^{13}C-NMR data for IV are listed in Table II.

Preparative-Scale Metabolism of Imipramine Using *A. flavipes* (ATCC 16795)—*A. flavipes* was grown in 4.0 liters of medium contained in 40 500-ml erlenmeyer flasks. After a 24-hr incubation of the Stage II cultures, a total of 800 mg of I·HCl was distributed evenly among the cultures; incubation was continued for 13 days (200 rpm, room temperature). The cultures were harvested by homogenization of the whole culture, followed by filtration (through büchner funnel). The aqueous culture filtrate (pH 8) was extracted with 10 500-ml portions of chloroform, and the combined chloroform layers were dried (sodium sulfate) and evaporated *in vacuo* to leave 663 mg of a brown oily residue.

Preparative TLC of 330 mg of the residue was carried out using silica gel G plates (2.0 mm thick, 20 × 20 cm) developed in ethyl acetate–methanol–ammonium hydroxide (81:15:4). The major band, corresponding to the *N*-oxide, was located by UV light and scraped off, and the silica gel was extracted exhaustively with 10% methanol–chloroform. Evaporation of the solvent afforded 112 mg of crude V, which was crystallized from benzene–hexane as white needles, mp 76–78° [lit. (24) mp 75–79°]. Direct comparison of V with an authentic sample of imipramine-*N*-oxide showed the two samples to be identical (melting point, mixed melting point, TLC, co-TLC, and superimposable IR spectra).

Preparative-Scale Metabolism of Imipramine (I) Using *M. griseo-cyanus* (ATCC 1207a)—A total of 1 g of I·HCl was fed to *M. griseo-cyanus* as described for *C. blakesleeana* and worked up as described for *F. oxysporum*. The chloroform residue (664 mg) was chromatographed over alumina[9] (90 g) using chloroform followed by an increasing percentage of methanol in chloroform. A total of 496 mg of I and 45 mg of III was obtained with chloroform as the eluent (identities established by TLC and HPLC). Elution with 1% methanol–chloroform gave 30 mg of V (TLC and HPLC); 2% methanol–chloroform gave 49 mg of VI, which was converted to the hydrochloride salt (acidic ether) and crystallized from acetone as needles (10 mg), mp 211–212° [lit. (24) mp 206–208° and 214–218°]. Direct comparison of VI·HCl with an authentic sample of desipramine hydrochloride showed the two samples to be identical (melting point, mixed melting point, TLC, co-TLC, and superimposable IR spectra). The ^{13}C-NMR data for VI are listed in Table II.

REFERENCES

(1) W. Charney and H. L. Herzog, "Microbial Transformations of Steroids, A Handbook," Academic, New York, N.Y., 1967.

(2) H. Iizuka and A. Naito, "Microbial Transformations of Steroids and Alkaloids," University Park Press, State College, Pa., 1967.

(3) K. Kieslich, "Microbial Transformations of Non-Steroid Cyclic Compounds," George Thieme, Stuttgart, West Germany, 1976.

(4) R. V. Smith and J. P. Rosazza, *Arch. Biochem. Biophys.*, **161**, 551 (1974).

(5) R. V. Smith and J. P. Rosazza, *J. Pharm. Sci.*, **64**, 1737 (1975).

(6) R. V. Smith and J. P. Rosazza, *Biotechnol. Bioeng.*, **17**, 795 (1975).

(7) R. V. Smith, D. Acosta, Jr., and J. P. Rosazza, *Adv. Biochem. Eng.*, **5**, 69 (1977).

(8) J. V. Dingell, F. Sulser, and J. R. Gillette, *J. Pharmacol. Exp. Ther.*, **143**, 14 (1964).

(9) J. L. Crammer and B. Scott, *Psychopharmacologia*, **8**, 461 (1966).

(10) M. H. Bickel and M. Baggioline, *Biochem. Pharmacol.*, **15**, 1155 (1966).

(11) M. H. Bickel and H. J. Weder, *Arch. Int. Pharmacodyn. Ther.*, **173**, 433 (1968).

(12) J. Christiansen and L. F. Gram, *J. Pharm. Pharmacol.*, **25**, 604 (1973).

