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METABOLISM OF DRUGS—LXXVIII

THE FORMATION *IN VITRO* OF OXOPROLINTANE FROM PROLINTANE BY RABBIT LIVER

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Abstract—The metabolism *in vitro* of prolintane was investigated with rabbit liver preparations. It was found that microsomes metabolized prolintane to an unidentified compound in the presence of NADPH under aerobic conditions, but not to oxoprolintane. Prolintane was, however, converted to oxoprolintane, if it was preincubated with microsomes in the presence of NADPH aerobically and then incubated with soluble fraction, the activity of which was almost equivalent to that of the 9000 *g* supernatant.

IN THE previous paper[1] from this laboratory, it was reported that, in rabbits, the major metabolic pathway of prolintane (1-phenyl-2-pyrrolidinopentane), a recently developed CNS stimulant, was oxidation of the α-carbon atom of the pyrrolidine ring to form oxoprolintane, 1-phenyl-2-(2-oxopyrrolidino)pentane. It is also known that such oxidation takes place in the metabolism of nicotine[2,3] and tremorine,[4,5] both of which are derivatives of pyrrolidine. Concerning the mechanism of this lactam formation, Hucker *et al.*[3] presented evidence that nicotine was initially oxidized at the α-carbon atom of the pyrrolidine ring, and the resulting hydroxynicotine was then dehydrogenated to produce cotinine, a lactam compound. In the metabolism of tremorine, Hammer *et al.*[5] recently identified a new metabolite to be *N*-(4-pyrrolidino-2-butynyl)-γ-aminobutyric acid and showed that this metabolite could be converted *in vivo* to oxotremorine. The first and obligatory step of these sequential reactions can also be considered to be formation of hydroxytremorine, because the amino acid described above must be derived from amino aldehyde, a ring-opened tautomer of hydroxytremorine.

This paper will present evidence that prolintane is also metabolized to oxoprolintane in rabbit liver by the following pathway: initial hydroxylation of prolintane in the α-position of the pyrrolidine ring by the mixed-function oxidase system in microsomes and successive dehydrogenation of "hydroxyprolintane" to oxoprolintane by an enzyme system in the soluble fraction.

METHODS

Tissue preparation and enzyme assay. The preparation of tissue samples was carried out at 0–4°. Male albino rabbits (2·5–3·0 kg) starved for 24 hr prior to preparation, were killed by a blow on the head. Livers were removed and homogenized with 2 vol. of ice-cold 1·15% KCl solution containing 25 μmoles/ml of nicotinamide.

The 9000 *g* supernatant was obtained by centrifugation of the homogenate for 20 min at 9000 *g*. Microsomes and 105,000 *g* supernatant (soluble fraction) were prepared from the 9000 *g* supernatant by centrifugation at 105,000 *g* for 1 hr. The microsomal pellet was suspended in 1·15% KCl and recentrifuged at 105,000 *g* for 1 hr. Washed microsomes thus obtained were resuspended in 1·15% KCl. Three ml of each preparation was equivalent to 1 g wet wt. of liver. No decrease of activity in these preparations was observed on 2 weeks of storage in a deep freezer (−5°).

A typical incubation mixture, unless otherwise mentioned, consisted of 0·6 ml enzyme solution (equivalent to 0·2 g liver), 1·0 μmole of unlabeled prolintane or uniformly labeled ^{3}H-prolintane (sp. act., 1·29 μCi/mg), 20 μmoles nicotinamide, 10 μmoles $MgCl_2$ and 0·1 M phosphate buffer, pH 7·4, to a final volume of 2 ml. If necessary, 3 μmoles NADPH or NADH was added as cofactor. A reaction mixture without prolintane was used as control. Incubation was performed aerobically for 1 hr at 37°. The enzyme reaction was stopped by acidification with 0·2 ml of 2 N HCl. The reaction mixture, after addition of 0·1 g NaCl, was extracted with 5 ml $CHCl_3$ by a mechanical shaker for 20 min. After centrifugation, 4 ml of the $CHCl_3$ phase was pipetted into a glass-stoppered tube. Chloroform was evaporated to dryness, and the residue was dissolved in 0·2 ml acetone. An aliquot of this acetone solution was applied to gas chromatography for quantitative analysis. The recoveries of unchanged prolintane and formed oxoprolintane were demonstrated to be 95 ± 5 per cent in this procedure. All of the results shown in the figures and tables are given as mean values of duplicate experiments.

Gas–liquid chromatography (GLC) and thin-layer chromatography (TLC). Gas chromatographic analysis was performed with a Shimadzu model GC-1C gas chromatograph equipped with a hydrogen flame ionization detector. The column used was a glass U-shaped tube (4 mm × 2·625 m), packed with 1·5% OV-1 on Shimalite W (100–200 mesh). The column temperature was maintained at 180°, the sample chamber and detector temperature at 210°. Nitrogen was used as the carrier gas with a flow rate of 40 ml/min (2·8 kg/cm^2).

