

NEW SECONDARY METABOLITES OF PHENYLBUTYRATE IN HUMANS AND RATS

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ABSTRACT:

Phenylbutyrate is used to treat inborn errors of ureagenesis, malignancies, cystic fibrosis, and thalassemia. High-dose phenylbutyrate therapy results in toxicity, the mechanism of which is unexplained. The known metabolites of phenylbutyrate are phenylacetate, phenylacetylglutamine, and phenylbutyrylglutamine. These are excreted in urine, accounting for a variable fraction of the dose. We identified new metabolites of phenylbutyrate in urine of normal humans and in perfused rat livers. These metabolites result from interference between the metabolism of phenylbutyrate and that of carbohydrates and lipids. The new metabolites fall into two

categories, glucuronides and phenylbutyrate β -oxidation side products. Two questions are raised by these data. First, is the nitrogen-excreting potential of phenylbutyrate diminished by ingestion of carbohydrates or lipids? Second, does competition between the metabolism of phenylbutyrate, carbohydrates, and lipids alter the profile of phenylbutyrate metabolites? Finally, we synthesized glycerol esters of phenylbutyrate. These are partially bioavailable in rats and could be used to administer large doses of phenylbutyrate in a sodium-free, noncaustic form.

Sodium phenylbutyrate (PB¹) is a highly effective drug for the treatment of patients with hyperammonemia resulting from inborn errors of urea synthesis (Batshaw et al., 1981, 2001; Brusilow, 1991). These patients excrete nitrogen as phenylacetylglutamine (PAGN) (Batshaw et al., 1981). The latter is also formed when the patients are treated with phenylacetate (PA). However, PB is preferred as a prodrug of PA because it does not have the foul smell of the latter. In addition, PB shows promise for the treatment of cystic fibrosis because it increases *trans*-membrane chloride conductance (Rubenstein and Zeitlin, 2000; Zeitlin et al., 2002). Also, PB is used in clinical trials for the treatment of sickle-cell anemia and thalassemia because it induces the formation of fetal hemoglobin (Dover et al., 1994; Hoppe et al., 1999). Lastly, PB is used in clinical trials as a cytostatic antineoplastic agent, because it inhibits histone deacetylases and potentiates the effect of cytotoxic agents on tumors (Samid et al., 1997; Gilbert et al., 2001).

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¹ Abbreviations used are: PB, 4-phenylbutyrate; PAGN, phenylacetylglutamine; PA, phenylacetate; PBGN, 4-phenylbutyrylglutamine; PHB, 3-hydroxy-4-phenylbutyrate; PrC, 4-phenyl-*trans*-crotonate; PKB, 4-phenyl-3-ketobutyrate; TMS, trimethylsilyl; GC-MS, gas chromatography-mass spectrometry; SW, sweep width; TD, data points; COSY, correlation spectroscopy; HSQC, heteronuclear single-quantum coherence; AUC, area under the curve.

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The clinical effectiveness of PB in some of these situations is limited by occasional incidences of toxicity at high doses (Carducci et al., 2001; Gore et al., 2002). Concerns have been raised by clinical investigators who treat patients with large doses of PB as a sodium salt. First, the total amount of PB and its known metabolites excreted in urine (PA, PAGN) is less than the administered PB dose, sometimes as low as 50%. Some of the unknown metabolites might contribute to PB toxicity at high doses. Second, the large sodium load of the treatment is potentially dangerous for patients with impaired cardiac and/or renal function. Third, the causticity of sodium PB can result in esophageal and/or gastric distress, even when it is administered as a powder suspended in water (this has been extensively debated on the Internet discussion group Metab-L at <http://lists.franken.de/mailman/listinfo/metab-L>). Neither the biochemical mechanism(s) of PB toxicity nor the identity of the missing metabolites of PB is known. Also, it is not clear whether the metabolism of PB (a modified fatty acid) interferes with, or is influenced by, the metabolism of fatty acids and carbohydrates present in foodstuffs. Lastly, it is not known whether stimulation of lipolysis under stress conditions interferes with PB metabolism.

Our original interest in PB metabolism was related to it being a precursor of PAGN, which can be used as a noninvasive probe of the ¹⁴C- or ¹³C-labeling pattern of citric acid cycle intermediates in human liver (Magnusson et al., 1991; Yang et al., 1996). As part of these investigations, we recently identified phenylbutyrylglutamine (PBGN) as a new metabolite of PB (Comte et al., 2002). PBGN is presumably formed from the reaction of PB-CoA with glutamine, by analogy with the formation of PAGN from the reaction between PA-CoA and glutamine (Webster et al., 1976). In normal adult sub-

