Demethylation of Imipramine by Enteric Bacteria

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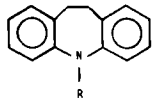
Abstract □ The ability of a number of aerobic and anaerobic bacteria to *N*-demethylate imipramine (I) to desipramine (II) has been investigated. Of the bacteria investigated, almost half were known inhabitants of the human GI tract. More than half of the enteric bacteria studied were capable of *N*-demethylating imipramine (I) to desipramine (II) to some extent in at least one medium. It was found that the medium in which the organism was grown had a significant effect on the *N*-demethylase activity observed.

Keyphrases □ Imipramine—*N*-demethylation to desipramine, microbial transformation, enteric bacteria □ Microbial transformation—imipramine, *N*-demethylation to desipramine, enteric bacteria □ Desipramine—microbial *N*-demethylation of imipramine, enteric bacteria

Imipramine (I) is a widely used tricyclic antidepressant whose metabolism, distribution, and excretion has been studied in detail (1–5). Desipramine (II) is a biologically active metabolite of imipramine which is formed by hepatic as well as by extrahepatic metabolism (6). A major site of extrahepatic metabolism has been shown to be the GI contents (6). It has also been shown that demethylation of I does not occur during passage across the intestinal wall (7). The role of the GI microflora in drug metabolism has been reviewed and has been shown to be a significant factor with many drugs (8–11). The microbial metabolism of imipramine using a number of fungi has also been shown to parallel the mammalian metabolism of imipramine (12). This study examined the metabolism of imipramine by bacteria, many of which are known to be inhabitants of the human GI tract.

EXPERIMENTAL

Imipramine hydrochloride (I)[1] was shown to be free of desipramine (II) and iminodibenzyl (III), known contaminants of some commercial samples of imipramine (13), by high-performance liquid chromatographic (HPLC) and TLC analyses. Desipramine hydrochloride was also shown to be pure by HPLC and TLC analyses. All solvents used for HPLC analysis were reagent grade quality.



I: R = $(CH_2)_3N(CH_3)_2$
II: R = $(CH_2)_3NHCH_3$
III: R = H

Fermentation Screening Procedures for Aerobic Bacteria—Aerobic bacterial stock cultures were maintained on nutrient agar[2] and eugon agar[3] at 4° and transferred every 4–6 months. Screening for the demethylation of imipramine to desipramine was carried out in four different media. Three of the media used were complex media; the fourth was a defined medium. The media used were: eugon broth[3] (medium A); 5 g of dextrose, 2 g of corn steep liquor, 5 g of yeast extract, 1 g of peptone, and 1 liter of distilled water (medium B); 20 g of dextrose, 5 g of peptone, 5 g of tryptone, 2.5 g of $CaCO_3$, and 1 liter of distilled water (medium C); and 780 ml of basal salts solution (14) [containing 1.5 g of KH_2PO_4, 7.0 g of $(NH_4)_2HPO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of $CaCl_2 \cdot 2H_2O$, 0.04 g of $MnSO_4 \cdot 4H_2O$, 0.025 g of $FeSO_4 \cdot 7H_2O$, 0.002 g of ammonium molybdate, and 1 liter of distilled water], 100 ml of amino acid solution (containing 0.38 g of DL-alanine, 0.89 g of DL-aspartic acid, 0.30 g of L-arginine hydrochloride, 0.02 g of L-cystine, 0.140 g of L-glutamic acid, 0.17 g of glycine, 0.24 g of L-histidine hydrochloride, 0.76 g of DL-isoleucine, 0.57 g of L-leucine, 0.24 g of L-lysine hydrochloride, 0.06 g of DL-methionine, 0.11 g of L-proline, 0.12 g of DL-serine, 0.10 g of DL-threonine, 0.06 g of L-tyrosine, 0.15 g of DL-valine, and 100 ml of distilled water), 20 ml of vitamin solution (15) (containing 5.0 μg of biotin, 10.0 μg of folic acid, 0.5 mg of riboflavin, 2.5 mg of thiamine hydrochloride, 2.5 mg of pyridoxal hydrochloride, 2.5 mg of calcium pantothenate, 2.5 mg of nicotinic acid, and 100 ml of distilled water), and 100 ml of glucose solution (30%, w/v) (medium D).

