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Reductive Debromination of (α-Bromoiso-valeryl)urea by Intestinal Bacteria

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Abstract

The reductive debromination of the hypnotic (α-bromoiso-valeryl)urea to (3-methylbutyryl)urea by intestinal bacteria has been studied.

The caecal contents of rats, mice, hamsters, guinea-pigs and rabbits had significant debrominating activity toward (α-bromoiso-valeryl)urea. The cell-free extract of intestinal bacteria from the caecal contents of rats had debrominating activity in the presence of both flavin mononucleotide (FMN) and NADH (or NADPH) under anaerobic conditions. Seven pure strains of intestinal bacteria were also tested and the highest activity was observed with *Clostridium sporogenes*. The cell-free extract of *Clostridium sporogenes* had debrominating activity in the presence of both FMN and NADH (or NADPH), and this activity was inhibited by sodium arsenite and potassium cyanide. The activity of the cell-free extract was also supported by the photochemically reduced form of FMN. The debromination in intestinal bacteria seems to proceed in two steps—reduction of flavins by bacterial flavin reductase(s) in the presence of NADPH or NADH, and then the reductive debromination of (α-bromoiso-valeryl)urea to (3-methylbutyryl)urea by bacterial dehalogenase(s) using the reduced flavins as an electron donor.

These results indicate that intestinal bacteria play a role in the reductive debromination of (α-bromoiso-valeryl)urea to (3-methylbutyryl)urea in animals. The debromination is inhibited by oxygen and dependent on flavins.

(α-Bromoiso-valeryl)urea (BVU) has been used as a mild sedative or hypnotic since 1906. The pharmacology of BVU has been reviewed (Meyer 1974). Acute poisoning by BVU caused by overdose or misuse is not uncommon because of the free availability of the drug, but death is rare because BVU is readily detoxified in the body (Maguchi 1961; Sticht & Käferstein 1973; Hayashida et al 1985).

BVU has been shown to be metabolized to (3-methylbutyryl)urea (MBU) by reductive debromination, and also to [2-(2-methylthio)-3-methylbutyryl]urea, α-(cystein-*S*-yl)isovalerylurea, α-(*N*-acetylcystein-*S*-yl)isovalerylurea and α-(cysteamin-*S*-yl)-isovaleric acid by glutathione conjugation in animals and man (Narafu 1967; Isono & Kozuka 1975; Isono 1978; Niederwieser et al 1978; Polhuijs et al 1986; Te Koppele et al 1988). In-vitro metabolism of BVU to MBU has been reported in liver microsomes of rabbits and rats (Narafu 1969; Oka et al 1996). Our recent experiments have demonstrated a definite role for cytochrome P450 1A1 and 2B1 in the microsomal reduction in rat liver (Oka et al 1996). We have also shown that BVU is reduced to MBU by rat blood (Kitamura et al 1999). However, the metabolic site involving the reductive debromination of BVU has been limited to liver microsomes and blood.

Because the reduction of certain xenobiotics is also mediated by intestinal bacteria (Scheline 1973), the current study was undertaken to investigate the role of intestinal bacteria in the reductive debromination of BVU.

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Materials and Methods

Chemicals

BVU was purchased from Nippon Shinyaku, NADPH and NADH from Oriental Yeast, and fla-

vin mononucleotide (FMN) and methyl viologen from Sigma. MBU was synthesized as described by Narafu (1967). Reduced FMN was prepared from FMN photochemically by the method of Yubisui et al (1980).

Bacterial strains

Clostridium sporogenes (IFO 14292), *Bifidobacterium bifidum* (IFO 14252), *Escherichia coli* K-12 (IFO 3301), *Salmonella typhimurium* (IFO 12529), *Pseudomonas fluorescens* (IFO 3081), *Lactobacillus rhamnosus* (IFO 3425) and *Streptococcus faecalis* (IFO 3971) were obtained from the Institute for Fermentation (Osaka, Japan). Facultative anaerobic bacteria were grown in l-broth at 37°C and harvested by centrifugation at 9000 *g* for 10 min. Cells were washed twice with sterilized 0·1 M K,Na-phosphate buffer (pH 7·4), and resuspended in the phosphate buffer. Anaerobic bacteria were grown in GAM broth (Nissui, Tokyo, Japan) using a BBL Gas Pack anaerobic jar (Becton-Dickinson, Cockeysville, MD). Cells harvested by centrifugation were suspended in GAM broth. The concentration of the cell suspensions was adjusted on the basis of the absorbance at 600 nm. When the absorbance was 0·1, the concentration was estimated to be 2×10^8 cells mL^{-1}.