(13) R. J. Abraham, L. J. Kricka, and A. Ledwith, *J. Chem. Soc. Perkin Trans. II*, **1974**, 1648.

(14) J. K. Baker and C. Y. Ma, *J. Chromatogr.*, **169**, 107 (1979).

(15) J. K. Baker, *Anal. Chem.*, **51**, 1693 (1979).

(16) J. K. Baker, R. E. Skelton, T. N. Riley, and J. R. Bagley, *J. Chromatogr. Sci.*, **18**, 153 (1980).

(17) M. S. Tute, *Adv. Drug Res.*, **6**, 1 (1971).

(18) J. K. Baker, R. E. Skelton, and C. Y. Ma, *J. Chromatogr.*, **168**, 417 (1979).

(19) K. C. Wang and T. Yeh, *J. Taiwan Pharm. Assoc.*, **25**, 45 (1973).

(20) M. H. Bickel, *Prog. Drug. Res.*, **11**, 121 (1967).

(21) C. D. Hufford, C. C. Collins, and A. M. Clark, *J. Pharm. Sci.*, **68**, 1239 (1979).

(22) A. M. Clark and C. D. Hufford, *J. Chem. Soc. Perkin Trans. I*, **1979**, 3022.

(23) W. Schindler, *Helv. Chim. Acta*, **43**, 35 (1960).

(24) "The Merck Index," 9th ed., Merck & Co., Rahway, N.J., 1976, pp. 383, 652.

ACKNOWLEDGMENTS

Supported in part by the Committee on Faculty Research, University of Mississippi, and the Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi.

The authors are grateful for the samples of 2-hydroxyimipramine hydrochloride, 10-hydroxyimipramine, and imipramine-*N*-oxide provided by Ciba-Geigy Corp. and desipramine hydrochloride provided by Merrell-National Laboratories. They also thank Ms. Ann Calloway and Ms. Sue Johnson for technical assistance.

Metabolism of Phencyclidine by Microorganisms

CHARLES D. HUFFORD *ˣ, JOHN K. BAKER ‡, and ALICE M. CLARK *

Received June 13, 1980, from the **Department of Pharmacognosy and the* ‡*Department of Medicinal Chemistry, School of Pharmacy, University of Mississippi, University, MS 38677.* Accepted for publication July 18, 1980.

Abstract □ A number of microorganisms were screened for their ability to metabolize phencyclidine. Two microorganisms, *Beauveria sulfurescens* and *Cunninghamella echinulata*, produced hydroxylated metabolites, which were identified as 1-(1-phenylcyclohexyl)-4-hydroxypiperidine and 4-phenyl-4-piperidinocyclohexanol by high-pressure liquid chromatographic analysis.

Keyphrases □ Phencyclidine—microbial metabolism, identification of metabolites □ Metabolites, microbial—phencyclidine, identification □ Microbial metabolites—phencyclidine, identification of metabolites

Phencyclidine (I) is a commonly abused drug whose metabolism has received limited attention in mammals. Ober *et al.* (1) conducted a study of the metabolism of I in rhesus monkeys and reported some unchanged I but larger amounts of metabolites. The major metabolite present in the urine was identified as a nonphenolic dihydroxyphencyclidine. Specific structures were not proposed, but a later report (2) gave structures for those metabolites, although details were not provided. Glazko (2) also reported considerable species variation of these metabolites,

