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# Debromination of (α-Bromoiso-valeryl)urea Catalysed by Rat Blood

SHIGEYUKI KITAMURA, MIE KUWASAKO, KAZUMI SUGIHARA, KIYOSHI TATSUMI AND SHIGERU OHTA

*Institute of Pharmaceutical Science, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8551, Japan*

**Abstract**

(α-Bromoiso-valeryl) urea, a sedative or hypnotic, is metabolized to (3-methylbutyryl)urea by reductive debromination. This study was designed to evaluate the role of blood in the debromination of (α-bromoiso-valeryl) urea.

Rat blood containing an electron donor had significant debrominating activity toward (α-bromoiso-valeryl)urea. This debromination proceeded by enzymatic and non-enzymatic processes which required both NADH (or NADPH) and flavin mononucleotide (FMN), under anaerobic conditions. The debrominating activity was sensitive to inhibition by carbon monoxide, and the pH optimum was 8·5. When FMN was replaced by flavin adenine dinucleotide (FAD) or riboflavin, similar results were obtained. The optimum concentration of flavins was $10^{-4}$ M. The reductive debromination was also mediated by rat erythrocytes, but not by plasma. When the blood or erythrocytes were boiled, the debrominating activity was not abolished, but was enhanced, suggesting that the activity arises from the haemoglobin in erythrocytes, and haemoglobin had debrominating activity when supplemented with both a reduced pyridine nucleotide and a flavin. Furthermore, haematin had significant debrominating activity in the presence of these cofactors. The activity of haematin was also observed with the photochemically reduced form of FMN.

The results imply that the debromination proceeds in two steps—enzymatic or non-enzymatic reduction of a flavin such as FAD, FMN or riboflavin by NADPH or NADH, then non-enzymatic reductive debromination of (α-bromoiso-valeryl)urea to (3-methylbutyryl)urea catalysed by the haem group of rat haemoglobin in the presence of the reduced flavin.

(α-Bromoiso-valeryl)urea (BVU) has been used for many years as a mild sedative or hypnotic, either alone or jointly with an antifebrile. Acute BVU poisoning is frequently observed as a result of overdose, but rarely causes death because the drug is easily detoxified (Maguchi 1961; Hayashida et al 1985). BVU is metabolized to (3-methylbutyryl) urea (MBU), [2-(2-methylthio)-3-methylbutyryl] urea, α-(cystein-*S*-yl)isovalerylurea, α-(*N*-acetyl-cystein-*S*-yl)isovalerylurea and α-(cysteamin-*S*-yl) isovaleric acid in rabbits, rats and man (Narafu 1967; Isono & Kozuka 1975; Isono 1978; Niederwieser et al 1978; Polhuijs et al 1989). It has recently been used as a model substrate for the characterization of glutathione conjugation, and roles were suggested for the α and μ classes of glutathione *S*-transferases in the conjugation of BVU (te Koppele et al 1988; Mulders et al 1993; Ouwerkerk-Mahadevan et al 1996). The reductive debromination of the drug to MBU has been demonstrated in-vitro using rabbit-liver microsomes (Narafu 1969). Our recent experiments have established that rat-liver microsomes can transform BVU to the debrominated metabolite, MBU, through a reductive reaction, perhaps involving formation of a BVU free-radical intermediate. A definite role for cytochrome P450 1A1 and 2B1 in the microsomal reduction has been established (Oka et al 1996).

In the current study the reductive debromination of BVU to MBU by rat blood was investigated. This study provides the first evidence that BVU can be reduced to MBU by blood in the presence of both a reduced pyridine nucleotide and a flavin.

Correspondence: S. Kitamura, Institute of Pharmaceutical Science, Hiroshima University School of Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8551, Japan.

## Materials and Methods

*Materials*

BVU was purchased from Nippon Shinyaku (Kyoto, Japan), NADPH and NADH from Oriental Yeast (Tokyo, Japan), and flavin mononucleotide (FMN), bovine blood haemoglobin, horse skeletal muscle myoglobin, bovine liver catalase, horse heart cytochrome c and bovine blood haematin ($Fe^{3+}$) from Sigma (St Louis, MO). MBU was synthesized as described by Narafu (1967). Reduced FMN was prepared from FMN photochemically by the method of Yubisui et al (1980).