Animals

Experiments were performed on male Wistar rats, 180–230 g, ddY mice, 25–32 g, Syrian golden hamsters, 75–90 g, Hartley guinea-pigs, 215–260 g, and Japanese albino rabbits, 2·1–2·4 kg. The first three and the last two species were fed standard MM-3 and RM-4 pellets (Funabashi Farm, Japan), respectively.

Caecal contents

The caecal contents of animals were suspended in 4 vols GAM broth and gently centrifuged at 500 *g* for 5 min to exclude food materials. The supernatant was used as the caecal contents.

Preparation of cell-free extract

Caecal contents (approx. 6 g) or *Clostridium sporogenes* cells (approx. 4 g) were suspended in 15 mL sterilized K,Na-phosphate buffer (0·1 M, pH 7·4; 15 mL), transferred to a glass vessel placed in an ice bath and sonicated for 20 cycles of 30 s on-180 s off at maximum power using a Tomy Ultrasonic disruptor (UR-200p). Any undisrupted cells in the suspension were removed by centrifugation at 9000 *g* for 10 min. The supernatant was used as cell-free extract from *Clostridium sporogenes* or intestinal bacteria in the caecal contents. The protein content of the cell-free extracts from *Clostridium sporogenes* and caecal contents were 4·6–5·5 and 14·8–16·6 mg protein mL^{-1}, respectively.

Assays of debrominating activity

Incubation was performed at 37°C for 60 min under an atmosphere of nitrogen in a Thunberg tube unless otherwise stated. A typical incubation mixture consisted of 1 μmol BVU in 10 μL methanol, 1 μmol electron donor, 0·2 μmol FMN or methyl viologen and an enzyme source in a final volume of 2 mL of 0·1 M K,Na-phosphate buffer (pH 7·4). When caecal contents or bacteria were used, the electron donor and FMN were omitted. After incubation, the mixture, after addition of HCl (1 N; 0·2 mL) and 2,4-dinitrochlorobenzene (100 μg) as internal standard, was extracted with chloroform (5 mL) and the extract was evaporated to dryness in-vacuo. The residue was dissolved in methanol (0·5 mL) and analysed by HPLC. HPLC was performed with an Hitachi L-6000 chromatograph fitted with a 125 mm × 4 mm LiChrosphere 100 RP-18(e) (5 μm) column (Merck, Darmstadt, Germany). The mobile phase was methanol–H_2O, 7 : 3, at a flow rate of 0·2 mL min^{-1} at room temperature. The detector wavelength was 210 nm. The elution times of MBU, BVU and 2,4-dinitrochlorobenzene were 7·0, 8·4 and 12·3 min, respectively. The amount of the metabolite (MBU) was determined from the peak area.

Determination of protein

Protein concentration was determined by the method of Lowry et al (1951) with bovine serum albumin as the standard protein.

Results

The caecal contents of rats had significant debrominating activity toward BVU under anaerobic conditions although little activity was observed under aerobic conditions. The time-course of the reductive debromination of BVU to MBU by caecal contents was essentially linear for 2 h under anaerobic conditions (Figure 1A). The activity increased linearly with increasing amounts of caecal contents up to 0·2 g (Figure 1B). When the caecal contents were sonicated or boiled, the activity was abolished (data not shown).