0022-3549/81/0200-0155$01.00/0

Table I—Microorganisms Used for Screening Phencyclidine

Culture (Number)[a]	Metabolite Production[b]
Arthrobacter simplex (6946)	–
Aspergillus flavipes (1030)	–
Aspergillus flavipes (11013)	–
Aspergillus flavipes (16795)	–
Aspergillus flavus (9170)	
Aspergillus flavus (24741)	–
Aspergillus niger (10549)	–
Aspergillus niger (11394)	–
Aspergillus niger (16888)	–
Aspergillus ochraceus (18500)	–
Aspergillus ochraceus (22947)	–
Aspergillus parasiticus (15517)	–
Beauveria bassiana (13144)	–
Beauveria sulfurescens (7159)	+
Botrytis allii (9435)	–
Chaetomium cochloides (10195)	–
Cladosporium resinae (22712)	–
Cunninghamella blakesleeana (8688a)	–
Cunninghamella echinulata (NRRL 3655)	–
Cunninghamella echinulata (9244)	+
Cunninghamella echinulata (11585a)	–
Cunninghamella echinulata (11585b)	–
Cunninghamella elegans (9245)	–
Curvularia lunata (12017)	–
Cylindrocarpon radicicola (11011)	–
Fomes pinicola (15341)	–
Fusarium oxysporum (7601)	–
Fusarium solani var. *coeruleum* (24389)	–
Mucor griseo-cyanus (1207a)	–
Nocardia corallina (19070)	–
Nocardia corallina (19071)	–
Nocardia corallina (19148)	–
Nocardia minima (19150)	–
Rhizopus arrhizus (11145)	–
Rhizopus stolonifer (6227b)	–
Rhizopus stolonifer (15441)	–
Streptomyces griseus (13968)	–
Streptomyces griseus (23337)	–
Streptomyces spectabilis (27465)	–
Syncephalastrum racemosum (18192)	–
Whetzelinia sclerotiorum (18015)	–
Whetzelinia sclerotiorum (24156)	–

[a] All cultures were obtained from the American Type Culture Collection in Rockville, Md., unless otherwise specified. [b] Metabolite production denoted by a + indicates one or more metabolites were produced, as evidenced by TLC.

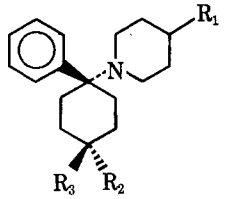
with humans showing relatively large amounts of the monohydroxy metabolite. Wong and Biemann (3) tentatively identified 11 metabolites by GLC–mass spectrometry in the rat. Three of these metabolites (II*b*, III, and IV) were detected in human urine samples. Metabolites II*a* or II*b* and III also were detected in human urine samples by Lin *et al.* (4). Thus, hydroxylation of the cyclohexyl and piperidyl rings in I seems to be the major mode of mammalian metabolism. It remains to be determined whether metabolism plays a significant role in the mechanism of action and/or toxicity of I.

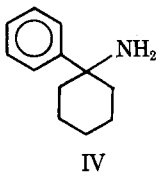
Smith and Rosazza (5–8) suggested that mammalian metabolism might be studied by using microbial models and cited numerous examples that show parallels between

Table II—Chromatographic Properties of Phencyclidine and Its Metabolites on HPLC System A

Compound	t_R[a]	Retention Index	A_{254}/A_{280}[b]
I	1.00	763	44.0
II*a*	0.36	614	60.8
II*b*	0.48	662	57.3
III	0.64	707	48.1

[a] Retention time relative to I (11 min). [b] Ratio of absorbances at 254 and 280 nm.





IV

I: $R_1 = R_2 = H$
II*a*: $R_1 = R_3 = H$, $R_2 = OH$
II*b*: $R_1 = R_2 = H$, $R_3 = OH$
III: $R_1 = OH$, $R_2 = R_3 = H$

mammalian and microbial metabolism. This report describes studies on the microbial metabolism of phencyclidine (I).

RESULTS AND DISCUSSION

Of the 44 microorganisms (Table I) incubated with phencyclidine (I), only two organisms, *B. sulfurescens* (ATCC 7159) and *C. echinulata* (ATCC 9244), showed any capability to metabolize this substrate. Since TLC showed that *B. sulfurescens* produced only one metabolite, this organism was chosen for preparative-scale study.

Phencyclidine hydrochloride[1] was distributed evenly among submerged cultures of *B. sulfurescens*. After incubation for 13 days, the cultures were combined and filtered, and the aqueous culture broth was extracted with chloroform. Chromatography of the chloroform extract over alumina afforded the recovered starting material and a crystalline residue. This residue gave one spot on TLC whose R_f value corresponded to that of the known hydroxylated piperidine metabolite (III). However, the ^{13}C-NMR spectrum showed the metabolite to be a mixture of two components, which was confirmed by high-pressure liquid chromatographic (HPLC) analysis. It appeared that these two components would be difficult to separate on a preparative scale and, since authentic samples of the hydroxylated phencyclidine metabolites were available, detailed HPLC analyses were conducted.