A two-stage fermentation procedure was utilized as previously described (12). For screening in media A–C, the cultures were grown in 25 ml of medium held in 125-ml Erlenmeyer flasks. The cultures were incubated at 37° and 250 rpm. Imipramine hydrochloride was added to 24-hr-old stage II cultures as a 5% solution in dimethylformamide to give a final concentration of 200 μg/ml of medium. The cultures were incubated for 6 days and assayed for desipramine formation at 3 and 6 days after substrate addition. The samples were adjusted to pH 8 and extracted with three equal volumes of chloroform. The combined chloroform layers were dried and evaporated, and the residues were analyzed by TLC and HPLC.

For screening in medium D, the cultures were grown in 10 ml of medium held in 50-ml Erlenmeyer flasks, incubated at 250 rpm and 37°. Imipramine hydrochloride was added to sterile medium D prior to inoculation of stage II cultures. The substrate was added as a 1% dimethylformamide solution to give a final concentration of 100 μg/ml of medium. The stage II cultures, containing substrate, were incubated for 48 hr. Each whole culture was adjusted to pH 8 and extracted with an equal volume of dichloromethane, and the dichloromethane extracts were evaporated and analyzed by TLC and HPLC.

Fermentation Screening Procedures for Anaerobic Bacteria—Stock cultures of anaerobic bacteria were maintained in appropriate agar or broth media in disposable anaerobic systems[4]. Screening for desipramine production was carried out in 10 ml of either brain heart infusion broth[3] or medium E which consisted of 20 g of peptone, 10 g of yeast extract, 5 g of glucose, 5 g of maltose, 0.5 g of cysteine, 40 ml of salts solution, and 4 ml of a 0.025% aqueous solution of resazurin per liter of distilled water. The salts solution consisted of 0.1 g of K_2HPO_4, 0.1 g of KH_2PO_4, 1.0 g of $NaHCO_3$, 0.2 g of NaCl, 0.02 g of $CaCl_2$, 0.02 g of $MgSO_4$, trace amounts of ammonium molybdate and $CoCl_2 \cdot 6H_2O$, and 0.3 ml of 50% H_2SO_4 per 200 ml of distilled water. Each medium was dispersed into 16 × 125-mm screw-cap test tubes (10 ml/tube) and sterilized by autoclaving at 121° and 15 psi for 20 min. After sterilization each medium was supplemented with 100 μg of imipramine hydrochloride/ml of medium.

The sterile medium containing imipramine was inoculated from stock cultures of anaerobic bacteria (0.05 ml broth culture/10 ml sterile medium). The cultures were incubated at 37° under anaerobic conditions[4] for 7 days, at which time the whole cultures were extracted and analyzed as described for aerobic cultures grown in medium D.

Control Studies—Culture controls consisted of fermentation blanks in which each organism was grown under identical conditions as biotransformation cultures, but without substrate. The cultures were extracted and analyzed as described for the biotransformation cultures.

Substrate controls were necessary to establish the stability of the substrate to the fermentation conditions used. Therefore, substrate controls were obtained for each set of medium and incubation conditions used. Substrate controls for aerobic cultures grown in media A–C were

[1] Sigma Chemical Co.
[2] Difco Laboratories, Detroit, Mich.
[3] BBL Microbiology Systems, Cockeysville, Md.
[4] GasPak Disposable Anaerobic System; BBL Microbiology Systems, Cockeysville, Md.

