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# OXIDATIVE DEAMINATION OF CYCLOHEXYLAMINE AND ITS HOMOLOGS BY RABBIT LIVER MICROSOMES

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**Abstract**—Cyclohexylamine (CHA) and its homologs, cyclopentylamine (CPA) and cycloheptylamine (CHPA), which formed the type II spectral changes in hepatic microsomes, were deaminated to the corresponding ketones by rabbit liver microsomes in the presence of NADPH and molecular oxygen. The alicyclic ketones were then reduced to the alcohols, of which average percentages in the deaminated products were approximately 75 (CHA), 3 (CPA) and 14 (CHPA). The apparent $K_m$'s for these amines were 5.0 mM (CHA), 4.2 mM (CPA) and 2.1 mM (CHPA), and $V_{max}$'s were 11.0 (CHA), 42.1 (CPA) and 16.4 (CHPA) nmoles/mg protein/30 min. The activity of deamination of these alicyclic primary amines was dependent on both NADPH and oxygen, and inhibited by carbon monoxide, SKF 525A, metyrapone, potassium cyanide and mercuric chloride. These experiments indicate that the deamination of the alicyclic primary amines is catalyzed by a microsomal cytochrome P-450-dependent monooxygenase system in the rabbit liver. Cyclohexanone oxime and other oximes were also identified from the incubation mixtures, and these oximes are suggested as possible intermediates of microsomal deamination of alicyclic primary amines.

Sodium cyclamate (CHS), which had been widely used as an artificial sweetening agent for various foods and drugs, was found to be partially metabolized to cyclohexylamine (CHA), cyclohexanol and cyclohexanone in animals and man [1–3], and was banned in 1969 for general use in foods and medicines in Japan owing to the chromosome-damaging effect of CHA [4] and the suspicion of carcinogenicity [5]. At present it is well known that CHS is converted to CHA by gut flora [6,7], and Niimura *et al.* reported that CHS was hydrolysed to CHA and inorganic sulfate by cyclamate sulfamatase [EC 3.10.1.2] of *Pseudomonas* species isolated from the feces of guinea pigs [8].

The metabolism of CHA was studied in detail by Renwick and Williams, who detected cyclohexanol, 1,2-cyclohexanediol, 3-aminocyclohexanol and so forth in the urine of man and certain animals [9]. CHA is known to be a weak inhibitor of mammalian monoamine oxidase [10] and can not be deaminated by this enzyme, but by CHA oxidase, which was prepared from the above bacterial strain in our laboratory and catalyzed the deamination of alicyclic primary amines specifically [11].

On the other hand, there is no report on the deamination of alicyclic primary amines such as CHA and its homologs by the enzyme in mammalian tissues, although amphetamine and its derivatives are known to be deaminated by rabbit liver microsomes [12]. In relation to our studies on CHA metabolism in mammals, the present paper deals with the deamination of CHA and its congeners in rabbit liver microsomes.

## MATERIAL AND METHODS

*Reagents.* Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, $NADP^+$, NADPH and NADH were purchased from Boehringer Manheim GmbH. Nicotinamide, cyclohexylamine hydrochloride, cyclohexanone oxime, 2,5-diphenyloxazole (DPO) and 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) were obtained from Wako Pure Chemical Industries, Ltd.; cyclohexanone, cyclohexanol, cyclopentanone, cyclopentanol, cycloheptanone, cycloheptanol and cycloheptylamine from Tokyo Chemical Industry Co. Ltd.; and cyclopentylamine from Aldrich Chemicals Co. [$^{14}$C]Cyclohexylamine hydrochloride (uniformly labelled) with a specific activity of 12.6 μCi/mg was purchased from Mallinckrodt Chemical Works. SKF 525A* (Smith Kline and French, Ltd.) and metyrapone† (Ciba–Geigy Ltd.) were gifts from the Department of Pharmacology in this institute. Cyclopentanone oxime and cycloheptanone oxime were synthesized by the usual method for the preparation of oxime using ketone and hydroxylamine. Cyclopentylamine and cycloheptylamine were purified as hydrochloride salts.

*Preparation of microsomal fraction.* Male albino rabbits (1.8–3.0 kg) were starved for 24 hr, stunned on the head and exsanguinated. The liver was immediately removed and homogenized with 4 vol. ice-cold 0.15 M KCl in 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA in a Potter–Elvejem type teflon glass homogenizer. The homogenate was centrifuged at 10,000 *g* for 10 min and the supernatant fluid was sedimented at 105,000 *g* for 60 min. The pellets could be stored at −20° without any appreciable loss of the deamination activity for a month. The protein concentration was determined by the method of Lowry *et al.* [13] with bovine serum albumin as standard.

*Assay of deamination activity.* The reaction mixtures containing 0.4 mM NADP, 10 mM nicotinamide, 5 mM glucose 6-phosphate, 5–20 mM substrates, 5 mM $MgCl_2$, 30 mM KCl, 50 mM potassium phosphate buffer (pH 7.4), microsomal fraction (2–6 mg protein/ml) and 16 units of glucose 6-phosphate dehydrogenase in a final volume of 16 ml were incubated at

* β-Diethylaminoethyl-2, 2-diphenylvalerate.
† 2-Methyl-1,2-di(3-pyridyl)-1-propane.