Thin-layer plates of Silica gel (Kieselgel G, Merck; 0·25 mm thick, activated at 105° for 30 min) were used in the present experiments. Solvent systems used were: (A) benzene–acetone–methanol (7:1:2), and (B) $CHCl_3$–diethylamine (9:1). Dragendorff reagent was used for detection of the metabolites.

The reference compounds, M–1 [1-phenyl-2-(2-oxopyrrolidino) pentane], M–2 [1-(4-hydroxyphenyl)-2-pyrrolidinopentane] and M–3 [1-(4-hydroxyphenyl)-2-(2-oxopyrrolidino)pentane] were the same as those described in the previous paper.[1]

Radioactivity measurements. An Aloka LSC-502 liquid scintillation counter was used for measurement of the radioactivity. Radioactivity of the extract, which was dissolved in methanol, was measured in 15 ml of a toluene scintillator containing 0·4% 2,5-diphenyloxazole (PPO) and 0·01% 1,4-bis-2-(4-methyl 5-phenyloxazolyl) benzene (POPOP). Radioactivity of aqueous samples was counted in the above scintillator, to which 5% BBS-3(Beckman, Bio-Solv) and two drops of saturated ascorbic acid solution were added. Activity on the TLC plate was counted by scraping the area along a 1-cm length of the plate into a scintillation vial containing 10 ml of the toluene scintillator described above. In some experiments, radioactivity on the TLC plate was determined by a thin-layer chromato scanner, model JTC 201.

RESULTS

Enzymatic formation of oxoprolintane. One μmole ^{3}H-prolintane was incubated with rabbit liver 9000 *g* supernatant or microsomes under aerobic conditions, and the $CHCl_3$ extract was applied to TLC with solvent system A and scanned. As shown in Fig. 1, the rabbit liver 9000 *g* supernatant produced M–1 (R_f 0·66) as the major

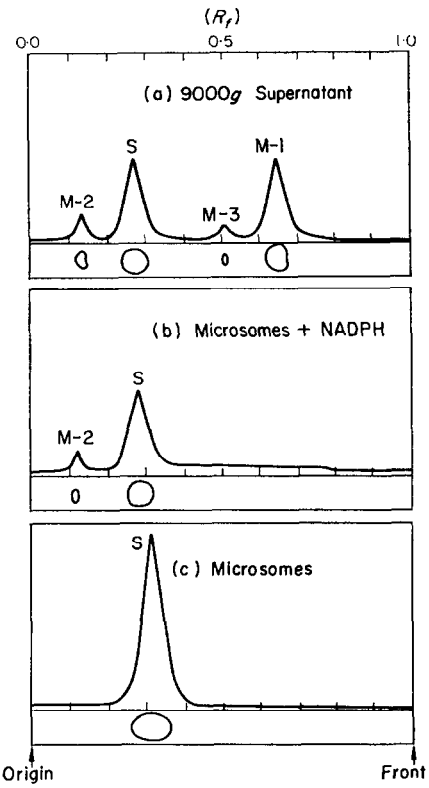

FIG. 1. Radiochromatograms of extracts from 9000 *g* supernatant and microsomal systems incubated with ^{3}H-prolintane. S, prolintane; M–1, oxoprolintane; M–2, 1-(4-hydroxyphenyl)-2-pyrrolidinopentane; M–3, 1-(4-hydroxyphenyl)-2-(2-oxopyrrolidino)pentane; solvent system A, detection of metabolites, Dragendorff reagent.

metabolite, andM–2 (R_f 0·14) and M–3 (R_f 0·52) as minor metabolites. The R_f values of these metabolites were the same as those of authentic samples of 1-phenyl-2-(2-oxopyrrolidino)pentane (oxoprolintane), and 1-(4-hydroxyphenyl)-2-pyrrolidinopentane and 1-(4-hydroxyphenyl)-2-(2-oxopyrrolidino)pentane, respectively, isolated from the urine of a rabbit given prolintane. The identity of these metabolites with the reference samples was further proved by additional TLC with solvent system (B), showing R_f values of 0·80 (M–1), 0·50 (M–2) and 0·40 (M–3), and by GLC giving retention times of 4·0, 4·8 and 11·5 min respectively.

Microsomal fraction alone, if it was fortified with NADPH, was able to metabolize prolintane, and the activity was almost the same as that with the 9000 *g* supernatant. But no metabolite was detected in this system, except a small amount of M–2 (Fig. 1). Without NADPH, no disappearance of prolintane was observed with microsomes.