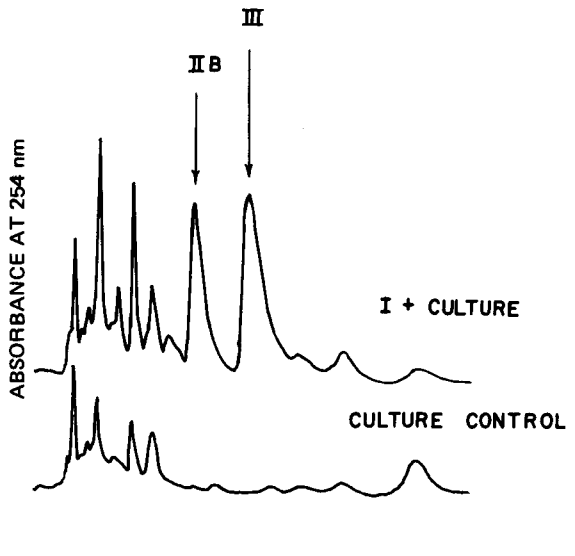


Figure 1—*Chromatogram of* B. sulfurescens *extract using HPLC System A. Although dual 254- and 280-nm UV detectors were used, only the response of the 254-nm detector is shown. The upper curve was obtained from the extract of the incubation mixture of I and the culture. The lower curve was obtained from the extract of the culture not containing I.*

[1] Phencyclidine (I), 1-(1-phenylcyclohexyl)-4-hydroxypiperidine (III), and 4-phenyl-4-piperidinocyclohexanol (II*a* and II*b*) were obtained from the National Institute of Drug Abuse.

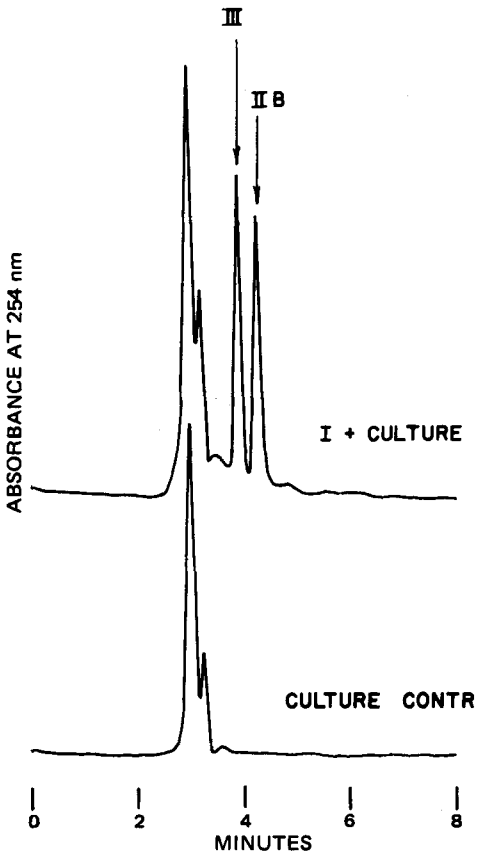


Figure 2—*Chromatogram of the* B. sulfurescens *extract using HPLC System B. The upper curve was obtained from the extract of the incubation mixture of I and the culture. The lower curve was obtained from the extract of the culture not containing I.*

HPLC analysis of an authentic sample of III[1] using System A showed one peak with a relative retention time of 0.64 (Table II). However, analysis of an authentic sample of 4-phenyl-4-piperidinocyclohexanol[1] using System A showed two distinct peaks with relative retention times of 0.36 and 0.48, respectively (Table II). The cyclohexyl metabolite, 4-phenyl-4-piperdinocyclohexanol, was available as a mixture of two epimers[2] (II*a* and II*b*), but their relative stereochemistry has not yet been established. The major component of this mixture corresponded to a relative retention time of 0.36 in HPLC System A and was arbitrarily assigned as II*a*. The minor component, assigned as II*b*, had a relative retention time of 0.48 in HPLC System A.