37° for 30 min under an atmosphere of air with moderate shaking, unless otherwise stated. At the end of incubation periods the vessels were immersed in ice-cold water and the reaction products were extracted with ethyl ether (22 ml) after the addition of sodium chloride (5 g). The ether extracts were separated after centrifugation at 2,000 r.p.m. and concentrated to about 3 ml at room temperature. The concentrate was subjected to gas chromatography. The deamination activity was expressed as the sum of the alicyclic ketone and alcohol produced (nmoles/mg protein/30 min).

*Gas chromatography.* Gas-chromatographic analyses were performed on a Shimadzu model GC-5A gas chromatograph equipped with hydrogen flame ionization detectors using nitrogen as carrier gas at a flow rate of 60 ml/min. The following columns were employed. Column I was 2-m glass tubing 3-mm i.d., packed with 20 % Carbowax 20 M plus 2.5% NaOH on acid-washed, DMCS-treated Chromosorb G, 60–80 mesh. Column II was 1-m glass tubing 3-mm i.d., packed with 7.5 % Carbowax 20 M on acid-washed, DMCS-treated Chromosorb W, 80–100 mesh. Column III was 1.5-m glass tubing 3-mm i.d., packed with 10 % Apiezon Grease L on acid-washed, DMCS-treated Chromosorb W, 80–100 mesh. The standard samples of ketones, alcohols and ketone oximes were used to quantify the amount of the extracted products by comparison with each peak height.

*Gas chromatography–mass spectrometry (g.c.–m.s.).* LKB 9000 and Hitachi RMU-6MG gas chromatograph–mass spectrometer fitted with similar g.c. columns were utilized, employing helium as carrier gas at ionization potential of 20 or 70 eV.

*Thin-layer chromatography (t.l.c.).* Silica gel spot films were used for t.l.c. of CHA and related compounds. Chromatograms were scanned with a radioscanner (TRM-1B, Aloka). The solvent system; $CHCl_3$:EtOH:HAc(8:2:1), was mainly used: $R_f = 0.92$ for cyclohexanone oxime; $R_f = 0.14$ for CHA. Radioactivity of each sample was counted in toluene scintillator containing 5 g DPO and 0.3 g POPOP per liter with a scintillation counter (LSC-651, Aloka).

*Inhibition experiment with carbon monoxide.* The air in Warburg-type flasks was replaced by a mixture of $O_2$, CO and $N_2$, in which the content of $O_2$ was always kept at 5 % (v/v) and that of CO was varied from 5 to 10, 15, 20 and 35 % (v/v), so that the remaining part was occupied with $N_2$. The control experiment was conducted with a mixture of $O_2$ and $N_2$ (5:95, v/v). The reactions were carried out in the dark under the same conditions as described above.

*Measurements of substrate-induced difference spectra.* Spectral changes produced by the addition of substrates to the microsomal suspension were determined in a Hitachi model 356 two-wavelength double-beam spectrophotometer according to the method of Schenkman *et al.* [16]. A microsomal suspension in 0.1 M potassium phosphate buffer, pH 7.4, containing 2.0 mg of microsomal protein per ml was equally divided into the sample and reference cuvettes. Various amounts of CHA, CPA and CHPA were added to the sample cuvettes, and the spectral changes produced were recorded between 370 and 500 nm.

* Mean value ± S.D.

## RESULTS

*Identification of metabolites.* The ether extracts of the reaction mixture for enzyme assay were used for the identification of metabolites by gas chromatography and g.c.–m.s. The data of metabolites were identical with those of authentic samples (Table 1). The alicyclic ketones and their oximes have prominent molecular ion peaks ($M^+$): cyclohexanone 98, cyclohexanone oxime 113, etc. The alicyclic alcohols have small molecular ion peaks and relatively prominent peaks ($M^+-18$): cyclohexanol 82, cycloheptanol 96, etc. In the case of a small amount of metabolites, we confirmed them by mass fragmentography and gas chromatography.

Isotope dilution methods were also used for the identification of cyclohexanol and cyclohexanone oxime. [$^{14}$C]CHA (10 $\mu$Ci) was incubated in the same manner as cold run. [$^{14}$C]Cyclohexanol in the concentrate was diluted with non-radioactive cyclohexanol and identified as its phenylurethane derivative, m.p. 84°, whose specific activity (307. 5 ± 1.5 d.p.m./mg)* remained constant during 4-recrystallizations from pet–ether (b.p. 40–60°) within the limits of experimental errors. A portion of ether concentrate was subjected to t.l.c. to separate [$^{14}$C]cyclohexanone oxime on the chromatogram, and the spot fraction ($R_f = 0.92$) corresponding to cyclohexanone oxime was eluted with ethanol. The concentrate of the ethanol eluate was used for dilution analysis. [$^{14}$C]Cyclohexanone oxime, m.p. 88°, was also recrystallized to the constant specific activity (41.7 ± 4.6 d.p.m./mg)* from pet–ether (b.p. 40–60°), although its radioactivity was low.

