J. Pharm. Pharmac., 1976, **28**, 815–821 *Received December 29, 1975*

In vitro metabolism of 1-phenyl-2-(n-propylamino) propane (*N*-propylamphetamine) by rat liver homogenates

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In vitro incubation of (±)-*N*-(n-propyl)amphetamine (NPA) with the 12 000 *g* supernatant fraction of rat liver homogenate resulted in the formation of two *N*-oxygenated products identified as *N*-hydroxy-1-phenyl-2-(n-propylamino)propane and *N*-[(1-methyl-2-phenyl) ethyl]-1-propanimine *N*-oxide by g.l.c., g.l.c.-m.s. and t.l.c. Amphetamine, phenylacetone, benzyl methyl ketoxime, and a previously unreported metabolite *N*-(2-hydroxy-n-propyl) amphetamine were also isolated. Increasing the pH of the incubation mixture from 7·4 to 8·4, or *in vivo* pretreatment of rats with 3-methylcholanthrene caused an increase in the *in vitro* metabolic *N*-oxidation of NPA, whereas pretreatment with phenobarbitone or NPA did not. The presence of SKF 525-A in incubation mixtures reduced the amount of *C*-oxidation and to a lesser degree of *N*-oxidation. When (+)-NPA or (−)-NPA was used as substrate, *C*-oxidation was favoured with the former and *N*-oxidation with the latter.

Current research (Beckett, Coutts & Ogunbona, 1973a; Gorrod, 1973; Beckett & Bélanger, 1974: Cho, Lindeke & Sum, 1974) has established that many aliphatic amines are metabolized *in vitro* to hydroxylamines. These metabolites are extremely reactive and can be metabolically, chemically or physically converted to other *N*-oxygenated products including oximes, nitrones, nitroso and nitro compounds. In most instances, *C*-oxygenated metabolites are also formed concomitantly. Studies (Beckett & Al-Sarraj, 1972; Gorrod, 1973; Beckett & Midha, 1974) have also revealed that, whereas microsomal preparations of guinea-pig and rabbit liver possessed significant *N*-oxidative properties, similar preparations of rat liver were much less active.

We wished to study the metabolism of selected aliphatic secondary amines in greater detail for four reasons: (a) to see if rat liver homogenates were generally low in *N*-oxidation activity; (b) to determine quantitatively the extent of metabolic *N*-oxidation; (c) to see whether rat liver could be induced to *N*-oxidize the chosen substrate; (d) to determine whether the presence of nicotinamide in the incubation mixture had an activating or inhibiting effect on metabolic *N*-oxidation of secondary amines. Of the substrates we have investigated to date, the most promising one appears to be 1-phenyl-2-(n-propylamino)propane (i.e., *N*-propylamphetamine, NPA, I).

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MATERIALS AND METHODS

Reagents and animals. Glucose-6-phosphate (G-6-P), nicotinamide adenine dinucleotide phosphate ($NADP^+$), nicotinamide, and 3-methylcholanthrene (3-MC) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. SKF 525-A was supplied by Smith, Kline and French Laboratories. Other chemicals were of reagent grade. Male Wistar rats (350–450 g) were used throughout. They were housed in wire suspension cages and allowed free access to food and water.

Instrumentation. Nuclear magnetic resonance spectra were recorded in $CDCl_3$ on a Varian model A-60D spectrometer with tetramethylsilane as internal standard. Specific rotation measurements were obtained with a Zeiss Circle Polarimeter (0·01° model), using a sodium lamp source (589 nm). Samples were dissolved in distilled water (2 % w/w) and rotation determined in a 10 dm tube.

Gas-liquid chromatography (g.l.c.). A Perkin-Elmer Model 990 chromatograph with dual flame ionization detectors was used. Column 1 was 1·2 m glass tubing 6 mm o.d., packed with 7½ % Carbowax 20 M on acid washed, DMCS treated Chromosorb 750, 80–100 mesh, and column 2 was 1·2 m glass tubing 6 mm o.d. packed with 10 % Apiezon L and 5 % KOH on the same solid support as column 1. Operating conditions were: injection port, 210°; oven, 155°; manifold, 210°; helium, 60 ml min^{-1}; air, 30 lb in^{-2}; hydrogen,

25 lb in^{-2}. Peak areas were determined with an electronic peak integrator (Infotronics Model CRS-208), using *p*-chloropropiophenone (PCP) as an internal standard.