Purified extracts of *B. sulfurescens* incubated with I in a manner analogous to the preparative-scale procedure were examined using HPLC Systems A and B. In addition, the extracts of *B. sulfurescens* cultures grown under the same conditions, but without I (culture control), were analyzed and used as controls to identify any interfering peaks due to the organism or medium. With System A, two peaks not present in the culture control samples were evident (Fig. 1), with relative retention times of 0.48 and 0.64. These peaks corresponded in retention time to one isomer of the hydroxylated cyclohexyl metabolite (II*b*) and the hydroxylated piperidine metabolite (III). The identity of these metabolites was confirmed further using System B, which also showed the presence of two components, corresponding in retention times to II*b* and III (Fig. 2). In addition, the ratios of the absorbances at 254 and 280 nm for each peak in the chromatograms were consistent with the values observed for the standard samples in both systems (Table II).

The only other culture shown by TLC to produce metabolites was *C. echinulata*. Chloroform extracts of both substrate-containing and nonsubstrate-containing cultures of *C. echinulata* were concentrated on an alumina column and eluted with 1% methanol in chloroform to remove I and some of the medium constitutents. The chromatogram of the purified *C. echinulata* extract on System A (Fig. 3) clearly showed that

[2] The sample was prepared as described in the literature (4) and apparently results in an epimeric mixture, which was not separated further.

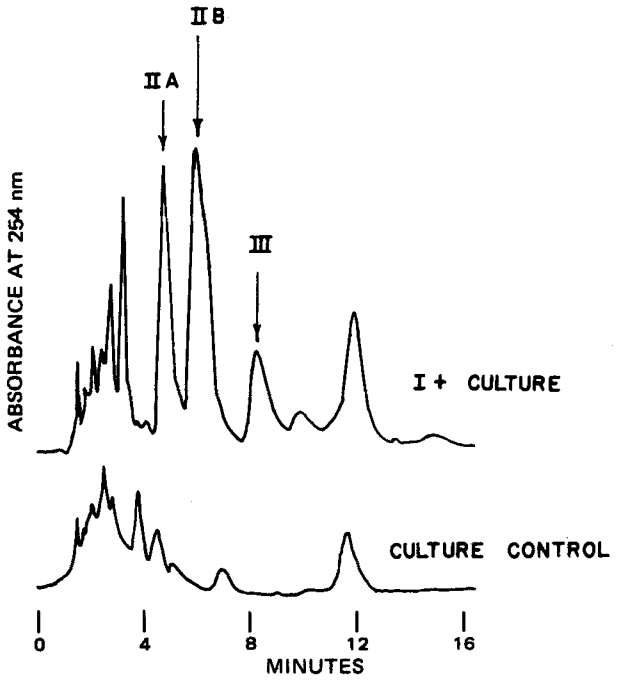


Figure 3—*Chromatogram of the* C. echinulata *extract using HPLC System A. The upper curve was obtained from the extract of the incubation mixture of I and the culture. The lower curve was obtained from the extract of the culture not containing I.*

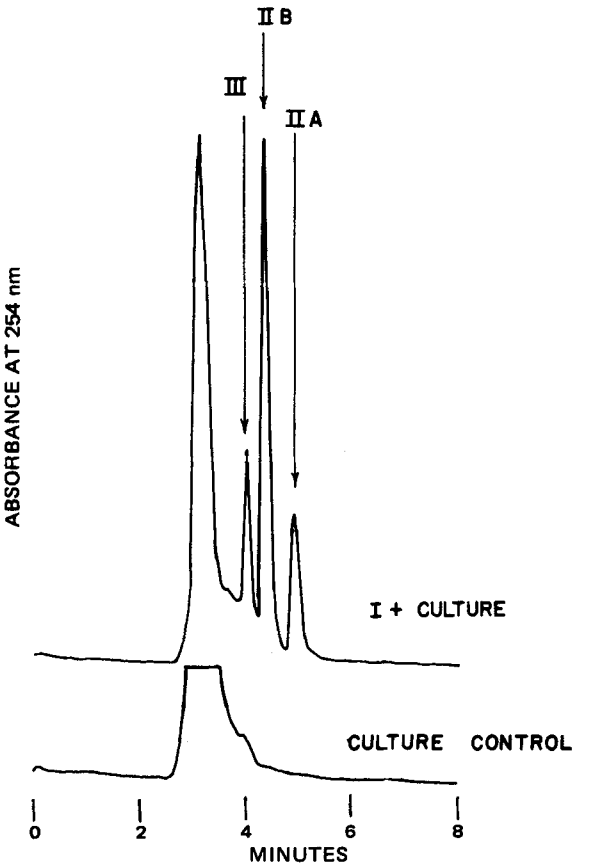


Figure 4—*Chromatogram of the* C. echinulata *extract using HPLC System B. The upper curve was obtained from the extract of the incubation mixture of I and the culture. The lower curve was obtained from the extract of the culture not containing I.*

metabolites II*a*, II*b*, and III were produced. This result was established further by analysis on System B (Fig. 4).