*Requirements of deamination in rabbit liver microsomes.* The deamination rates of CPA, CHA and CHPA at 20 mM are compared in Table 2. In microsomal incubations with NADPH-generating system, the deamination rate of CPA was about 4 times higher than that of CHA, and nearly twice as high as that of CHPA. Without NADPH-generating system, the deamination did not occur in these amines, indicating the requirement for NADPH. NADH took the place of NADPH by 1/3–2/3 of the activity at higher concentration (4 mM). The deamination activity of these amines decreased with decreasing oxygen contents in nitrogen. This fact demonstrates a requirement for molecular oxygen in this deamination system.

*Percentages of alicyclic alcohols in the deaminated products.* In microsomal incubation mixtures of alicyclic primary amines, two deaminated products, alicyclic ketone and alcohol, were found. The percentages of the alcohols in the deaminated products are shown in Table 2. When these amines were incubated with NADPH-generating system, the percentages were 75 (cyclohexanol), 14 (cycloheptanol) and 2 (cyclopentanol). It is noticeable that this result is the reverse of the order of deamination rates. The percentages of the alcohols were higher in the incubation with NADH than with NADPH, and also higher under nearly anerobic condition and at 5 % oxygen in nitrogen than under air with NADPH-generating system.

*Time course of CHA deamination.* The time course of CHA deamination was followed by determining the formation of cyclohexanone, cyclohexanol and cyclohexanone oxime (Fig. 1). The amounts of cyclohexanone and its oxime increased linearly for about 10 min and then reached the apparent constant state at lower

Table 1. Identification of *in vitro* metabolites of alicyclic primary amines

| | Gas chromatography | | | Gas chromatography–mass spectroscopy | |
|---|---|---|---|---|---|
| | Retention time (min) column | | | Relative abundance of the molecular ion and prominent fragment ions | |
| | I | II | III | $M^+$ (%) | *m/e* (%) |
| | (105°) | (105°) | (80°) | | |
| Cyclopentanone | 5.0 | 0.9 | 2.7 | 84 (52) | 56 (33) 55 (100) 42 (14) 41 (41) 39 (18) |
| Cyclopentanol | 8.5 | 1.5 | 2.7 | 86 (25) | 68 (12) 67 (13) 58 (23) 57 (100) 44 (42) 41 (19) |
| Cyclopentanone oxime | — | 13.2 | 9.0 | 99 (29) | 84 (10) 82 (39) 80 (6) 67 (22) 57 (9) 55 (100) 54 (56) 53 (11) 44 (16) 42 (38) 41 (66) 39 (56) |
| | (128°) | (130°) | (90°) | | |
| Cyclohexanone | 5.2 | 1.1 | 2.6 | 98 (45) | 83 (9) 70 (23) 69 (28) 56 (11) 55 (100) 42 (50) 41 (17) |
| Cyclohexanol | 7.5 | 2.6 | 2.65 | 100 (5) | 82 (44) 67 (15) 57 (100) 56 (15) 54 (10) 44 (26) |
| Cyclohexanone oxime | — | 6.8 | 9.0 | 113 (100) | 98 (31) 96 (35) 85 (31) 81 (36) 72 (56) 69 (40) 68 (51) 67 (31) 59 (75) 55 (76) 54 (99) 42 (33) |
| | (145°) | (145°) | (100°) | | |
| Cycloheptanone | 5.7 | 0.8 | 4.9 | 112 (70) | 84 (48) 83 (23) 69 (41) 68 (100) 56 (48) 55 (52) |
| Cycloheptanol | 8.7 | 1.3 | 5.6 | 114 (0.2) | 96 (35) 81 (86) 71 (24) 70 (21) 68 (62) 67 (25) 57 (100) |
| Cycloheptanone oxime | — | 6.7 | 13.3 | 127 (56) | 112 (29) 110 (37) 99 (100) 98 (25) 95 (63) 83 (30) 82 (52) 73 (41) 68 (27) 67 (42) 59 (39) 54 (41) |

The columns I, II, and III are described in Materials and Methods. The column temperatures are described in parentheses. The detector and injection port temperatures were adjusted to about 20–30° above the column temperatures.

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Table 2. Effects of various incubation conditions on the microsomal deamination of alicyclic primary amines and the percentages of alicyclic alcohols in the deaminated products

| Incubation condition | Deamination rate and percentage of the alcohol nmoles/mg protein/30 min (% of alcohol/ketone+alcohol) | | |
|---|---|---|---|
| | Cyclopentylamine | Cyclohexylamine | Cycloheptylamine |
| Complete system* | 34.8 (2) | 8.8 (75) | 14.9 (14) |
| −NADPH-generating system | 0.2 | 0.1 | 0.1 |
| Boiled microsomes† | 0 | 0 | 0 |
| 5 % $O_2$ in nitrogen | 15.3 (3) | 4.9 (83) | 12.7 (16) |
| Nearly anaerobic condition‡ | 4.7 (3) | 2.9 (91) | 3.8 (23) |
| NADPH§, 4 mM | 40.2 (1) | 6.7 (22) | 12.1 (6) |
| NADH§, 4 mM | 17.7 (16) | 2.6 (96) | 7.9 (23) |

* The concentration of each amine was 20 mM. Other reaction conditions were as described in Materials and Methods.
† Microsomes were denatured by immersing in boiling water (97°) for 10 min.
‡ Air was replaced by pure nitrogen gas (purity, 99.99 %).
§ 4 mM NADPH or NADH was used instead of NADPH-generating system.

levels. On the other hand, the formation of cyclohexanol increased constantly for about 30 min at higher levels and continued to increase slowly up to about 90 min.