Gas-liquid chromatography-mass spectrometry (g.l.c.-m.s.). Mass spectra of authentic reference compounds and samples were obtained by combined g.l.c.-m.s. using columns 1 and 2 described above in a Hewlett-Packard Model 5700A gas chromatograph coupled to a Hewlett-Packard Model 5981A mass spectrometer at an ionizing potential of 70 eV.

Thin-layer chromatography (t.l.c.). Chromatograms were run on glass plates (20 × 20 cm) spread to 0·5 mm with a slurry of silica gel G-PF254 (Brinkman) and activated at 100° for 1 h. The solvent system used was chloroform–acetone (9 : 2). Spots were detected by short-wave ultraviolet light (254 nm), iodine vapour, or ammoniacal silver nitrate spray.

Substrates and reference compounds. (+)- and (−)-*NPA (I)* were prepared from (+)- or (−)-amphetamine by the methods described by Leonard, Adamcik & others (1958) for the related *N*-ethylamphetamines, except that propionic anhydride was substituted for acetic anhydride. (±)-NPA was obtained from 1-phenyl-2-propanone and n-propylamine by the literature method (Temmler, 1952), except that ethanol was used as a solvent. All three products were converted to colourless hydrochlorides in the usual way, and recrystallized from ethanol-ether to constant melting point.

(±)-NPA hydrochloride had a m.p. of 155·5–157°, higher than the m.p. 148–149° previously reported by us (Coutts, Dawson & others, 1976); it gave a characteristic mass spectrum which has been interpreted (Coutts & others, 1976); nmr (in D_2O): δ 0·97 (t, 3H, J = 6·5, CH_2CH_3); 1·25 (d, 3H, J = 6·5, $CHCH_3$); 1·68 (sextet, 2H, J = 6·5, $CH_2CH_2CH_3$); 2·60–3·90 (m, 5H, $PhCH_2$, NCH_2 and $CHCH_3$) 7·38 (s, 5H, C_6H_5). The free base chromatographed as a single peak on two different g.l.c. columns (Table 1).

(+)-NPA hydrochloride, $[\alpha]_D^{25}$ = +19·0° (c = 2, H_2O), had m.p. 178–180°. Its nmr and mass spectra, and its g.l.c. behaviour were identical to those of the racemate. (Calcd for $C_{12}H_{20}ClN$: C, 67·43; H, 9·43; N, 6·55. Found: C, 67·29; H, 9·55; N, 6·63.

(−)-NPA hydrochloride, $[\alpha]D_D^{25}$ = −16·5° (C = 2, H_2O) had m.p. 176·5–178·5°. Its nmr and mass spectra, and its g.l.c. behaviour were also identical to those of the racemate. (Calcd for $C_{12}H_{20}ClN$: C, 67·43; H, 9·43; N, 6·55. Found: C, 67·48; H, 9·31; N, 6·41.

The metabolites, *N*-hydroxy-*N*-(n-propyl)amphetamine (II), *N*-[(1-methyl-2-phenyl)ethyl]-1-propanimine *N*-oxide (III) and 1-phenyl-2-propanone oxime (VI) have been described previously (Beckett, Coutts & Ogunbona, 1973b, 1973c, 1974). Phenylacetone (1-phenyl-2-propanone, IV) was supplied by Aldrich Chemical Co. and distilled before use, and (±)-amphetamine (V) sulphate was obtained from Smith, Kline and French Laboratories. Both compounds gave single peaks when gas chromatographed on columns 1 and 2 (see Table 1), and each gave an nmr spectrum consistent with its structure.