*Preparation of blood and erythrocytes*

The study was performed on male Wistar (Slc : Wistar/ST) rats, 160–220 g. Blood samples were collected from the jugular vein into polystyrene tubes containing heparin as an anticoagulant and was centrifuged at 1000 *g* for 5 min at 4°C. The plasma was removed and the pellet (erythrocyte fraction) was washed with 2 vols cold isotonic saline. Erythrocytes were suspended and haemolysed in 1 vol. K,Na-phosphate buffer (0·01 M, pH 7·4).

*Assay of debrominating activity*

The incubation mixture consisted of 1 μmol BVU, 1 μmol NADPH or NADH, 0·2 μmol FMN, and blood (10 μL) or blood-equivalent (erythrocytes equivalent to 10 μL blood, 5 nmol haemoglobin, or 5 nmol haematin) in a final volume of 2 mL K,Na-phosphate buffer (0·01 M; pH 7·4). In some experiments the incubation mixture except substrate and cofactors was heated in a boiling water bath for 5 min. Incubation was performed using a Thunberg tube under anaerobic conditions. The side-arm contained NADPH or NADH, and the body contained all other components. The tube was gassed for 3 min with deoxygenated nitrogen, evacuated with an aspirator for 10 min and again gassed with the nitrogen. The reaction was started by mixing the components of the side arm and the tube body, was continued for 1 h at 37°C, and was stopped by addition of HCl (1 N; 0·2 mL). The mixture, after addition of 2,4-dinitrochlorobenzene (100 μg) as internal standard, was extracted once 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 high-performance liquid chromatography (HPLC) with an Hitachi L-6000 chromatograph equipped with an ultraviolet absorption detector, operated at a wavelength of 210 nm, and fitted with a 4 mm × 125 mm LiChrospher 100 RP-18(e) column, 5 μm particles (Merck, Darmstadt, Germany). The mobile phase was methanol-water, 7 : 3 (v/v) at a flow rate of 0·2 mL $min^{-1}$ at room temperature. The elution times of MBU, BVU and 2,4-dinitrochlorobenzene were 7·0, 8·4 and 12·3 min, respectively. The amount of MBU formed was determined from its peak area.

*Measurement of haem content*

The haem content of blood and erythrocytes was determined by the pyridine haemochrome method with haematin as standard (De Duve 1948).

*Measurement of protein content*

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

## Results

The time-course of the reductive debromination of BVU to MBU by rat blood in the presence of both NADH and FMN was essentially linear for 2 h under anaerobic conditions (Figure 1); no increase with time was observed in air (data not shown). When BVU was incubated with rat blood in the presence of these cofactors for 1 h at different pH values, the pH optimum for the formation of MBU was observed at pH 8·5 (Figure 2). However, the

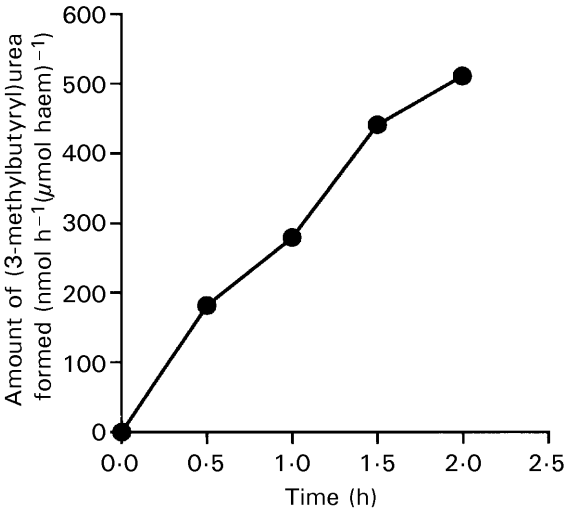


Figure 1. Time-course of the reductive debromination of (α-bromoiso-valeryl)urea to (3-methylbutyryl)urea by rat blood in the presence of both NADH and FMN. Reactions were conducted at 37°C with 10 μL rat blood under anaerobic conditions.