*Effect of* pH. The influence of pH on CHA deamination in rabbit liver microsomes is shown in Fig. 2. Cyclohexanone and its oxime were produced in alkaline pH range preferentially. The amount of the oxime was smaller than that of other two products, and nearly proportional to that of the ketone. The amount of cyclohexanol was larger than other two products, and maximum at about pH 7.4.

We assumed the activity of this deamination system to be the sum of alicyclic ketone and alcohol, so the optimum pH seemed to be the range from 7.4 to 7.8 (Fig. 2). Therefore, most experiments were conducted at pH 7.4 for 30 min.

*Kinetic studies.* Lineweaver–Burk plots of deamination of alicyclic primary amines were linear between 2 and 50 mM of the amines. At higher concentrations of the amine (200 and 400 mM), substrate inhibition was observed. The apparent $K_m$'s for these amines obtained from the linear plots were 5.0 mM for CHA, 4.2 mM for CPA and 2.1 mM for CHPA. There was little difference in the apparent $K_m$'s. The maximum rate of deamination, $V_{max}$, was 11.0 (CHA), 42.1 (CPA) and 16.4 (CHPA) nmoles/mg protein/30 min. Their ratios were similar to those of the rates in Table 2. and the $V_{max}$ of CPA was the largest among the three amines.

*Conversion of alicyclic ketone to alicyclic alcohol.* In order to determine the metabolic sequence of two deaminated products in this system, each alicyclic ketone and each alcohol was independently incubated in a similar way as described in Materials and Methods. Fig.

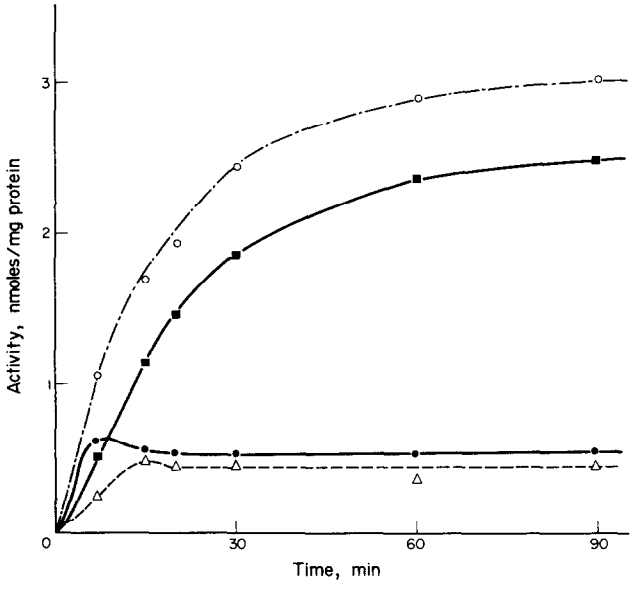


Fig. 1. Time course of deamination of cyclohexylamine. The reactions were carried out at 5 mM cyclohexylamine. Other reaction conditions were as indicated in Materials and Methods. ●——●, Cyclohexanone formed; ■——■, cyclohexanol formed; △- - - -△, cyclohexanone oxime formed; ○—·—·—○, the sum of cyclohexanone and cyclohexanol.

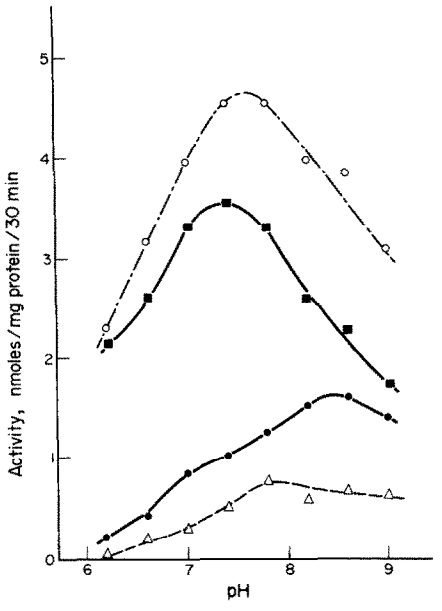


Fig. 2. Effect of pH on deamination of cyclohexylamine. The reactions were carried out at 5 mM cyclohexylamine in a mixture of 50 mM potassium phosphate buffer (pH 6.2–7.8) and 50 mM Tris–hydrochloride buffer (pH 8.2–9.0). Other reaction conditions were as indicated in Materials and Methods. ●——●, Cyclohexanone formed; ■——■, cyclohexanol formed; △- - - -△, cyclohexanone oxime formed; ○—·—·—○, the sum of cyclohexanone and cyclohexanol.