(±)-N-*2-(Hydroxypropyl) amphetamine (VII) hydrochloride.* A suspension of platinum oxide (150 mg) in dry ethanol (25 ml) was hydrogenated for 0·5 h at 2 atm. (guage pressure) in a Parr hydrogenator. A solution of 1-amino-2-propanol (8·2 ml) and 1-phenyl-2-propanone (20 ml) in dry ethanol (50 ml) was then added and the mixture was hydrogenated for 33 h at an initial pressure of 3 atm. The mixture was filtered and the filtrate evaporated *in vacuo* to a pale yellow oil which was dissolved in dry ether and treated with an excess of HCl in dry ether. The white solid which formed was crystallized from ethanol–ether and gave the title compound (15·5 g), m.p. 135–136°; nmr (in $CDCl_3$): δ 1·20 and 1·28 (overlapping doublets, 6H, J = 6·5, 2 CH_3 groups); 2·10–4·00 (m, 7H, 2 CH_2 groups, $CHOH$, $CHCH_3$ and NH); 4·60 (s, 1H, D-exchangeable, OH); 7·12 (s, 5H, C_6H_5). Mass spectrum: *m/e* (% relative abundance) 193(5), 178(7), 148(30), 119(10), 102 (100), 91(49), 84(65), 77(8), 65(10), 56(19). (Calcd for $C_{12}H_{20}ClNO$: C, 62·73; H, 8·77; N, 6·10. Found: C, 62·74; H, 9·14; N, 6·08). The free base chromatographed as a single peak on g.l.c. column 1 (Table 1).

Pretreatment of animals. A. (±)-*N*-(n-Propyl) amphetamine (±)-NPA. Rats were given daily injections of (±)-NPA (10 mg kg^{-1}, i.p.) in pH 7·4 phosphate buffer for two weeks. The rats received the last injection 18 h before death. B. Phenobarbitone. Daily injections of sodium phenobarbitone (75 mg kg^{-1}, i.p.) in pH 7·4 phosphate buffer were given for five days before death. C. 3-Methylcholanthrene. A single dose of 20 mg kg^{-1} (i.p.) in corn oil was administered 18 h before death. D. Controls. Control animals received 1 ml kg^{-1} (i.p.) of the appropriate vehicle for the same time periods as those indicated in the respective treatments A-C.

Preparation of liver microsomal fraction. Animals were fasted overnight and killed by cervical dislocation. Livers were removed and immediately placed into ice cold 1·15 % KCl. Subsequent operations were performed at 4°. A 20 % w/v homogenate corresponding to 200 mg wet weight liver ml^{-1} in isotonic KCl was prepared in a Potter homogenizer equipped with a Teflon pestle. The microsomal supernatant was obtained by centrifugation of the liver homogenate at 12 000 *g* for 20 min (0·3°).

Determination of cytochrome P_{450} and microsomal protein. For these determinations 3·5 and 1 ml, respectively, of the 12 000 *g* supernatant were transtransferred to separate ultracentrifuge tubes and diluted to 10 ml with 1·15 % KCl. After centrifugation at 105 000 *g* for 1 h, the superantant was discarded and the pellets resuspended either in distilled water (25 ml) for estimation of protein, or in 50 mM phosphate buffer pH 7·4 containing 10^{-3} μM $litre^{-1}$ EDTA (6 ml) for cytochrome P_{450} determination. Protein was determined according to Lowry, Rosebrough & others (1951) as modified by Miller (1959). The method of Omura & Sato (1964) was used for cytochrome P_{450}.

Incubation. One ml of the 12 000 *g* supernatant was added to 25 ml incubation flasks containing G-6-P (20 μmol), $NADP^+$ (4·4 μmol), $MgCl_2$ (20 μmol), substrate (10 μ mol) and sufficient 0·1 M phosphate or tris HCl buffer, pH 7·4, to make 6 ml. Samples were then incubated aerobically for 60 min at 37° in a Dubnoff shaking metabolic incubator (120 oscillations min^{-1}). At the end of the incubation period, the flasks were immersed in ice, before addition of the internal standard (*p*-chloropropiophenone, 2 μmol) and extraction three times with freshly distilled ether, initially at pH 7·4 then after basifying to pH 12–13. The ether extracts were concentrated at 45° on a water bath to approximately 250 μl, isobutanol (50 μl) was added to prevent the sample going to dryness and consequent loss of the internal standard, and the mixture finally concentrated to about 20–50 μl, before analysis.