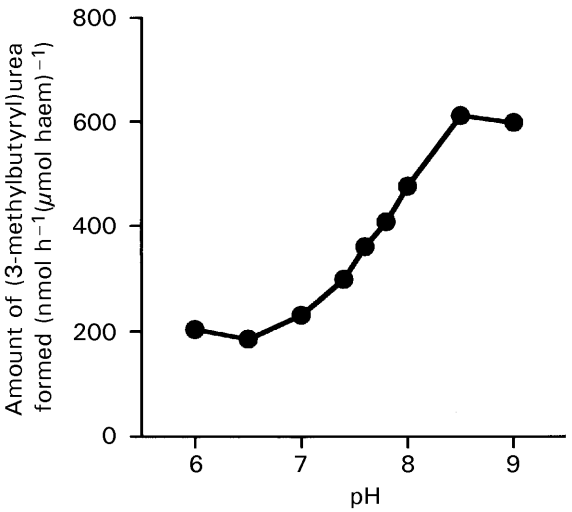


Figure 2. pH Profile of the reductive debromination of (α-bromoiso-valeryl)urea to (3-methylbutyryl)urea by rat blood in the presence of both NADH and FMN. Reactions were conducted at 37°C with 10 μL rat blood for 1 h under anaerobic conditions in 0·01 M phosphate buffer.

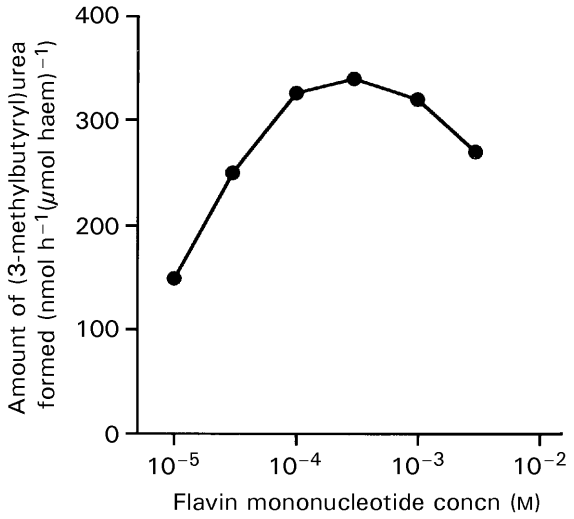


Figure 3. Dependence on flavin concentration of the debromination of (α-bromoiso-valeryl)urea to (3-methylbutyryl)urea. Reactions were conducted at 37°C with 10 μL rat blood in the presence of NADH and different concentrations of FMN.

other experiments in this study were performed at the physiological pH, 7·4.

The rate of reductive debromination of BVU by rat blood was determined in the presence of different cofactors. Rat blood had the highest debrominating activity when supplemented with both NADPH (or NADH) and FMN. Addition of NADH, NADPH or FMN alone resulted in only marginal activity. The activity was sensitive to inhibition by carbon monoxide. When the blood was boiled, the activity was not abolished, but was markedly enhanced. The activity of boiled blood was also markedly inhibited by carbon monoxide (Table 1).

Table 1. Reductive debromination of (α-bromoiso-valeryl)urea to (3-methylbutyryl)urea by rat blood.

| Sample | Amount of (3-methylbutyryl) urea formed (nmol $h^{-1}$ (μmol haem)$^{-1}$) |
|---|---|
| None | 2 ± 3 |
| NADH | 5 ± 3 |
| NADPH | 4 ± 5 |
| NADH + flavin mononucleotide | 313 ± 86 |
| NADPH + flavin mononucleotide | 244 ± 57 |
| NADH + flavin mononucleotide + carbon monoxide | ND |
| Flavin mononucleotide | 10 ± 7 |
| NADH + flavin mononucleotide* | 2380 ± 290 |
| NADH + flavin mononucleotide + carbon monoxide* | ND |

Each value is the mean ± standard deviation of results from three experiments. *Boiled blood was used. Flavin mononucleotide was added at $1 \times 10^{-4}$ M. ND = not detected.

When FMN was replaced by FAD or riboflavin in the presence of NADH or NADPH, similar results were obtained in the debromination of BVU by rat blood, but addition of methyl viologen or benzyl viologen was not effective (Table 2). The effect of flavin concentration was examined by changing the concentration of FMN. FMN was most effective at a concentration of $3 \times 10^{-4}$ M. The activity decreased when the concentration of FMN was greater than $3 \times 10^{-4}$ M (Figure 3).

