldehyde reductase), the ^{18}O incorporation into the recovered benzyl alcohol was 78 per cent, indicating that exchange of the oxygen into the medium in the original benzaldehyde was facile [29]. Thus, it appears that oxygen exchange in neutral aqueous media is rapid into carbonyl oxygen and that the source of oxygen for microsomal oxidations of both VIII and 1-benzyl-4-cyano-4-phenylpiperidine is molecular oxygen.

To determine the metabolic sequence of the metabolite productions in this system, we incubated *N*-hydroxyamphetamine (II), in rabbit liver micro-

* EC_{50} = concentration that inhibits the reaction by 50 per cent.

Table 3. Microsomal concentrations of metabolic products of VIII*

Incubation time (min)	Benzyl alcohol (V)	Benzoic acid (XV)	Phenylacetone (VII) and ketol (IX)	Glycols† (XI)
10	2.6 ± 0.8	4.9 ± 1.8	55.6 ± 3.6	0.6 ± 0.2
20	2.9 ± 1.0	5.1 ± 2.0	70.2 ± 16.5	5.4 ± 1.0
30	3.4 ± 1.1	5.5 ± 2.1	49.2 ± 11.2	8.6 ± 2.3

* Expressed as nmoles/mg protein ± S.D.; minimum of four experiments with each point determined in duplicate. Microsomes were from phenobarbital-pretreated animals. Substrate concentration = 0·05 mM. Protein concentration was approximately 1.8 mg/ml in a 5-ml incubation.

† Both erythro and threo isomers.

somes, and found that the major metabolites were the nitro compound (VIII), ketone (VII), oxime (III), and small amounts of benzyl alcohol (V), benzaldehyde (IV), alcohol (X), and glycols (XI). Separate incubation of the ketone (VII) yielded alcohol (X), glycols (XI), the ketol (IX), and small amounts of benzyl alcohol (V). A plot of the ketol (IX) formation versus incubation time (Fig. 3) indicates that the ketol was disappearing and forming some other metabolite. A preliminary plot of the formation of benzoic acid over time during denitrification (not shown) showed a rapid rise in the level of benzoic acid, which reached a maximum and plateaued at 20 min. Thus, it seems unlikely that the ketol (IX) was disappearing to form benzoic acid. Separate incubations of the ketol (IX) should answer the question of its ultimate fate. A plot of the glycol (XI) formation versus time (Fig. 4) from phenylacetone (VII) indicates that the glycols were terminal metabolites and build up slowly with time.

Kinetic studies. A plot of V versus S for the conversion of the nitro derivative (VIII) to the ketone (VII) (Fig. 5) describes typical saturation kinetics with half-maximum velocity occurring at 0.047 mM and a V_{max} of 8.0 nmoles·(mg protein)$^{-1}$·min^{-1}. A plot of V versus V/S (not shown) was not linear, so

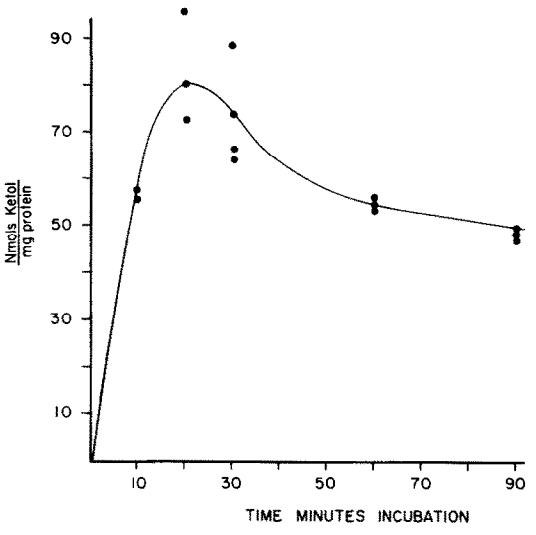

Fig. 3. Time course of the formation of 1-phenyl-2-propanon-1-ol (IX) from phenylacetone (VII) incubated at a concentration of 0.5 mM in rabbit liver microsomes from phenobarbital-pretreated animals (4 × 60 mg/kg, i.p.). The data are plotted as nmoles IX/mg protein against incubation time.