These results showed that 9000 *g* supernatant was required for oxoprolintane formation from prolintane *in vitro*.

Effect of substrate concentration on oxoprolintane formation. Preliminary studies were carried out with rabbit liver homogenate to approximate optimal incubation conditions for oxoprolintane formation. The maximum activity of 0·4 μmole product/hr was obtained when the incubation was carried out with a substrate concentration of 1 μmole prolintane/2 ml. Higher substrate concentrations were inhibitory; for example, the activity described above was reduced by about 30 per cent at the concentration of 2 μmoles prolintane/2 ml. Therefore, as a standard condition, 0·2 g liver was incubated with 1 μmole prolintane in 0·1 M phosphate buffer, pH 7·4.

Intracellular localization of and cofactor requirement for prolintane metabolism. All of the activity for oxoprolintane formation from prolintane in liver homogenate was found in the supernatant fraction (Table 1). Either microsomes or soluble fraction

TABLE 1. INTRACELLULAR LOCALIZATION OF AND COFACTOR REQUIREMENT FOR PROLINTANE METABOLISM*

Enzyme system	Prolintane recovered (%)	Oxoprolintane formed (%)
Homogenate	75·4	18·6
Supernatant	46·3	27·3
Microsomes	90·5	0·0
Soluble fraction	98·2	0·0
Microsomes + soluble fraction	71·4	10·3
Supernatant (in N_2)	68·0	0·0
Microsomes + NADPH	53·2	0·0
Microsomes + NADH	80·3	0·0
Microsomes + NADP	100·3	0·0
Microsomes + NAD	95·7	0·0
Microsomes + NADPH +NADH	45·8	0·0
Boiled microsomes + NADPH	101·3	0·0

* The incubations were carried out as described in Methods. A total of 3·0 μmoles cofactor was added at 10-min intervals to the incubation system.

alone was inactive, but when they were recombined the activity was restored. Microsomes plus NADPH were capable of increasing the disappearance of prolintane, although no lactam formation could be observed. NADH was also active for prolintane metabolism, but it could take the place of NADPH only in part. Simultaneous addition of both reduced cofactors showed an additive effect on this metabolism. On the other hand, oxidized cofactors did not show significant effect. In nitrogen atmosphere prolintane could not be metabolized to oxoprolintane by the supernatant fraction, although a considerable amount of added prolintane disappeared during the incubation.

These results strongly suggested that prolintane might not be oxidized directly to

oxoprolintane, but to some precursor by the enzyme system in microsomes and, in turn, this precursor could be converted to oxoprolintane by soluble fraction.

Role of microsomes and soluble fraction in oxoprolintane formation. It was evident from the above results that both microsomes and soluble fraction were indispensable for oxoprolintane formation in rabbit liver. However, the role of these subcellular fractions in this metabolic reaction was still obscure, although the process was assumed to consist of two sequential reactions, namely, initial oxidation of the α-carbon atom of the pyrrolidine ring with microsomes to form "hydroxyprolintane", which was in turn followed by dehydrogenation with soluble fraction to produce oxoprolintane.

Therefore, an attempt was made to prove the above assumption as follows (see Table 2). In the first case (system A), incubation was carried out with microsomes, and after inactivation by boiling, the incubation mixture was reincubated with soluble

TABLE 2. EFFECT OF STEPWISE INCUBATION WITH MICROSOMES AND SOLUBLE FRACTION ON OXOPROLINTANE FORMATION

System*	Prolintane recovered (%)	Oxoprolintane formed (%)
A	45·8	20·3
B	39·4	0·0
C	33·0	19·5

* All systems were preincubated with microsomes + NADPH for 1 hr as described in Methods. In the case of systems A and B, preincubation mixtures were inactivated by boiling for 1 min and then reincubated as follows: system A, the cooled mixture was reincubated with soluble fraction for another hour; system B, the $CHCl_3$ extract of the preincubation mixture was dissolved in 0·1 M phosphate buffer and reincubated with soluble fraction. In the case of system C, the preincubation mixture was reincubated at once with soluble fraction.

fraction. In this system, prolintane was metabolized to oxoprolintane as in non-inactivating system C. However, if the $CHCl_3$ extract of the preincubation mixture with microsomes was further incubated with soluble fraction, oxoprolintane was no longer produced (system B). In this case, the recovery of unchanged prolintane was decreased as well. From these observations, it was assumed that the intermediary product of the microsomal system could not be extracted with $CHCl_3$ or was very labile.