Rat erythrocytes haemolysed by addition of 0·01 M phosphate buffer, also had significant debrominating activity in the presence of both NADH and FMN under anaerobic conditions. When the erythrocytes were boiled, the activity was enhanced, as had been found with blood. The activity was abolished in an atmosphere of air or

Table 2. Flavins required for reduction of (α-bromoiso-valeryl) urea by rat blood.

| Addition* | Amount of (3-methylbutyryl) urea formed (nmol $h^{-1}$ (μmol haem)$^{-1}$) | |
|---|---|---|
| | NADH | NADPH |
| Flavin mononucleotide | 325 ± 55 | 285 ± 25 |
| Flavin adenine dinucleotide | 251 ± 40 | 174 ± 28 |
| Riboflavin | 435 ± 68 | 378 ± 22 |
| Methyl viologen | ND | ND |
| Benzyl viologen | ND | ND |

Each value is the mean ± standard deviation of results from three experiments. *Amount added, $1 \times 10^{-4}$ M. ND = not detected.

carbon monoxide. Plasma had little activity in the presence of the cofactors (Table 3). These results implied that haemoglobin is involved in the debromination of BVU in rat erythrocytes.

When haemoglobin was used instead of blood, debrominating activity, which was sensitive to carbon monoxide, was observed in the presence of both NADH and FMN. The debrominating activity of boiled haemoglobin with both NADH and FMN was twice that of native haemoglobin (Table 4). When myoglobin, catalase and cytochrome c were used as haemoproteins instead of haemoglobin, the debrominating activity was also observed but to different extents (Table 5).

Haematin also had significant debrominating activity in the presence of both NADH and FMN, or NADPH and FMN. The non-enzymatic debromination of BVU by haematin was also observed in the presence of the photochemically reduced form of FMN (Table 6). In this system the reaction proceeded within minutes. Protoporphyrin, ferric chloride or ferrous chloride had no effect on the debromination (data not shown).

Table 3. Debromination of (α-bromoiso-valeryl)urea by erythrocytes and plasma of rats in the presence of NADH and FMN.

| Sample | Amount of (3-methylbutyryl) urea formed (nmol $h^{-1}$ (mg protein)$^{-1}$) |
|---|---|
| Erythrocytes | 7·6 ± 1·6 |
| Boiled erythrocytes | 84·2 ± 4·5 |
| Plasma | 3·1 ± 0·9 |
| Boiled plasma | 5·0 ± 4·0 |

Each value is the mean ± standard deviation of results from four experiments. Reactions were conducted at 37°C with 10 μL erythrocytes or 50 μL plasma in the presence of both NADH and FMN under anaerobic conditions. Ten microlitres of erythrocytes was equivalent to 1·5–1·9 mg protein and 15·3–15·7 nmol haem.

Table 4. Debromination of (α-bromoiso-valeryl)urea to (3-methylbutyryl)urea by haemoglobin.

| Sample | Amount of (3-methylbutyryl) urea formed (μmol $h^{-1}$ (μmol haem)$^{-1}$) |
|---|---|
| None | 0·5 ± 0·1 |
| NADH | 0·3 ± 0·1 |
| NADH + flavin mononucleotide | 14·7 ± 2·2 |
| NADH + flavin mononucleotide + carbon monoxide | 0·2 ± 0·1 |
| NADH + flavin mononucleotide (boiled haemoglobin) | 27·8 ± 7·7 |

Each value is the mean ± standard deviation of results from three experiments.

Table 5. Debromination of (α-bromoiso-valeryl)urea to (3-methylbutyryl)urea by haemoproteins.

| Protein | Amount of (3-methylbutyryl) urea formed (μmol $h^{-1}$ (μmol haem)$^{-1}$) |
|---|---|
| Myoglobin (5 nmol) | 2·0 ± 0·4 |
| Cytochrome c (5 nmol) | 1·7 ± 0·2 |
| Catalase (5 nmol) | 16·9 ± 1·5 |

Each value is the mean ± standard deviation of results from three experiments.

Table 6. Debromination of (α-bromoiso-valeryl)urea to (3-methylbutyryl)urea by haematin.

| Sample | Amount of (3-methylbutyryl) urea formed (μmol $h^{-1}$ (μmol haem)$^{-1}$) |
|---|---|
| None | 1·3 ± 0·2 |
| NADH | 1·7 ± 0·6 |
| NADPH | 1·7 ± 0·3 |
| NADH + flavin mononucleotide | 121·0 ± 41·7 |
| NADPH + flavin mononucleotide | 77·3 ± 18·6 |
| Flavin mononucleotide | 4·0 ± 0·9 |
| Photochemically reduced flavin mononucleotide | 470·0 ± 200·0 |

Each value is the mean ± standard deviation of results from three experiments.