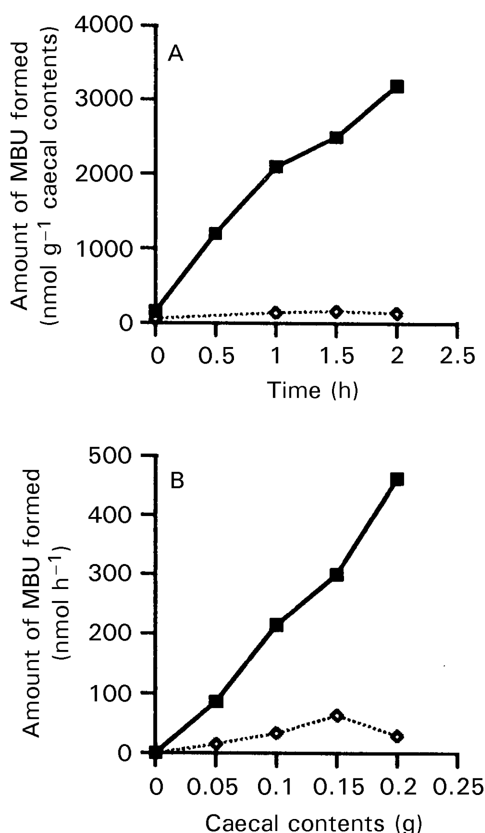

Figure 1. The reductive debromination of (α-bromoiso-valeryl)urea to (3-methylbutyryl)urea (MBU) by rat caecal contents. (A) Time-course of the debromination and (B) dependency on the amount of caecal contents. Reactions were conducted at 37°C with caecal contents under anaerobic (■) or aerobic (◇) conditions. In (A), 0·1 g caecal contents was added. In (B), the reaction mixture was incubated for 1 h.

The caecal contents of mice, hamsters, rabbits and guinea-pigs, like those of rats, catalysed the debromination of BVU to MBU under anaerobic conditions. Among the mammalian species examined, the highest activity was observed with mice and the lowest with guinea-pigs (Table 1). The capacity of the sonicated cell-free extract of intestinal bacteria from the caecal contents of rats to debrominate BVU to MBU was examined. When NADH, NADPH or FMN was added to the incubation mixture limited debrominating activity was observed. The activity was enhanced by addition of both NADH (or NADPH) and FMN under anaerobic conditions. The activity was inhibited by oxygen (Table 2). Similar results were obtained when FMN was replaced with flavin adenine dinucleotide (FAD) or riboflavin (data not shown).

The caecal contents contain a variety of bacteria. In this study, seven pure strains of intestinal bacteria were also tested for debrominating activity toward BVU under anaerobic conditions. The highest activity was observed for *Clostridium sporogenes*, followed by *Salmonella typhimurium*

Table 1. Debromination of (α-bromoiso-valeryl)urea by the caecal contents from several mammalian species.

Species	Amount of (3-methylbutyryl)urea formed (nmol h^{-1} (g caecal contents)$^{-1}$)	
	Anaerobic	Aerobic
Mouse	5764 ± 1177	108 ± 215
Rabbit	2793 ± 210	ND
Hamster	2449 ± 1283	214 ± 224
Rat	2147 ± 365	335 ± 201
Guinea-pig	764 ± 85	28 ± 15

Each value is the mean ± standard deviation of results from four experiments. ND = not detected. Incubation was performed with 0·1 g of caecal contents for 1 h.

Table 2. Debromination of (α-bromoiso-valeryl)urea to (3-methyl-butyryl)urea by the cell-free extract obtained from sonicated caecal contents of rats.

Sample	Amount of (3-methyl-butyryl)urea formed (nmol h^{-1} (mg protein)$^{-1}$)
None	–
NADH	13 ± 13
NADPH	19 ± 6
NADH + flavin mononucleotide	134 ± 13
NADPH + flavin mononucleotide	143 ± 71
Flavin mononucleotide	5 ± 5
NADH + flavin mononucleotide under aerobic conditions	4 ± 4

Each value is the mean ± standard deviation of results from four experiments. Reactions were conducted at 37°C for 1 h with 0·5 mL of the cell-free extract (approx. 8 mg protein) in the presence of cofactors under anaerobic conditions in 0·1 M phosphate buffer.

Table 3. Debromination of (α-bromoiso-valeryl)urea by different intestinal bacteria.