In order to obtain further evidence, large-scale incubations were carried out with supernatant and microsomal fractions, using ^3H-prolintane as substrate. The results indicated that radioactivity extracted with $CHCl_3$ was almost identical in both incubation systems (per cent recovery of added radioactivity was 65 and 70 per cent, respectively, in the supernatant and microsomal fractions). In contrast, a great difference was observed in the results of the GLC determination of extracts (Table 3). In the

TABLE 3. GAS LIQUID CHROMATOGRAPHIC DETERMINATION OF RECOVERED PROLINTANE AND FORMED OXOPROLINTANE IN EXTRACTS* OF SUPERNATANT AND MICROSOMAL SYSTEMS

System†	Prolintane recovered‡ (%)	Oxoprolintane formed‡ (%)
Supernatant	48·8	16·7
Microsomes + NADPH	59·5	0·0

* Extractions were carried out three times with 20 ml $CHCl_3$.
† Incubation systems used were 5-fold greater than standard system. Five μmoles (1530·5 mμ) ^{3}H-prolintane and 9·26 μmoles NADPH were used.
‡ Values are expressed as per cent of added prolintane.

supernatant system, about 49 per cent of added prolintane was recovered as the unchanged form and about 17 per cent was converted to oxoprolintane, whereas in the microsomal system, there was no metabolite peak, other than that of unchanged prolintane showing about 60 per cent recovery.

From these results, the extractable radioactivity in the supernatant system was shown to consist of prolintane and oxoprolintane. However, in the microsomal system, the per cent recovery of unchanged prolintane determined by GLC seemed to be a little less than the value calculated from the radioactivity. This possibly suggested that the intermediate formed in the microsomal system was extractable, but not measurable with GLC because of its instability.

DISCUSSION

The present studies indicate that prolintane is metabolized to oxoprolintane by two sequential reactions in rabbit liver. The first step, hydroxylation in the α-position of the pyrrolidine ring, is catalyzed by liver microsomes and requires NADPH and O_2. The second step, dehydrogenation to oxoprolintane, is catalyzed by soluble fraction.

Hammer *et al.*[5] proposed two possible mechanisms for oxotremorine formation from tremorine as follows: (1) through direct dehydrogenation of "hydroxytremorine", and (2) through cyclization of *N*-(4-pyrrolidino-2-butynyl)-γ-aminobutyric acid.

In previous experiments *in vivo*,[1] the authors isolated the ring-opened metabolite, *N*-(1-phenyl-2-pentyl)-γ-aminobutyric acid (PPGABA), from the urine of rabbits given prolintane. In order to elucidate the possibility of participation of PPGABA as an obligatory intermediate in oxoprolintane formation, PPGABA was incubated with rabbit liver homogenate or 9000 *g* supernatant. The result, however, did not show any formation of oxoprolintane. On the basis of the results obtained here, the transformation of prolintane to oxoprolintane may be proposed as shown in Fig. 2.

The postulated "hydroxyprolintane" has not been identified definitively, beause it may be unstable enough to exhibit aldehyde properties. If once an aldehyde formed, it would probably not be detectable with GLC because of polymerization at the injection port temperature (210°); additional incubation of the aldehyde with soluble fraction should no longer give oxoprolintane, but PPGABA. This idea was also

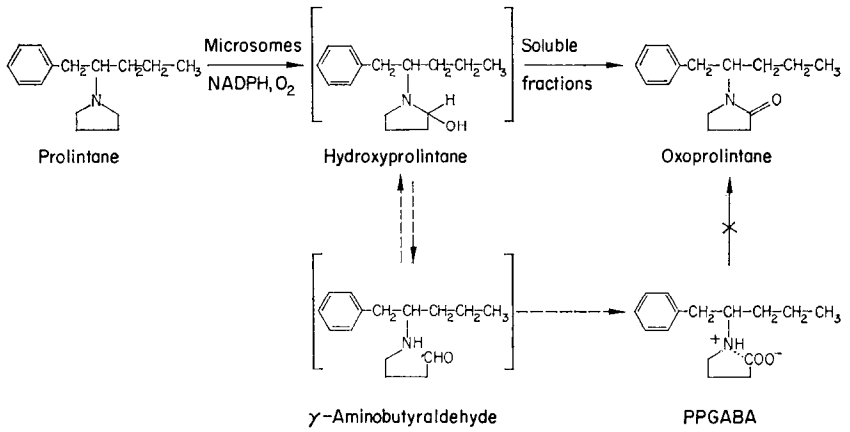

FIG. 2. Possible mechanism of oxoprolintane formation by rabbit liver.

supported by our preliminary observation that the Ag_2O-oxidation product of the aldehyde, which was derived from acid treatment of the microsomal product ("hydroxyprolintane"), is identical chromatographically with PPGABA.

These results strongly suggest that PPGABA is not necessarily an obligatory intermediate, and direct dehydrogenation of "hydroxyprolintane" must be more important in the formation of oxoprolintane from prolintane in rabbit liver.

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