Quantitative analysis

Known but varying quantities of compounds I–VII (0·1–10 μmol) were added to flasks containing 1 ml of aged 20 % w/v rat liver homogenate and 5 ml pH 7·4 phosphate buffer. Internal standard (*p*-chloropropiophenone, 2 μmol) was added and the solutions were extracted three times with equal volumes of freshly distilled ether at pH 7·4. The ether extracts were combined. The extraction procedure was repeated after adjusting the pH of the aqueous solutions to pH 12–13 (1N NaOH). Each combined extract was concentrated to about 1 ml, isobutanol (50 μl) was then added to prevent volalitization of contents, and each extract was concentrated to 50–100 μl on a water bath before g.l.c. analysis.

Calibration curves were constructed by plotting weight of each compound (I–VII) added *vs* the g.l.c. peak area ratio of the compound: internal standard. These calibration curves were linear throughout the concentration ranges encountered during the *in vitro* experiments.

Calibration curves and quantitation were done as follows: compounds II, III and VI at pH 7·4 on column 1; compounds I and V at pH 12–13 on column 2; compound IV at pH 7·4 on column 2; compound VII at pH 12–13 on column 1.

Calibration curves were reproducible provided g.l.c. analysis was performed within 24 h of extraction. In ether solution, some decomposition of II to III and VI was apparent after 48–72 h. Each g.l.c. peak was scanned by g.l.c.-m.s. to confirm that overlapping peaks were not present. Compounds I, IV and V, had almost identical retention times on column 1; column 2 was therefore used for their quantitation. Compounds II and VII had similar retention times on column 1. For the quantitation of VII in the presence of II, all of II was first converted to III and VI by basifying the aqueous solution with 5 % w/v NaOH and shaking at room temperature (30 min), under which condition VII was stable.

RESULTS AND DISCUSSION

In vitro metabolism of (±)-NPA at pH 7·4 using the 12 000 *g* supernatant of homogenized rat liver fortified with the usual cofactors but not nicotinamide, resulted in the formation of various metabolites (Table 1) including two *N*-oxygenated products identified as *N*-hydroxy-1-phenyl-2-(n-propylamino)-propane, i.e. *N*-hydroxy-NPA (II), and the related nitrone (III) by a comparison of their g.l.c., g.l.c.-m.s., and t.l.c properties with those of authentic samples of II and III. The mass spectra of II and III were virtually identical to reported (Beckett & others, 1973b) spectra of these compounds except for differences in the relative abundances of fragment ions which are expected (*cf* McFadden, 1973) when different instrumentation is used. Fragment ions of particular diagnostic value in the identification of II, and which permitted differentiation of this metabolite from the isomeric metabolite VII, are illustrated in

Table 1. In vitro *metabolism of* (±)-N-(*n-propyl*) *amphetamine by fortified 12 000* g *supernatant of rat liver homogenate—g.l.c. retention times* (Rt), *t.l.c.* R_F *values, and yields of recovered substrate and metabolites after 60 min incubations at 37°.*

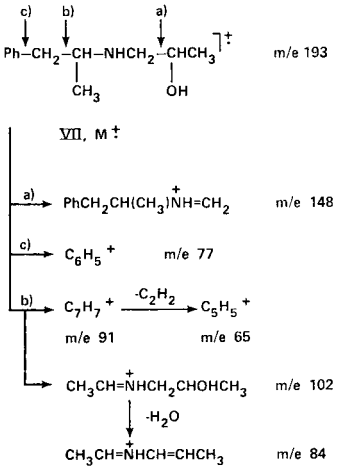
Compound*		Yield (%)[1]	Rt (Min) Col 1	Col 2	R_F
R-NH(CH_2)$_2$Me	(I)	91·9–94·5	1·9	9·7	0·2
R-N(CH_2)$_2$Me | OH	(II)	0·3–0·6	13·2	—	0·65
R-N = $CHCH_2$Me | O–	(III)	1·0–2·0	22·1	—	0·3
$PhCH_2COMe$	(IV)	0·2–0·5	1·8	3·2	0·08
$PhCH_2CH(Me)NH_2$	(V)	2·5–3·0	1·8	3·8	0·15
$PhCH_2C(Me)$ = NOH	(VI)	0·1–0·2	16·5	—	0·8
R-$NHCH_2CHOHMe$	(VII)	0·2–0·4	13·0	—	0·1