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obtained by growing stage II cultures of two representative organisms (*Escherichia coli* 10536 and *Bacillus cereus* v. *fluorescens* 13024) in each medium for 7 days (250 rpm, 37°). The cultures were then sterilized by autoclaving. Imipramine hydrochloride (200 μg/ml of medium) was added to the sterilized cultures, which were incubated an additional 6 days at 37° and 250 rpm. The sterilized substrate control cultures were then extracted and analyzed as described for aerobic cultures grown in media A–C. Five cultures of *E. coli* 10536 and two cultures of *B. cereus* v. *fluorescens* 13824 were used to determine medium A control values. For medium B control values, five cultures of *E. coli* 10536 and three cultures of *B. cereus* v. *fluorescens* 13824 were used. For medium C five cultures of each organism were used. Three cultures of *B. cereus* v. *fluorescens* grown in medium A and two cultures grown in medium B became contaminated after sterilization and addition of imipramine, and therefore could not be used in calculating control values. In addition, substrate controls consisting of sterile media (A–C) containing imipramine (200 μg/ml) were prepared and incubated at 250 rpm and 37° for 6 days (five determinations/medium). Each substrate control was extracted and analyzed as described for growing cultures. In addition, substrate controls consisting of sterile medium D containing imipramine (100 μg/ml of medium) were prepared and incubated at 250 rpm and 37° for 48 hr (five determinations). Each substrate control was extracted and analyzed as described for aerobic cultures grown in medium D.

Substrate controls for anaerobic media were obtained by growing each of the seven anaerobic bacteria in media without imipramine for 7 days. The cultures were then sterilized by autoclaving. After cooling, imipramine hydrochloride was added (100 μg/ml of medium) and incubation under anaerobic conditions[4] (37°) was continued an additional 7 days. The substrate control cultures were then extracted and analyzed as described for aerobic cultures grown in medium D.

TLC Analyses—Culture extracts were redissolved in 100 μl of chloroform or dichloromethane, and 6-μl aliquots of the solutions were spotted on precoated silica gel G TLC plates[5]. The plates were developed in ethyl acetate–methanol–ammonium hydroxide (81:15:4) and visualized by UV light and by spraying with diazotized *p*-nitroaniline followed by spraying with concentrated hydrochloric acid. The R_f values and colors for imipramine and desipramine are 0.57 (blue) and 0.29 (blue), respectively.

HPLC Analyses—Quantitative analyses of the culture extracts were accomplished on a silica column[6] using an HPLC pump[7], a microsyringe-loaded loop injector[8], and a UV detector[9]. The mobile phase consisted of methanol–2 *N* ammonia–1 *N* ammonium nitrate (27:2:1) and was used at a flow rate of 1.0 ml/min. Culture extracts were redissolved in 400 μl of methanol, and 5-μl aliquots of the solutions were injected for HPLC. Quantitation of desipramine in culture extracts was accomplished by comparison of the peak heights in culture extracts with the peak heights of standard samples of desipramine at known concentrations.

For aerobic cultures grown in complex media (A–C), desipramine levels were considered significant if the level exceeded the sterilized control value plus four times the standard deviation for that medium. Thus, for medium A, levels <0.56% were not considered significant and for media B and C, levels <0.59% and 0.64%, respectively, were not considered significant. For aerobic cultures grown in medium D, a sterilized control value was established for each organism. In these cases, yields of desipramine that exceeded 3 times the control value were considered significant. The same criterion was used for determining significant *N*-demethylase activity among anaerobic bacteria.

RESULTS AND DISCUSSION

Imipramine (I) was subjected to biotransformation screening using 29 aerobic and 7 anaerobic bacteria. Of the aerobic bacteria, nine are known to be inhabitants of the human GI tract; these organisms were screened in three complex media (media A–C) and one defined medium (D). The remaining 20 aerobic bacteria were screened in the three complex media (A–C). All seven anaerobic bacteria were screened for their ability to *N*-demethylate imipramine in two complex media: brain heart infusion broth and medium E. Cultures grown in the presence of imipramine were extracted with either dichloromethane or chloroform, and the extracts were analyzed by TLC and HPLC. The results of these studies are summarized in Tables I–III.

[5] Brinkmann Instruments, Inc., Westbury, N.Y.
[6] μPorasil; Waters Associates, Milford, Mass.
[7] Model M-6000; Waters Associates, Milford, Mass.
[8] Model U6-K; Waters Associates, Milford, Mass.
[9] Model 440; Waters Associates, Milford Mass.