Bacteria	Amount of (3-methylbutyryl)urea formed (nmol h^{-1}/10^{10} cells) Anaerobic	Aerobic
Clostridium sporogenes	44·2 ± 5·2	13·1 ± 2·5
Bifidobacterium bifidum	2·2 ± 0·8	1·5 ± 0·7
Salmonella typhimurium	6·1 ± 2·7	2·7 ± 1·2
Escherichia coli	5·1 ± 1·8	4·1 ± 1·1
Lactobacillus rhamnosus	3·5 ± 0·9	1·5 ± 1·1
Pseudomonas fluorescens	1·9 ± 0·5	1·3 ± 0·7
Streptococcus faecalis	1·1 ± 0·1	0·9 ± 0·2

Each value is the mean ± standard deviation of results from four experiments. Incubation was performed with 3–10 × 10^{10} cells for 1 h.

Table 4. Debromination of (α-bromoiso-valeryl)urea to (3-methylbutyryl)urea by the cell-free extract from *Clostridium sporogenes*.

Sample	Amount of (3-methylbutyryl) urea formed (nmol h^{-1} (mg protein)$^{-1}$)
None	17 ± 3
NADH	28 ± 3
NADPH	21 ± 2
NADH + flavin mononucleotide	91 ± 42
NADPH + flavin mononucleotide	105 ± 69
NADH + methyl viologen	130 ± 83
NADPH + methyl viologen	139 ± 40
Flavin mononucleotide	53 ± 22
Methyl viologen	40 ± 8
NADH + flavin mononucleotide under aerobic conditions	9 ± 1

Each value is the mean ± standard deviation of results from four experiments. Reactions were conducted at 37°C for 1 h with 0·5 mL of the cell-free extract (approx. 3 mg protein) in the presence of cofactors under anaerobic conditions in 0·1 M phosphate buffer.

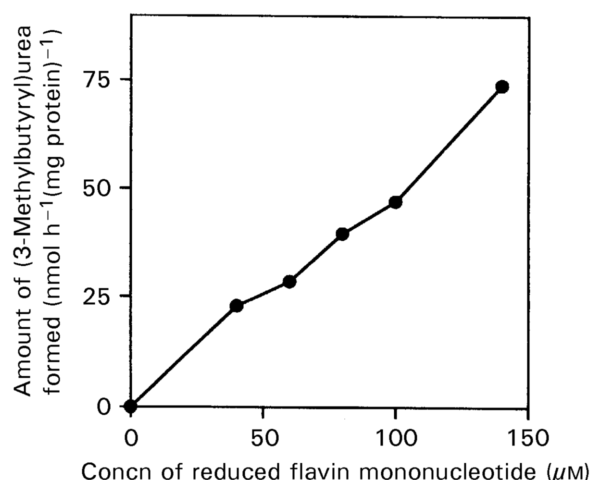

Figure 2. Debromination of (α-bromoiso-valeryl)urea to (3-methylbutyryl)urea by cell-free extract from *Clostridium sporogenes* with various concentrations of reduced flavin mononucleotide. Each value is the mean from two experiments. The incubation mixture consisted of BVU, 0·5 mL of the cell-free extract and photochemically reduced FMN instead of NADH and FMN. Incubation was performed for 1 h at 37°C under anaerobic conditions.

Table 5. Influence of some chemicals on the debromination of (α-bromoiso-valeryl)urea by the cell-free extract from *Clostridium sporogenes*.

Sample	Amount of (3-methylbutyryl)urea formed (nmol h^{-1} (mg protein)$^{-1}$)
None	85 ± 47
Cupric sulphate	82 ± 21
Sodium arsenite	63 ± 3
Potassium cyanide	37 ± 21

Each value is the mean ± standard deviation of results from four experiments. Reactions were conducted at 37°C for 1 h with cell-free extract (0·5 mL) in the presence of NADH, flavin mononucleotide and the test chemicals (10^{-4} M).

and *Escherichia coli*; the lowest was for *Streptococcus faecalis* (Table 3).