[1] Total recovery of I-VII was 98·8 ± 0·4 % s.e.m.
* R = $PhCH_2CH(Me)$

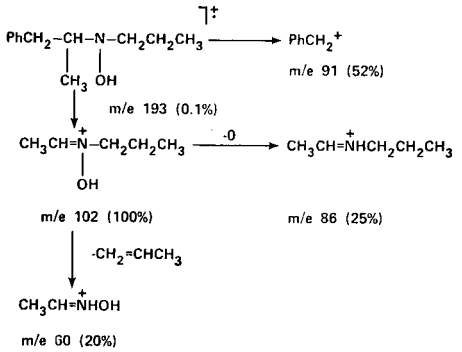
Scheme 1. Diagnostic fragment ions in the spectrum of III have already been discussed (Beckett & others, 1973b, 1974). Other components identified in the same manner were 1-phenyl-2-propanone (IV), amphetamine (V), 1-phenyl-2-propanone oxime (VI) and a previously unreported metabolite, 2-(2-hydroxypropylamino)-1-phenylpropane, i.e. *N*-(2-hydroxypropyl)amphetamine (VII). The mass spectrum of VII differed appreciably from that of the isomer II. The former had a weak molecular ion (*m/e* 193); it contained a fragment ion (*m/e* 148) of particular diagnostic value, and it lacked the ion of *m/e* 60 which was present in the spectrum of II. The major ions in the mass spectrum of VII are identified in Scheme 2.

No 1-phenyl-2-propanol ($PhCH_2CHOHCH_3$; VIII) was detected as a metabolic product of NPA although this alcohol is known to be an *in vitro* metabolite of the ketone (IV) in the rabbit (Hucker, 1973). On g.l.c. column 2, an authentic sample of VIII eluted between peaks IV and V. Mass spectrometry confirmed that there was no 1-phenyl-2-propanol in the tail of the phenylacetone (IV) peak. It is possible, however, that there is formed a very small amount of the alcohol (VIII) which elutes with much larger amounts of amphetamine (V) and remains undetected.

Although *p*-hydroxyamphetamine is a major *in vivo* metabolic product of amphetamine in the rat (Axelrod, 1954; Dring, Smith & Williams, 1966) and has been identified in trace quantities *in vitro* (Cho, Hodshon & others, 1975) its presence, or that of *p*-hydroxy-*N*-(n-propyl)amphetamine could not be



SCHEME 2



SCHEME 1

confirmed in this *in vitro* study. However, preliminary *in vivo* studies (Coutts & others, 1976) using male rats injected with (±)-NPA have established that a significant amount (i.e. 20 %) of the administered (±)-NPA is excreted as the *p*-hydroxy metabolite.

Initially, the quantitation of *N*-OH-NPA was based upon its direct g.l.c. analysis immediately after extraction at pH 7·4. On-column decomposition was later found to occur in significant amounts when acid washed, DMCS-treated Chromosorb W was used as a support for the Carbowax 20 M in Column 1. However, when Chromosorb 750 was subsituted for the Chromosorb W and the column prepared according to Leibrand & Dunham (1973), the decomposition was minimal.

Time course studies on the rate of production of II showed that the amount of this metabolite present in the incubation mixture increased linearly for the

first 15 min, but during the next 45 min of incubation, no further increase in the amount of II present could be demonstrated. At the same time the amount of nitrone (III) and oxime (XI) continued to increase linearly through the 60 min incubation period.

When II was used as the substrate in incubation mixtures containing microsomes but no cofactors, or boiled microsomes with cofactors, appreciable quantities of III and VI were obtained, which indicated that the conversion of II to III and VI was at least in part, non-enzymic. In view of this information, incubation samples were adjusted to pH 12–13 and allowed to stand at room temperature (20°) for 20 min to convert metabolite II to the nitrone (III) and small amounts of the oxime (VI) which were stable for longer periods of time than II under the analytical conditions employed. As a result, overall quantitation of the total amount of isolable *N*-oxygenated products was facilitated.