The identification of the metabolites was verified by a comparison of the absorbance ratios observed for the metabolites and the standards (Table II). The absolute quantity of each metabolite present in the alumina-purified extracts of both *B. sulfurescens* and *C. echinulata* cultures was calculated. When 15 mg of I·HCl was added to 100-ml cultures, the alumina-purified extracts of *B. sulfurescens* cultures contained 0.13 mg of II*b* and 0.19 mg of III, while purified *C. echinulata* extracts contained 0.15 mg of II*a*, 0.22 mg of II*b*, and 0.09 mg of III.

CONCLUSIONS

It appears that both *B. sulfurescens* and *C. echinulata* produce III, which is also a metabolite in humans. GLC–mass spectral studies (3) suggested that the major mammalian metabolite is II*b* rather than II*a*; however, those findings were not confirmed by comparison to standards of proven stereochemistry. It is not possible to determine if the isomer produced by *B. sulfurescens* is the same as the reported human metabolite since the standard sample was available only as a mixture of the two isomers. *C. echinulata* appears to be less selective and produces both isomers (II*a* and II*b*).

EXPERIMENTAL

Fermentation Procedures—Initial screening studies were performed to determine microorganisms capable of metabolizing phencyclidine. A total of 44 organisms (Table I) were selected[3] and were grown according to the usual two-stage fermentation procedure (9, 10) in 25 ml of medium held in 125-ml cotton-plugged erlenmeyer flasks. Phencyclidine hydrochloride was added to 24-hr second-stage cultures, which were incubated on a rotary shaker at 200 rpm and 25°. Samples of 5 ml were removed at various intervals after the substrate had been added.

Each sample was adjusted to pH ~8 with ammonium hydroxide and extracted with chloroform (3 × 5 ml), and the chloroform extracts were evaporated to dryness. The residues were redissolved in 100 μl of chloroform, and 6 μl of the solution was spotted on TLC plates. The medium used for all fermentations consisted of 10 g of dextrose, 10 g of corn steep liquor, 10 g of soya flour, 5 g of dry malt extract, 1 g of calcium chloride, and 5 g of sodium chloride in 1 liter of water. Culture controls consisted of fermentation blanks, in which the organisms were grown under identical conditions but without the substrate. Substrate controls consisted of the addition of the same amount of substrate to sterile media.

TLC and HPLC—Silica gel GF_{254} plates were developed in either 10% acetic acid in absolute ethanol or ethyl acetate–methanol–concentrated ammonium hydroxide (85:10:5). Alumina GF_{254} plates were developed in 1% methanol–chloroform. The plates were visualized under UV light and sprayed with iodoplatinate spray reagent. The R_f values of I, II*a*, II*b*, and III in the 10% acetic acid in ethanol system were 0.38, 0.22, 0.22, and 0.28, respectively. Their R_f values in the ethyl acetate–methanol–ammonium hydroxide (85:10:5) system were 0.80, 0.47, 0.55, and 0.55, respectively, and their R_f values in the 1% methanol in chloroform (alumina) system were 0.80, 0.28, 0.28, and 0.30, respectively.

HPLC System A consisted of a 3.9-mm × 30-cm octadecyl reversed-phase column[4] with 10-μm particles. The mobile phase was prepared using 6.6 g of dibasic potassium phosphate, 8.4 g of monobasic potassium phosphate, 1.6 liters of methanol, and 2.4 liters of water; the flow rate was 2.0 ml/min. An HPLC pump[5] and a microsyringe-loaded loop injector[6] also were used. Two UV detectors[7] were used in series (254-nm detector followed by a 280-nm detector) and were calibrated using a morphine reference standard (11).