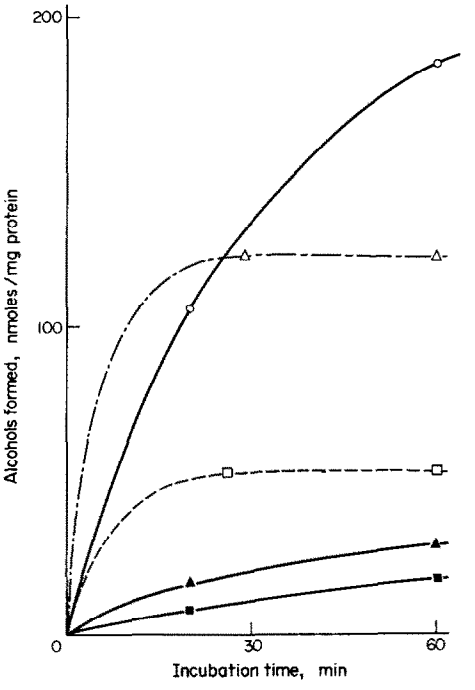


Fig. 3. Reduction of alicyclic ketones to alicyclic alcohols. The reactions were carried out at 37° with NADPH-generating systems as described in Materials and Methods, NADPH (3.7 mM), or NADH (4.3 mM). Twice-washed microsomes (1.4 mg protein/ml) were used for the incubations of cyclopentanone (4.1 mM), cyclohexanone (4.2 mM) and cycloheptanone (3.7 mM). With NADPH-generating system: ■——■, cyclopentanol formed from cyclopentanone; ○——○, cyclohexanol formed from cyclohexanone; ▲——▲, cycloheptanol formed from cycloheptanone. Without NADPH-generating system: □- - - -□, cyclohexanol formed from cyclohexanone with NADPH (3.7 mM); △—·—·—△, cyclohexanol formed from cyclohexanone with NADH (4.3 mM).

3 shows that cyclohexanone was rapidly reduced to cyclohexanol in the presence of NADPH or NADH. With NADPH-generating system, the reduction rate of cyclohexanone (about 5 nmoles/mg protein/min) was nearly 7-fold higher than that of cycloheptanone, and nearly 13-fold higher than that of cyclopentanone. The order of reduction rates of the ketones to the alcohols was the same as that of percentages of the alcohols in the deaminated products in Table 2 (cyclohexanol > cycloheptanol > cyclopentanol). The oxidation of alicyclic alcohols to the ketones was not observed in the same incubation mixture. These facts imply that alicyclic ketones are the precursors of the corresponding alcohols in the deamination steps.

*Effects of inhibitors.* The requirement for NADPH and oxygen in the deamination of alicyclic primary amines implied a participation of the mixed-function oxidase. So we further studied the involvement of cytochrome P-450, which plays an important role as the oxygen-activating enzyme in various microsomal oxidations.

NADPH-dependent deamination of alicyclic primary amines was inhibited by carbon monoxide (Table 3 and Fig. 4), which combines with the reduced form of cytochrome P-450 with high affinity and inhibits the reaction involving cytochrome P-450 [14]. The inhibitory effect of carbon monoxide on deamination of these amines, varying the ratios of carbon monoxide to oxy-

Table 3. Effects of inhibitors on the microsomal deamination of alicyclic primary amines

| | Relative activity of deamination (% of the control) | | |
|---|---|---|---|
| | Cyclopentylamine | Cyclohexylamine | Cycloheptylamine |
| Control | 100 | 100 | 100 |
| SKF 525A (0.2 mM) | 43 | 61 | 56 |
| SKF 525A (1.0 mM) | 25 | 28 | 36 |
| Metyrapone (1.0 mM) | 43 | 46 | 71 |
| $HgCl_2$ (0.2 mM) | 3 | 5 | 2 |
| KCN (1.0 mM) | 51 | 58 | 58 |
| $CO:O_2$ (95:5, v/v)* | 32 | 34 | 30 |

* The relative activity is expressed as percentages of the control ($N_2:O_2 = 95:5$, v/v).