Table I—Yields of Desipramine by Aerobic Bacteria in Complex Media [a]

Bacterium (ATCC number)	Medium A	Medium B	Medium C
Arthrobacter species (19140)	0.29	0.15	0.16
Arthrobacter species (21237)	0.90[b]	0.70[b]	0.70[b]
Bacillus cereus v. *fluorescens* (13824)	0.95[b]	1.20[b]	1.80[b]
Bacillus megaterium (9885)	1.10[b]	0.20	1.10[b]
Bacillus subtilis (6633)	0.20	0.05	0.40
Corynebacterium species (14887)	—[c]	—[c]	—[c]
Enterobacter aerogenes (13048)[d]	0.17	0.08	0.31
Escherichia coli (27165)[d]	0.34	0.11	0.23
E. coli (10536)	1.58[b]	0.22	1.78[b]
Flavobacterium oxydans (1245)	0.52	0.40	0.60
Klebsiella pneumoniae (27889)[d]	0.10	0.79[b]	0.35
K. pneumoniae (29016)[d]	0.70[b]	0.29	0.21
Mycobacterium cuneatum (21498)	—[c]	—[c]	—[c]
Mycobacterium smegmatis (607)	—[c]	—[c]	—[c]
M. smegmatis (14468)	0.05	0.10	0.20
Nocardia corallina (19070)	0.70[b]	0.05	0.05
N. corallina (19071)	0.62[b]	0.50	0.25
N. corallina (19148)	0.60[b]	0.40	0.20
Nocardia minima (19150)	0.46	0.71[b]	0.30
Nocardia petroleophila (15777)	0.55	0.70[b]	0.67[b]
Proteus vulgaris (27973)[d]	0.50	0.30	0.40
Pseudomonas aeruginosa (14205)[d]	0.80[b]	0.40	0.30
P. aeruginosa (15442)	0.35	0.35	0.23
Pseudomonas desmolytica (15005)	—[c]	—[c]	—[c]
Pseudomonas species (19286)	0.79[b]	0.61[b]	0.10
Staphylococcus aureus (6538)	0.79[b]	0.50	<0.10
S. aureus (14458)[d]	0.46	0.20	0.34
Streptococcus faecalis (6569)[d]	0.50	0.50	0.71[b]
Streptococcus faecium (6056)[d]	1.12[b]	0.13	0.36
Control (sterilized)[e]	0.32 ± 0.06	0.31 ± 0.07	0.28 ± 0.09
Control[f]	0.26 ± 0.08	0.10 ± 0.03	0.07 ± 0.02

[a] Yields are expressed as percent yield on a mole basis. [b] Considered as significant *N*-demethylase activity, based on a minimum value of the sterilized control value plus 4 times the standard deviation. [c] These cultures were not analyzed by HPLC since TLC analysis showed no desipramine. [d] Known inhabitants of the gut. [e] Sterilized control values are expressed as the average (±*SD*) of 7–10 determinations using sterilized cultures of two representative organisms, *E. coli* 10536 and *B. cereus* v. *fluorescens* 13824, grown in each of the three media. [f] Simple control values are expressed as the average (±*SD*) of five determinations in which imipramine was incubated in each of the sterile media for 6 days.

The quantitative results obtained from HPLC analyses of aerobic bacteria grown in complex media (A–C) containing imipramine are shown in Table I. The results obtained from HPLC analyses of aerobic gut bacteria grown in the defined medium (D) containing imipramine are shown in Table II. Twelve of the twenty-nine organisms screened were capable of *N*-demethylation in medium A. In media B and C, six of the bacteria screened could *N*-demethylate imipramine (Table I). In total, 16 of the 29 aerobic bacteria screened, including 5 of the 9 gut bacteria, were capable of *N*-demethylating imipramine to some extent in at least one of the complex media (Table I).