These results lead to the conclusion that BVU can be reduced to MBU non-enzymatically by the catalytic action of the haem group of haemoglobin in the presence of a flavin and a reduced pyridine nucleotide, both of which can be replaced with a reduced flavin.

## Discussion

Halogenated hydrocarbons are metabolized to dehalogenated metabolites by reductive dehalogenation. Previous studies have shown that dehalogenation of carbon tetrachloride, chloroform, halothane, 4-halogenated anilines and 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) and BVU is limited to the liver microsomal fraction, and that cytochrome P450 is involved in the microsomal dehalogenation (Esaac & Matsumura 1980; Kubic & Anders 1981; Ahr et al 1982; Kelner et al 1986; Cnubben et al 1995; Testai et al 1995; Oka et al 1996). In this study we have shown that blood is a site for the debromination of BVU, in addition to liver microsomes.

The reductive metabolism of xenobiotic *N*-oxides by blood or its components has been reported—for example the reductions of indicine *N*-oxide by denatured haemoglobin (Powis &

DeGraw 1980), trimethylamine *N*-oxide by haemoglobin in the presence of cysteine (Vaisey 1956), 4-dimethylaminoazobenzene *N*-oxide by methaemoglobin and haematin (Terayama 1963), and 4-bromo-*N*,*N*-dimethylaniline *N*-oxide by ferrihaemoglobin (Kiese et al 1971).

We recently briefly reported that the dechlorination of DDT to DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane) is mediated by rat blood, and involves reduced pyridine nucleotides and flavins. The rate of dechlorination in blood is equal to that of the reaction catalysed by rat-liver microsomes (Sugihara et al 1998). We propose that blood plays an important role in the dehalogenation of various halogenated compounds.

The debromination of BVU by blood presented in this study seems to proceed in two steps. The first is reduction of a flavin by a reduced pyridine nucleotide; in native blood a flavin is reduced by NADPH-flavin reductase with NADPH, as reported by Yubisui et al (1977), whereas in boiled blood the flavin seems to be reduced non-enzymatically by NADPH or NADH, as reported by Singer & Kearney (1950). The second step is non-enzymatic reduction of BVU to MBU catalysed by the haem group of rat haemoglobin in the presence of reduced flavin (Figure 4). In boiled blood or erythrocytes the activity was increased, possibly because the substrate and cofactor have easier access to the haem group of denatured haemoglobin.

We have previously reported that BVU is enzymatically debrominated by rat-liver microsomes in a reaction involving cytochrome P450 (Oka et al 1996). The reductive debromination operates under anaerobic conditions and is inhibited by carbon monoxide and oxygen, in the same way as the reaction in blood. The debrominating activity was 4–6 nmol/40 min (mg protein)$^{-1}$, being of the same order as the activity of blood observed in this study. The debromination system in liver microsomes seems to involve electron transfer from NADPH to NADPH-cytochrome P450 reductase, cytochrome P450 and then to BVU, although it is possible that the haem group of cytochrome P450 has non-enzymatic debrominating activity toward BVU as described in our report on *N*-oxide reduction (Takekawa et al 1997). An investigation of the non-enzymatic reductive debromination of BVU by liver microsomes is in progress in this laboratory.

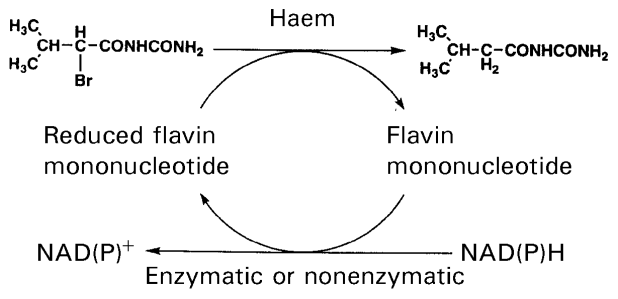


Figure 4. Proposed mechanism of the reductive debromination of (α-bromoiso-valeryl)urea by blood.

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