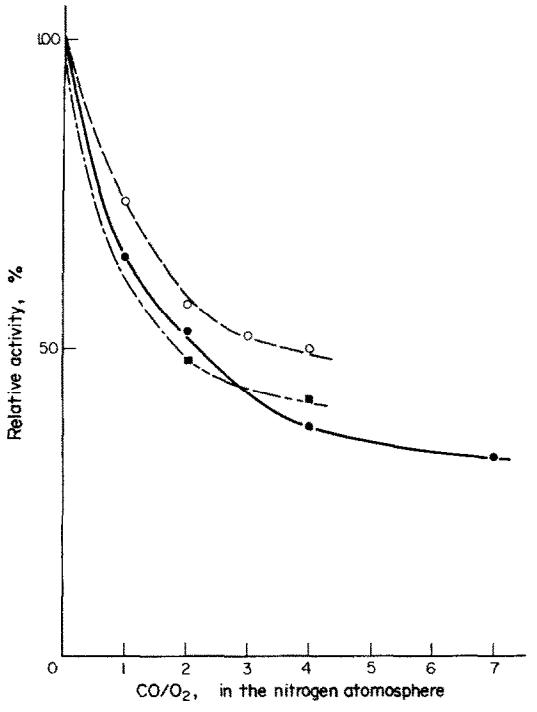


Fig. 4. Dependence of CO inhibition of deamination of alicyclic primary amines upon $CO/O_2$ ratio. Mixtures of $O_2$ (5 % v/v), CO and $N_2$ in various ratios were used as gas phases. The relative activities are expressed as percentages of the control in a gas mixture of $O_2$ and $N_2$ (5:95, v/v): ●——●, cyclopentylamine; ■——■, cyclohexylamine; ○——○, cycloheptylamine. Other reaction conditions were as indicated in Materials and Methods.

gen is shown in Fig. 4. In an atmosphere of 5 % oxygen and 5, 10, 15, 20 or 35 % carbon monoxide in nitrogen, the inhibition ratios became greater with increasing ratios of carbon monoxide to oxygen. In addition to the inhibitory effect of carbon monoxide, the deamination of these primary amines was inhibited by the microsomal oxidative inhibitors (Table 3); SKF 525A (39–57 per cent inhibition at 0.2 mM) and metyrapone (29–57 per cent inhibition at 1 mM). Further, 0.2 mM mercuric chloride almost completely inhibited the deamination and the addition of catalase (75–256 μg/ml) did not affect the deamination.

These results indicate that the deamination of alicyclic primary amines is catalyzed by microsomal cytochrome P-450-dependent monooxygenase system.

Table 3 indicates also the inhibitory effect of cyanide on the deamination (42–49 per cent inhibition at 1 mM KCN). This deamination system seemed to have lower sensitivity to cyanide than the fatty acid desaturation system in rat liver microsomes (50 per cent inhibition at about 0.1 mM KCN [15]) and higher sensitivity than many microsomal hydroxylation systems. We therefore investigated more precisely the inhibitory effect of cyanide with varying concentrations of CHPA. A Lineweaver–Burk plot of the deamination of CHPA in Fig. 5 appeared to be a noncompetitive inhibition of the deamination system by cyanide ($K_i$ = 1.5 mM KCN).

*Difference spectra of microsomes by alicyclic primary amines.* Oxidized cytochrome P-450 is known to interact with many nitrogen bases to produce spectral changes grossly characteristic of type II binding [16, 17]. Indeed, CHA, CPA and CHPA interacted with cytochrome P-450 and produced type II spectral changes in rabbit liver microsomes. The changes were characterized by the appearance of an absorption peak at 430 nm and a trough at about 410 nm in lower concentration of the amine and at about 393 nm in higher concentration of the amine. Typical spectral changes caused by the addition of CHA were shown in Fig. 6. The shift of a trough from 410 to 393 nm might be explained by the observation that a binding of each amine to the type a (high spin) P-450 was much weaker than the type b (low spin) P-450 in microsomes [17]. The spectral dissociation constant, $K_s$, for CHA calculated from the spectral changes from 430 nm was $K_1$ = 4 mM and $K_2$ = 60 mM.

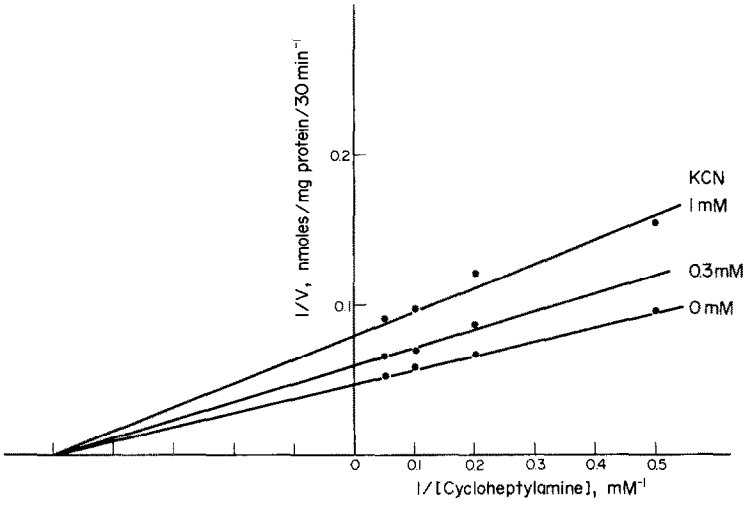


Fig. 5. Inhibitory effect of cyanide on deamination of cycloheptylamine (Lineweaver–Burk plot). The activities of microsomal deamination with varying concentrations of cycloheptylamine were estimated in the presence of 0, 0.3 and 1.0 mM KCN. The incubations were performed as described in Materials and Methods.