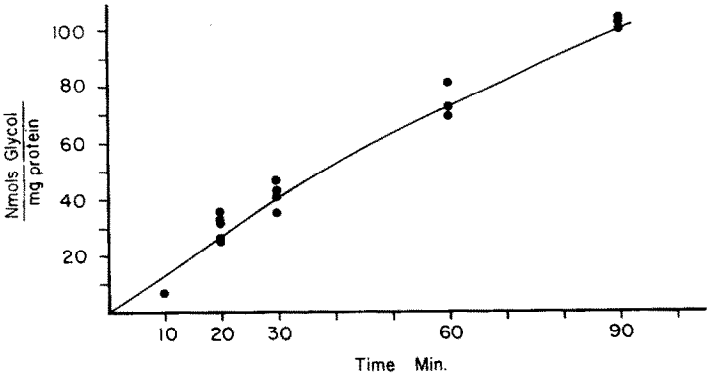

Fig. 4. Time course of the formation of 1-phenyl-1,2-propanediol (erythro) (XI) from phenylacetone (VII) incubated at a concentration of 0.5 mM in rabbit liver microsomes, from phenobarbital-pretreated animals (4 × 60 mg/kg, i.p.). The data are plotted as nmoles XI/mg protein against incubation time.

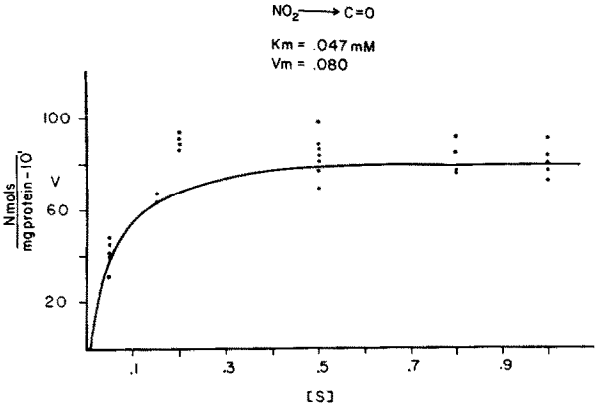

Fig. 5. V vs S plot for the conversion of 2-nitro-1-phenylpropane (VIII) to phenylacetone (VII).

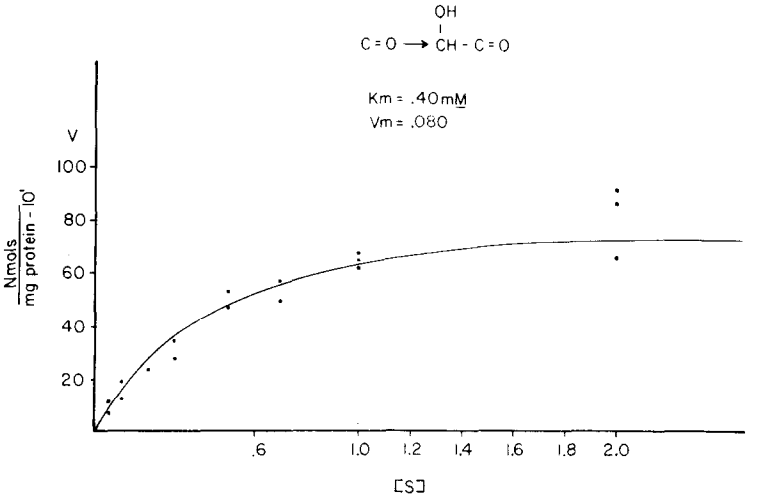

Fig. 6. V vs S plot for the conversion of phenylacetone (VII) to 1-phenyl-2-propanon-1-ol (IX).