The capacity of the cell-free extract from *Clostridium sporogenes* to debrominate BVU was enhanced by addition of NADH (or NADPH) and FMN, or NADH (or NADPH) and methyl viologen under anaerobic conditions. The NADH- and FMN-dependent activity was inhibited by oxygen (Table 4). The debrominating activity was not influenced by addition of cupric sulphate, but was inhibited by sodium arsenite and potassium cyanide (Table 5).

The debrominating activity of the cell-free extract was substantial when photochemically reduced FMN was added instead of FMN and NADH (or NADPH). The activity increased with increasing concentrations of reduced flavin mononucleotide up to 140 μM (Figure 2).

Discussion

Various halogenated compounds are metabolized by reductive dehalogenation. For example, carbon tetrachloride, chloroform, halothane, 4-halogenated anilines and 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) are dehalogenated in the liver microsomal fraction, and cytochrome P450 is involved in this process (Esaac & Matsumura 1980; Kubic & Anders 1981; Ahr et al 1982; Noguchi et al 1982; Kelner et al 1986; Cnubben et al 1995; Testai et al 1995). The debromination of BVU is also mediated by cytochrome P450 in rat liver microsomes (Oka et al 1996). The current study provides the first evidence that intestinal bacteria play a role in the reductive debromination of BVU to MBU, and also confirms that their cell-free extract has the debrominating activity in the presence of both a reduced pyridine nucleotide and a flavin.

The reductive metabolism of xenobiotics by intestinal bacteria includes reductions of the nitro group, azo linkage, sulphoxide, hydroxamic acid derivatives and double-bond compounds, and reductive cleavage of the benzisoxazole ring (Williams et al 1970; Gingell et al 1971; Levin & Dent 1982; Renwick et al 1982; Ishida et al 1996; Kitamura et al 1997). However, dehalogenation by

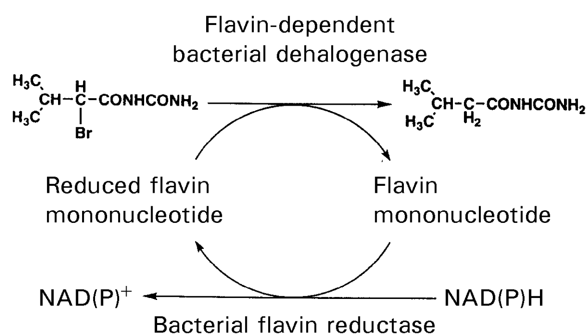

Figure 3. Proposed mechanism of the reductive debromination of (α-bromoiso-valeryl)urea by intestinal bacteria.

intestinal bacteria has been reported only for DDT. It was shown that DDT was transformed to DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane) by the intestinal bacteria of the northern anchovy, but the enzyme system involved was not identified (Malone 1970). The importance of intestinal bacteria in the metabolism of xenobiotics has been increasingly recognized, and the toxicological and pharmacological implications are of great concern (Scheline 1973). NADH- (or NADPH-) and FMN-dependent reductase activity toward BVU was present in the cell-free extract from *Clostridium sporogenes* or from intestinal bacteria in rat caecal contents. Therefore the debromination of BVU by intestinal bacteria presented in this study might proceed in two steps. The first step is reduction of FMN by a bacterial flavin reductase in the presence of a reduced pyridine nucleotide, as described by Fieschi et al (1995), or non-enzymatic reduction by NADPH or NADH, as reported by Singer & Kearney (1950). The second step is debromination of BVU to MBU by a flavin-dependent bacterial dehalogenase with the reduced flavin (Figure 3).

We have shown that BVU is enzymatically debrominated by rat liver microsomes (Oka et al 1996). The debromination is inhibited by oxygen, as is the reaction in the cell-free extract of intestinal bacteria. However, the microsomal debromination was not dependent on flavins. We have previously reported that 1-nitropyrene and zonisamide were reduced by a flavin-dependent enzyme system in cell-free extracts of *Escherichia coli* and *Clostridium sporogenes*, respectively (Narai et al 1984; Kitamura et al 1997). These systems resemble the debromination system in this study. Further investigation of the reducing enzyme system involved in the debromination of BVU by intestinal bacteria is in progress.

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