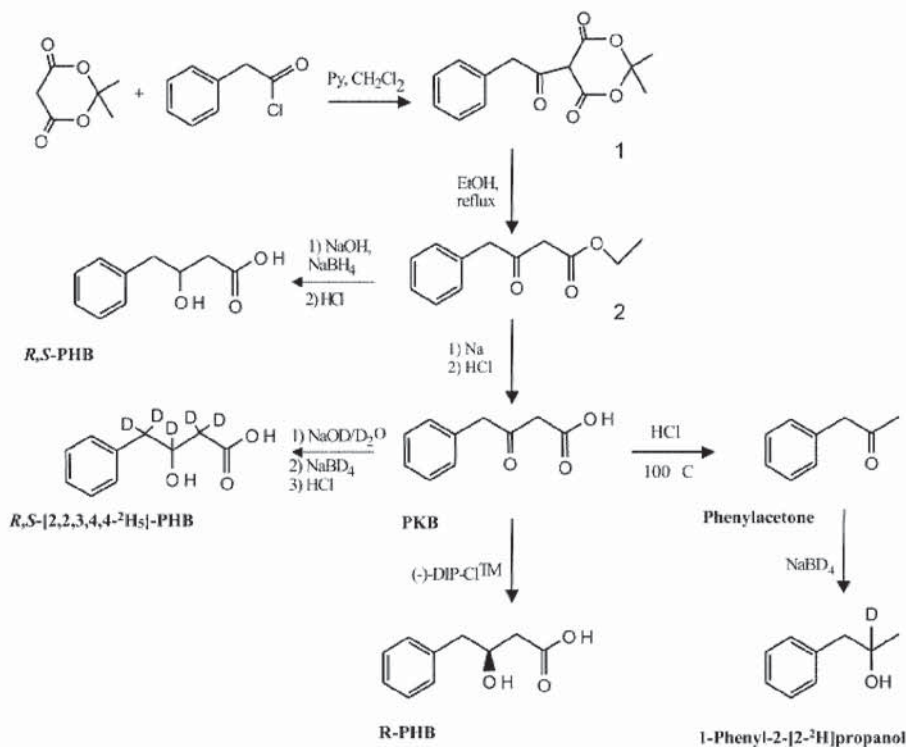


FIG. 1. Scheme for the synthesis of unlabeled and deuterated standards.

jects who ingested a fairly low dose of PB (5 g/75 kg), we found that the total excretion of PB + PA + PAGN + PBGN accounted for only half the ingested PB dose (Comte et al., 2002). The missing fraction of the dose may be disposed of either in urine as unknown metabolites, or in feces as unabsorbed PB and/or PB metabolites.

In the present study, we report the identification of additional metabolites of PB in humans, i.e., *R*- and *S*-3-hydroxy-4-phenylbutyrate (PHB), phenylacetone, and 1-phenyl-2-propanol, as well as PA and PB glucuronides. We studied the mechanism of PHB formation from PB in perfused rat livers and identified two additional metabolites, 4-phenyl-*trans*-crotonate (Pc) and 4-phenyl-3-ketobutyrate (PKB). Lastly, we prepared sodium-free esters of PB and investigated their bioavailability in rats. Glycerol-PB esters appear promising for the administration of large amounts of PB without the corresponding sodium load.

Materials and Methods

Materials. All chemicals used in syntheses, general chemicals, and solvents were obtained from Sigma-Aldrich (St. Louis, MO). All organic solvents were dried and distilled immediately before use. [²H₇]Phenylacetic acid (99%) and [²H₆]benzene were purchased from Isotec Inc. (Miamisburg, OH). The derivatization agent *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide was supplied by Regis Technologies, Inc. (Morton Grove, IL). All aqueous solutions were made with water purified with the Milli-Q system (Millipore Corporation, Bedford, MA).

Preparation of Unlabeled and Deuterated Standards. *S*-2-Phenylbutyryl chloride was prepared by reacting *S*-2-phenylbutyric acid with SOCl₂, and was vacuum-distilled and stored at 4°C as a 0.5 M solution in benzene. [²H₂]PB was prepared by aluminum chloride-catalyzed condensation of γ -butyrolactone with [²H₆]benzene as previously described (Comte et al., 2002). γ -Phenyl-*trans*-crotonic acid was prepared by a Friedel-Crafts reaction of benzene with ethyl γ -bromo-*trans*-crotonate, followed by acid hydrolysis of ethyl γ -phenyl-*trans*-crotonate (Löffler et al., 1970). The *trans* configuration of the product was confirmed by ¹H NMR. [²H₇]Phenylacetyl glycine was synthesized by reacting [²H₇]phenylacetyl chloride with glycine, as described previ-

ously for the unlabeled analog (Ramsdell and Tanaka, 1977). [²H₇]Phenylacetyl chloride was prepared by activation of [²H₇]phenylacetic acid with freshly distilled dichloromethyl methyl ether and used immediately after evaporation of excess dichloromethyl methyl ether and of the methyl chloroformate byproduct. The synthetic protocol for preparation of PHB, PKB, [²H₅]PHB, phenylacetone, and 1-phenyl-2-[2-²H]propanol is outlined in Fig. 1.