Adjustment of the buffer used in the incubation mixture from pH 7.4 to 8·4 resulted in a 2–3 fold increase in the overall metabolism of NPA (Table 2). The increase is most likely associated with an increase in the proportion of the non-ionized lipophilic form of NPA as the pH is adjusted toward the pKa of NPA. This in effect increases the amount of NPA available to react with microsomal *C*- and *N*-oxidative enzymes. At pH values higher than 8·4, metabolism began to decrease, presumably as a result of enzyme inactivation.

Similar *in vitro* experiments were performed using (+)-NPA and (−)-NPA as substances. The results are shown in Table 3. With the exception of metabolite VII, these results substantiate an earlier report (Henderson, Vree & others, 1974) that the (+)-isomer is *C*-oxidized at a faster rate than the (−)-isomer. In contrast, the rate of *N*-oxidation of (−)-NPA is faster than that of (+)-NPA.

An attempt was made to increase *in vitro* metabolic *N*-oxidation by injecting rats with (±)-NPA.HCl for fourteen days before preparation of the microsomal fraction. An ether extract of a portion of this liver homogenate (adjusted to pH 12) was examined by g.l.c. to confirm that it contained no residual quantities of compounds I–VII. When this microsomal preparation was used with normal cofactors (omitting nicotinamide) to metabolize (±)-NPA, (+)-NPA or (−)-NPA, no significant differences were observed from results obtained with non-pretreated animals.

Pretreatment of rats with 3-MC resulted in a 39 % increase in total *in vitro* production, at pH 8·4, of *N*-oxidized metabolites (III and VI) and a smaller increase (11 %) in the amounts of IV and V. It was noted that the increase in the sum of IV and V was due almost entirely to an increase in V. No increase in VII was observed.

Table 2. *Effect of pH on the* in vitro *metabolism of 10 μmol* N-*n-propyl-amphetamine by rat liver 12 000* g *supernatant*[1].

Substrate	pH	*N*-Oxidized products[2] μmol	*N*-Oxidized products[2] μmol g^{-1} protein^{-1} h^{-1}	*C*-Oxidized products[2] μmol	*C*-Oxidized products[2] μmol g^{-1} protein^{-1} h^{-1}
(+)-NPA	7·4	0·11 (0·02)	41·1 (3·9)	0·77 (0·07)	305·7 (23·6)
	8·4	0·26 (0·04)	98·0 (9·6)	1·05 (0·1)	418·3 (32·3)
(-)-NPA	7·4	0·26 (0·03)	101·9 (10·3)	0·70 (0·07)	278·6 (24·0)
	8·4	0·53 (0·14)	196·0 (34·8)	0·96 (0·09)	392·3 (48·1)
(±)-NPA	7·4	0·19 (0·01)	69·6 (5·5)	0·62 (0·04)	230·4 (13·4)
	8·4	0·40 (0·02)	149·6 (6·6)	0·89 (0·07)	330·1 (24·3)

[1]Values (with standard deviations) representing 3 experiments, each of which was done in duplicate.

[2]The term 'C-oxidized products' refers to compounds isolated and identified as possessing a C-O function introduced during either the metabolism reaction or the subsequent analytical procedure. Similarly the term '*N*-oxidized products' refers to isolated compounds shown to possess an N-O group. It is *not* implied that the total *C*-oxidized products are formed by metabolic *C*-oxidation reactions; *C*-oxidized compounds can be formed as a result of an initial metabolic *N*-oxidation (Beckett & Bélanger, 1975).

Phenobarbitone pretreatment of rats had no effect on the *in vitro* production at pH 8·4 of II, III or VI but did cause a 30 % increase in IV and V.

Incorporation of SKF 525-A (2 μmol) into the incubation mixture reduced the quantities of IV and V formed to negligible amounts and also reduced III and VI formation by approximately 25 % presumably by a competitive mechanism.