Two additional gut bacteria, which were incapable of *N*-methylation in the complex media, were shown to *N*-demethylate imipramine when grown in medium D (Table II). One organism, *Streptococcus faecalis* 6569 would not grow in medium D. Only two aerobic gut bacteria, *Klebsiella pneumoniae* 29016 and *Streptococcus faecium* 6056, were capable of *N*-demethylation in both complex (A) and defined media (D).

A number of anaerobic bacteria were also screened for their ability to *N*-demethylate imipramine (I). A total of seven anaerobes known to inhabit the GI tract of the human were screened for *N*-demethylase activity in two complex media. The results of these studies are shown in Table III. Only one anaerobe, *Fusobacterium fusiforme* 23726 showed signifi-

Table II—Yields of Desipramine by Aerobic Gut Bacteria in Defined Medium [a]

Bacterium (ATCC number)	Yields of Desipramine	
	Growing Cultures	Control Cultures[b]
Enterobacter aerogenes (13048)	0.72	0.60
Escherichia coli (27165)	2.02[c]	0.67
Klebsiella pneumoniae (27889)	2.19	1.23
K. pneumoniae (29016)	1.12[c]	0.22
Proteus vulgaris (27973)	1.77[c]	0.52
Pseudomonas aeruginosa (14205)	2.20	1.58
Staphylococcus aureus (14458)	0.55	0.19
Streptococcus faecalis (6569)	—[d]	—[d]
Streptococcus faecium (6056)	1.60[c]	0.52

[a] Yields are expressed as percent yield on a mole basis. [b] Simple substrate controls consisting of sterile medium containing imipramine showed an average percent yield of desipramine of 0.17 ± 0.05 after 48 hr of incubation. [c] Considered as significant *N*-demethylase activity, based on a minimum value of 3 times the control value. [d] No growth in this medium.

cant *N*-demethylase activity in medium E. However, in brain heart infusion medium, four of the seven anaerobes (*Bacteroides eggerthii* 27754, *Bifidobacterium longum* 15707, *Clostridium paraperfringens* 27640, and *Lactobacillus catenaforme* 25644) were capable of *N*-demethylation. It is interesting to note that *F. fusiforme* 23726 did not show *N*-demethylase activity in brain heart infusion medium. Therefore, as with the aerobic bacteria, it appears that the choice of medium is important in demonstrating *N*-demethylase activity in anaerobic bacteria.

Table III—Yields of Desipramine from Anaerobic Bacteria in Complex Media [a]

Bacterium (ATCC number)	Yield of Desipramine			
	Growing Cultures in Medium E	Control Cultures in Medium E	Growing Cultures in Brain Heart Infusion Medium	Control Cultures in Brain Heart Infusion Medium
Bacteroides eggerthii (27754)	0.58	0.55	1.28[b]	0.31
Bifidobacterium adolescentis (15703)	0.80	0.65	0.53	0.26
Bifidobacterium bifidum (15696)	0.66	0.34	0.68	0.47
Bifidobacterium longum (15707)	0.48	0.26	1.73[b]	0.45
Clostridium paraperfringens (27640)	0.52	0.42	0.78[b]	0.22
Fusobacterium fusiforme (23726)	0.78[b]	0.21	0.39	0.24
Lactobacillus catenaforme (25644)	0.60	0.42	0.81[b]	0.21

[a] Yields are expressed as percent yield on a mole basis. [b] Considered as significant *N*-demethylase activity, based on a minimum value of 3 times the control value.

Based on these studies, it appears that *N*-demethylase activity was common among bacteria. Of particular interest were the enteric bacteria, both aerobic and anaerobic. A high percentage of the gut bacteria screened were capable of *N*-demethylation of imipramine to desipramine. Among the anaerobic bacteria, five of seven were capable of *N*-demethylating imipramine to desipramine as were seven of nine aerobic gut bacteria. The medium in which the organism was grown appeared to have a significant influence on the *N*-demethylase activity. Since it is known that considerable variability exists in response to imipramine therapy, the variable *N*-demethylation of imipramine may be an important consideration (16). A similar situation occurs with digoxin in which the gut flora metabolizes the drug to biologically inactive metabolites in some patients, lowering their plasma levels of digoxin (17).

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