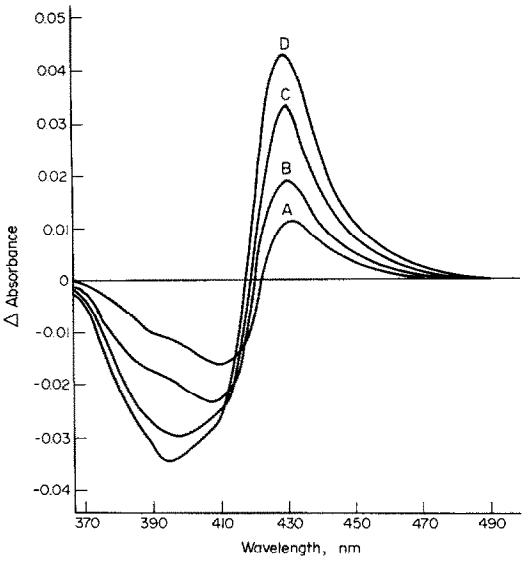


Fig. 6. Difference spectra of rabbit liver microsomes after addition of cyclohexylamine. The conditions are described in Materials and Methods. The spectra correspond to the following concentrations of cyclohexylamine: (A) 2.9 mM, (B) 10.9 mM, (C) 42.8 mM, (D) 70.2 mM.

## DISCUSSION

Since Axelrod firstly demonstrated the conversion of amphetamine to phenylacetone by rabbit liver microsomes [12], many papers have been published on the microsomal deamination of amphetamine and its derivatives [18–20]. However, to our knowledge, few reports have appeared concerning microsomal deamination of other kinds of amines.

In connection with CHA metabolism, Tokieda *et al.* have recently reported the deamination of CHA by CHA oxidase of *Pseudomonas* species isolated from the feces of guinea pigs and suggested the participation of intestinal bacteria in the deamination of CHA [11].

Renwick and Williams reported that some amounts of the deaminated metabolites of CHA were excreted in the urine of animals and man together with a large amount of unchanged CHA [9]. The excretion ratio (14 per cent) of deaminated metabolites of CHA in rabbit urine (dose, 500 mg/kg) [9] was lower than that (54 per cent) of amphetamine (dose, 10 mg/kg) [21]; however, the species differences of CHA metabolism were similar to those of amphetamine metabolism: the main metabolic reaction was deamination in rabbits and ring hydroxylation in rats [9, 21].

Based on these facts, we presumed that the intact liver would also play an important role in CHA metabolism. Indeed, we have confirmed here that alicyclic primary amines such as CHA and its homologs are deaminated to the corresponding ketones and alcohols by rabbit liver microsomes in the presence of NADPH and molecular oxygen.

Two deaminated products, ketones and alcohols, were found in microsomal incubation of these amines. In this work, we defined the deamination activity as the sum of the ketones and alcohols produced. The ratios of the alcohols decreased in the following order: cyclohexanol (75 per cent), cycloheptanol (14 per cent) and cyclopentanol (2 per cent). The reduction rates of the ketones by microsomal fractions with NADPH decreased in the same order, and seemed to be higher than the deamination rates of these amines (Fig. 3). Thus we might conclude that the ketones are initially produced in the deamination steps and subsequently reduced to the alcohols by microsomes with NADPH. Microsomal reduction of these ketones might be catalyzed by aromatic aldehyde ketone reductase [26] and/or NADPH-cytochrome *c* reductase with NADPH, and more rapidly by NADH-cytochrome $b_5$ reductase and/or alcohol dehydrogenase [27] with NADH. It is very interesting that the reduction rates of the ketones are the reverse of the order of deamination rates (CPA > CHPA > CHA).

Further, we have found that derivatives with an aromatic ring such as 1, 2, 3, 4-tetrahydro-1-naphthylamine and 1, 2, 3, 4-tetrahydro-2-naphthylamine were also deaminated to the corresponding ketones and alcohols by rabbit liver microsomes (unpublished results). The deamination rate of the latter derivative appeared to be similar to that of CHA.

The oxidative metabolism of a variety of nitrogenous compounds by hepatic microsomes can be catalyzed by either of two membrane-bound monooxygenase systems: the mixed function amine oxidase, a flavoprotein [22] or the cytochrome P-450-dependent monooxygenase system. Since the total activity of microsomal deamination of CHA and its homologs was inhibited by carbon monoxide, SKF 525A and metyrapone, we could conclude that the microsomal deamination of alicyclic primary amines was catalyzed by cytochrome P-450-dependent monooxygenase, although this deamination system was inhibited by cyanide.

On the other hand, it is now established that a prerequisite for hydroxylation reactions is a binding of the substrate to oxidized cytochrome P-450 of the monooxygenase system to form a complex. Two types of spectral changes, type I and type II, have most commonly been found to occur when substrates are added to a microsomal suspension [16, 17, 23]. It has been suggested that the type I spectral change is related to metabolism of the substrate, while the type II spectral change is caused by the direct interaction of the basic amine with the heme iron of cytochrome P-450 [16]. Only a few of the type II compounds are known to be substrates of the cytochrome P-450-dependent monooxygenase system. We have found here that alicyclic primary amines such as CHA and its homologs, which formed the type II spectral changes, are deaminated to the corresponding ketones by this system. However, we cannot deny the possibility that CHA and its homologs might be shown to produce a hidden type I component like aniline as studied by the hexobarbital saturation method [23].