The presence of $NADP^+$ was required to obtain maximum activity of the microsomal system employed. However, neither increasing its concentration beyond 20 μmol nor addition of NADPH improved the activity of the system. Some metabolic activity could be demonstrated in the absence of G-6-P but a maximum effect followed addition of 20 μmol. The requirement for $MgCl_2$ could not be verified.

Table 3. In vitro *metabolism (60 min incubation) of (+), (−), and (±) isomers of* 2-N-*(n-propylamino)-1-phenylpropane (NPA) by rat liver 12 000* g *supernatant.*

Metabolite[1]	(±)-NPA	% Yield[2] (+)-NPA	(−)-NPA
III	1·4 (0·1)	0·9 (0·1)	2·1 (0·2)
IV	0·7 (0·1)	1·6 (0·2)	0·6 (0·1)
V	3·7 (0·2)	4·3 (0·3)	3·7 (0·4)
VI	0·5 (0·1)	0·2 (0·04)	0·5 (0·1)
VII	1·37 (0·1)	0·5 (0·1)	1·32 (0·2)

[1]For identity of metabolites, see Table 1.
[2]Yields are given (with standard deviations) for 3 experiments each of which was done in duplicate.

Nicotinamide is often added to incubation mixtures in the belief that its presence prevents the loss of $NADP^+$ by inhibiting nucleotidase action. Schenkman, Ball & Estabrook, (1967) and Parli & Mannering (1971) refute this and claim that its presence *inhibits* metabolic *C*-oxidation of certain substrates. Since nicotinamide is itself metabolically *N*-oxidized to its *N*-oxide (Chaykin & Bloch, 1959; Sparthan & Chaykin, 1969) it may compete with substrate in metabolic *N*-oxidation studies. It would seem advisable to omit nicotinamide from all *in vitro* metabolism reactions. However, conflicting evidence is available. *In vitro* metabolic *C*-oxidation of nicotine to cotinine is not adversely affected by the presence of nicotinamide and the rate of metabolic *N*-oxidation of nicotine is actually *increased* (Booth & Boyland, 1970, 1971). The presence of nicotinamide in the incubation mixture also *increases* the rate of phentermine *N*-oxidation (Cho & others, 1974). Since these results were obtained from a tertiary (nicotine) and a primary (phentermine) amine substrate, it was of interest to see what the effect would be of including nicotinamide in the (±)-NPA (a secondary amine) incubation mixture.

A preliminary study was carried out using liver homogenates from less mature rats (250–260 g) which did not metabolically *N*-oxidize (±)-NPA to such an extent as liver preparations from 400–450 g rats. The results (Table 4) indicate that the presence of nicotinimide in the incubation mixture *reduced* significantly the amount of metabolic *N*-oxidation of (±)-NPA. Since nicotinamide is extracted during *in vitro* metabolism studies and is an interfering peak in qualitative and quantitative g.l.c. studies, its presence is undesirable in incubation mixtures. For this reason, and because of the results depicted in Table 4 it is apparent that the inclusion of nicotinamide in NPA incubation mixtures is unnecessary. This may also be the case with other medicinal secondary amine derivatives of amphetamine.

Table 4. *Effect of including nicotinamide in the* (±)-*NPA incubation mixture (pH 7·4).*

Concn of nicotinamide μmol/6 ml	Percentage of total *N*-oxidation[1–3] after an incubation of 30 min	60 min
0	1·83 (0·24)	3·12 (0·32)
30	1·74 (0·20)	3·09 (0·35)
60	1·30 (0·29)	2·66 (0·32)
120	1·22 (0·13)	2·34 (0·46)

[1]Sum of metabolites II, III and VI.
[2]Average of 4 determinations; values (with standard deviations).
[3]See footnote 2 to Table 2.

Although the amount of cytochrome P_{450} was determined for control and pretreated animals, no correlation between the levels of cytochrome P_{450} and *N*-oxidation of NPA could be established.

Acknowledgements

The authors thank Dr C. W. Kazakoff for assistance in the synthesis of substrates and reference compounds. The financial assistance of Medical Research Council of Canada (Grant MT-2993 to R.T.C.) is gratefully acknowledged.

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