The HPLC retention indexes with HPLC System A were measured essentially as reported previously (12, 13). The retention index scale was based on the retention of the drug in question relative to a series (C_3–C_{23}) of 2-keto alkanes[8]. The retention index of a given 2-keto alkane standard was, by definition, equal to 100 times the number of carbons in the compound. Thus, 2-butanone was assigned a value of 400. The HPLC retention index of I and its metabolites was determined by interpolation of the logarithm of the capacity factors observed for the test compound and the standards.

HPLC System B consisted of a 3.9-mm × 30-cm silica column[9] with 10-μm particles. The mobile phase was prepared using methanol, 2 *N* ammonia, and 1 *N* ammonium nitrate (27:2:1); the flow rate was 1.0 ml/min.

Preparative-Scale Metabolism of Phencyclidine (I) Using *B. sulfurescens* (ATCC 7159)—*B. sulfurescens* (ATCC 7159) was grown in 4.0 liters of medium held in 40 500-ml cotton-plugged erlenmeyer flasks. A total of 400 mg of phencyclidine hydrochloride[1] was distributed equally among the cultures. The flasks were kept at room temperature and 200 rpm for 13 days after substrate addition. The cultures were harvested by filtration, and the aqueous culture broth (pH 8) was extracted with chloroform (3 × 2 liters). The combined chloroform layers were dried (sodium sulfate) and evaporated to dryness, yielding 341 mg of extract.

The extract was chromatographed over alumina[10] (40 g) using chloroform as the solvent. Elution with chloroform yielded a fraction containing phencyclidine (I) (137 mg), identified by TLC and ^{13}C-NMR analyses. Elution with 1% methanol–chloroform gave a 30-mg fraction (crystalline residue) of the metabolite. The fraction showed one spot in all of the TLC systems tried but clearly was a mixture of two components (II*b* and III) as determined by HPLC and ^{13}C-NMR spectroscopy.

REFERENCES

(1) R. E. Ober, G. W. Gwynn, T. Chang, D. A. McCarthy, and A. J. Glazko, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, **22**, 539 (1963).

(2) A. J. Glazko, *Antimicrob. Agents Chemother.*, **3**, 660 (1966).

(3) L. K. Wong and K. Biemann, *Clin. Toxicol.*, **9**, 583 (1976).

(4) D. C. K. Lin, A. F. Fentiman, R. L. Foltz, R. D. Forney, and I. Sunshine, *Biomed. Mass Spectrom.*, **2**, 206 (1975).

(5) R. V. Smith and J. P. Rosazza, *Arch. Biochem. Biophys.*, **161**, 551 (1974).

(6) R. V. Smith and J. P. Rosazza, *J. Pharm. Sci.*, **64**, 1737 (1975).

(7) R. V. Smith and J. P. Rosazza, *Biotechnol. Bioeng.*, **17**, 75 (1975).

(8) R. V. Smith, D. Acosta, and J. P. Rosazza, *Adv. Biochem. Eng.*, **5**, 69 (1977).

(9) C. D. Hufford, C. C. Collins, and A. M. Clark, *J. Pharm. Sci.*, **68**, 1239 (1979).

(10) A. M. Clark and C. D. Hufford, *J. Chem. Soc. Perkin Trans. I*, **1979**, 3022.

(11) J. K. Baker, R. E. Skelton, and C. Y. Ma, *J. Chromatogr.*, **168**, 417 (1979).

(12) J. K. Baker and C. Y. Ma, *ibid.*, **169**, 107 (1979).

(13) J. K. Baker, *Anal. Chem.*, **51**, 1693 (1979).

ACKNOWLEDGMENTS

Supported in part by the Committee on Faculty Research, University of Mississippi, and the Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi.

[3] Obtained from University of Mississippi School of Pharmacy culture collection.

[4] μBondapak C_{18}, Waters Associates, Milford, Mass.

[5] Model M-6000, Waters Associates, Milford, Mass.

[6] Model U6-K, Waters Associates, Milford, Mass.

[7] Model 440, Waters Associates, Milford, Mass.

[8] Analabs Inc., North Haven, Conn.

[9] μ-Porasil, Waters Associates, Milford, Mass.

[10] Woelm neutral grade I.