the data were analyzed for their fit into a model of two enzymes, utilizing a weighted, iterative, nonlinear regression analysis. No consistent biphasic curve resulted, so that no consistent pattern of enzyme kinetics was thus derived for this reaction. A Michaelis–Menten plot of reduction velocity versus substrate concentration for the conversion of the ketone (VII) to the ketol (IX) (Fig. 6) gives values of 0.40 mM for the K_m and a V_{max} of 8.0 nmoles/mg·min^{-1}. A plot of V versus V/S of these data (not shown) gave a K_m of 0.46 mM and a V_{max} of 9.5 nmoles/mg·min^{-1}, which are in good agreement with the Michaelis–Menten values.

DISCUSSION

There is little known about the metabolism of aliphatic nitro compounds although an early reference reported the *in vivo* transformation of an aliphatic nitro derivative to a carbonyl compound in the rabbit [1]. Rat liver microsomes can transform both 2-nitropropane [30] as well as several other nitro derivatives [4] to the appropriate carbonyl compounds in a cytochrome P-450-dependent reaction. During microsomal oxidation, some compounds lead to intermediates able to react with reduced cytochrome P-450 to form complexes, which then cause an inactivation of cytochrome P-450 toward further mono-oxygenation reactions. It is known that both the microsomal oxidation products of *N*-hydroxyamphetamine (II) [31] and the oxidation products of other aliphatic hydroxylamines [32] do form complexes with cytochrome P-450. It has also been shown that both 2-nitroso-1-phenylpropane [33] and 2-nitro-1-phenylpropane (VIII) [34], in the presence of dithionite, readily form complexes with microsomal cytochrome P-450. As the concentration of VIII increases from 0.3 to 1 mM in the incubation media, the rate of formation of the primary metabolite (VII) declines from 0.091 (maximum) to 0.075 μmole/(mg·protein)$^{-1}$·10 min^{-1}. One means by which this mechanism of inhibition might occur is the conversion of nitro (VIII) to the nitroso derivative (not shown), and subsequent formation of a P-455 complex. Although no amphetamine (I) or hydroxylamine (II) was detected in the incubates, we cannot rule out the possibility that VIII was reduced metabolically to the nitroso compound which would then form a complex with cytochrome P-450, as reported earlier [33].

Although it is known that the nitro compound (VIII) is a microsomal metabolite of the hydroxylamine (II), and we have shown that the ketone (VII) and the ketol (IX) are microsomal metabolites of the nitro compound (VIII), the enzymatic nature of the two conversions is clearly different. It has been reported that the conversion of hydroxylamine (II) to nitro compound (VIII) is sensitive to both hemoglobin and catalase [14], suggesting the involvement of peroxide in the reaction. The conversion of the nitro compound (VIII) to the ketone (VII) and its metabolite is extremely sensitive to DPEA, and is insensitive to both catalase, a peroxide consumer, and azide, a catalase inhibitor. Thus, it appears that peroxide is not involved in this subsequent metabolic step. Since this oxidative denitrification reaction is also inhibited by carbon monoxide and greatly increased by phenobarbital pretreatment, the involvement of cytochrome P-450 is indicated.

The metabolic data suggest that at long incubation times, beginning at 30 min, new, less volatile, and as yet unidentified metabolites appear in the chromatograms. Possible structures of these peaks include β-hydroxylated derivatives, or perhaps β,*N*-bis-hydroxy derivatives, which rapidly decompose to benzaldehyde [35], nitrones which could result from condensation of benzaldehyde with this β,*N*-bis-hydroxy derivative [36], or β-hydroxy nitro derivatives, which should be very unstable. Other possibilities include a β-hydroxyoxime, which was found in the incubation of β-hydroxyamphetamine (XII) (norephedrine), in rabbit liver microsomes [37], or other as yet undescribed compounds.

In this study, we have shown that 2-nitro-1-phenylpropane (VIII) is rapidly denitrificated by microsomes in a cytochrome P-450 mediated reaction to phenylacetone (VII) and its metabolites, plus some unknown compounds.

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