The deamination system of alicyclic primary amines under our conditions seemed to be similar to an aniline hydroxylation system [24]. Firstly, both substrates were type II compounds. Secondly, their microsomal metabolic rates (deamination of alicyclic primary amines 0.3–1.2 nmoles/mg protein/min; aniline hydroxylation, about 1 nmole/mg protein/min [24]) were lower than those of the usual type I substrates. Thirdly, both systems were noncompetitively inhibited by cyanide [24, 25]. The lower rates of deamination might be

caused by weak affinity of these amines to cytochrome P-450, or by the inhibitory effects of these amines on the binding of oxygen with the common ligand of the cytochrome heme. For example, the apparent $K_m$'s for these amines (2–5 mM) were larger than those for other substrates in demethylation (0.3 mM for aminopyrine [16]) and hydroxylation (0.1 mM for hexobarbital [16]).

In this study, small amounts of the ketone oximes were always accompanied by the deaminated products. It thus remains an interesting problem whether the oximes are key intermediates or not in the deamination mechanism, as several workers have discussed in amphetamine [18–20]. If the oximes are possible intermediates, the following deamination steps would be presumed. The alicyclic primary amine might firstly be hydroxylated at the carbon atom adjacent to the nitrogen or at the nitrogen atom itself, and then the former (carbinolamine) might be converted to the oxime via imine [19] and the latter (*N*-hydroxylamine) might be oxidized to the oxime [20]. Finally, the ketone would result either from the hydrolysis of the oxime and the imine, or the ammonia elimination of the carbinolamine. Experimental evidence for these mechanisms is presently being sought through the use of $^{18}O_2$.

## REFERENCES


1. S. Kojima and H. Ichibagase, *Chem. pharm. Bull., Tokyo* **14**, 971 (1966).
2. S. Kojima and H. Ichibagase, *Chem. pharm. Bull., Tokyo* **16**, 1851 (1968).
3. S. Kojima and H. Ichibagase, *Chem. pharm. Bull., Tokyo* **17**, 2620 (1969).
4. S. Green, K. A. Palmer and M. S. Legator, *Fd. Cosmet. Tox.* **8**, 617 (1970).
5. J. M. Price, C. G. Biava, B. L. Oser and E. E. Vogin, *Science* **167**, 1131 (1970).
6. M. Asahina, T. Niimura, T. Yamaha and T. Takahashi, *Agr. biol. Chem.* **36**, 711 (1972).
7. B. S. Drasar, A. G. Renwick and R. T. Williams, *Biochem. J.* **129**, 881 (1972).
8. T. Niimura, T. Tokieda and T. Yamaha, *J. Biochem., Tokyo* **75**, 407 (1974).
9. A. G. Renwick and R. T. Williams, *Biochem. J.* **129**, 857 (1972).
10. S. Sarkar, R. Banerjee, M. S. Ise and E. A. Zeller, *Helv. chim. Acta* **43**, 439 (1960).
11. T. Tokieda, T. Niimura, F. Takamura and T. Yamaha, *J. Biochem., Tokyo* **81**, 851 (1977).
12. J. Axelrod, *J. biol. Chem.* **214**, 753 (1955).
13. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
14. O. Y. Cooper, S. Levin, S. Narasimhulu and O. Rosenthal, *Science* **147**, 400 (1965).
15. N. Oshino, Y. Imai and R. Sato, *Biochim. biophys. Acta.* **128**, 13 (1966).
16. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3**, 113 (1967).
17. C. R. E. Jefcoate, J. L. Gaylor and R. L. Calabrese, *Biochemistry* **8**, 3455 (1969).
18. H. B. Hucker, B. M. Michniewicz and R. E. Rhodes, *Biochem. Pharmac.* **20**, 2123 (1971).
19. C. J. Parli, N. Wang and R. E. McMahon, *Biochem. biophys. Res. Commun.* **43**, 1204 (1971).
20. A. H. Beckett and S. Al-sarraj, *J. Pharm. Pharmac.* **24**, 174 (1972).
21. L. G. Dring, R. L. Smith and R. T. Williams, *Biochem. J.* **116**, 425 (1970).
22. D. M. Ziegler and C. H. Mitchell, *Archs Biochem. Biophys.* **150**, 116 (1972).
23. J. B. Shenkman, *Biochemistry* **9**, 2081 (1970).
24. T. Matsubara and Y. Tochino, *FEBS Lett.* **52**, 77 (1975).
25. T. Matsubara and Y. Tochino, *Archs Biochem. Biophys.* **172**, 696 (1976).
26. H. W. Culp and R. E. McMahon, *J. biol. Chem.* **243**, 848 (1968).
27. N. J. Moir, W. L. Miller and J. L. Gaylor, *Biochem. biophys. Res. Commun.* **33**, 916 (1968).