Ethyl 4-Phenyl-3-ketobutyrate (2; Fig. 1) was synthesized by a method adapted from Capozzi et al. (1993). The commercial isopropylidene malonate (2,2-dimethyl-4,6-diketo-1,3-dioxane, also called Meldrum's acid; Aldrich Chemical Co., Milwaukee, WI) was reacted with phenylacetyl chloride in the presence of dry pyridine in anhydrous methylene chloride. The crude phenylacetylated Meldrum's acid (compound 1, Fig. 1) was refluxed in absolute ethanol until evolution of CO₂ ceased (about 3 h). After evaporation of the solvent, ethyl 4-phenyl-3-ketobutyrate was purified on a silica gel column.

4-Phenyl-3-ketobutyric acid (PKB). A 10% molar excess of 1 N NaOH solution was added slowly to ice-cooled ethyl 4-phenyl-3-ketobutyrate and stirred at room temperature until the organic phase disappeared (approximately 12 h). The solution was acidified with 1 N HCl to pH 2 and extracted three times with 3 volumes of ethyl ether. After drying over Na₂SO₄, the solvent was evaporated to give the white solid product (yield 94%, m.p. 70°C). ¹H NMR (300 MHz, δ , CDCl₃): keto, 3.33 (s, 2H, CH₂COO), 3.78 (s, 2H, CH₂Ph), 7.17–7.33 (m, 5H, Ph); enol, 4.82 (s, 1H, CH), 12.13 (s, 1H, OH); keto/enol = 7.8:1. ¹³C NMR (75 MHz, δ , CDCl₃): 48.36 (CH₂COO), 49.87 (CH₂Ph), 127.41 (C-4 Ph), 128.73 (C-3, C-5 Ph), 129.50 (C-2, C-6 Ph), 133.14 (C-*ipso*, Ph), 169.26 (COO), 201.57 (CO).

The identity of the product was also confirmed by 1) reduction of 4-phenyl-3-ketobutyrate with NaBH₄, 2) extraction of *R,S*-PHB with ethyl acetate, and 3) TMS derivatization and NH₃-positive chemical ionization GC-MS. The mass spectrum of the derivative was identical to that of PHB synthesized as described below. The 4-phenyl-3-ketobutyric acid was stored at -80°C to prevent decomposition. Just before use in liver perfusion experiments, the acid was dissolved in water and titrated to pH = 7.40.

***R,S*-3-Hydroxy-4-phenylbutyric acid (PHB).** Ethyl 4-phenyl-3-ketobutyrate (2.06 g, 10 mmol) was mixed with 10 ml of H₂O and a calculated amount of NaOH/H₂O was added dropwise with cooling to give 0.5M solution of [OH⁻].

NaBH_4 (0.37 g, 10 mmol) was added and the mixture stirred for 24 h. After the reaction mixture was cooled to 0°C, the pH was brought to 7.0 with HCl (6N) and more than half of water removed by lyophilizer. The pH was brought to 2 by HCl (6 N) and the solution extracted 3 times with 3 volumes of ethyl ether. After drying the combined ether extract, the ether was evaporated giving a white solid product. Yield 79%. Purity of product was assayed by GC-MS after derivatization with TMS.

R-3-Hydroxy-4-phenylbutyrate (*R*-PHB) was prepared by reducing the corresponding 4-phenyl-3-ketobutyric acid with *B*-chlorodiisopinocampheylborane [(−)-DIP-Cl] (Wang et al., 1999). The yield was 89% from PKB; enantiomeric excess was 97% [GC-MS of the methyl *S*-2-phenylbutyryl derivative (see below), and purity was confirmed by NMR].

R,S-3-Hydroxy-4-phenyl[2,2,3,4,4- $^2\text{H}_5$]butyrate (*R,S*-[$^2\text{H}_5$]PHB). 4-Phenyl-3-ketobutyric acid (0.267 g, 1.5 mmol) was suspended in 3 ml of $^2\text{H}_2\text{O}$ (99.9%) to which 0.36 ml of 40% of NaO^2H (3.6 mmol) in $^2\text{H}_2\text{O}$ was slowly added at 0–5°C. This procedure exchanges ^1H for ^2H atoms on the methylene groups adjacent to the carbonyl. The solution was stirred at room temperature overnight and then lyophilized. The residue was dissolved in 3 ml of $^2\text{H}_2\text{O}$ and stirred for another 5 h at room temperature. The solution was cooled on ice, treated with NaB^2H_4 (63 mg, 1.5 mmol), and stirred overnight at room temperature (Des Rosiers et al., 1988). After acidification to pH 1 to 2 with HCl (6 N), the solution was saturated with NaCl and extracted three times with 3 volumes of diethyl ether. Solvent evaporation yielded *R,S*-[$^2\text{H}_5$]PHB (yield 74%, purity 99% by NMR, M5 isotopic enrichment 95% by GC-MS of the TMS derivative). The free acid was titrated to pH 8 with NaOH and stored frozen as a 0.5 M solution until use.

Phenylacetone was prepared by decarboxylating phenylketobutyrate in acid at 100°C. It was reduced to 1-phenyl-2-[^2H]propanol with NaB^2H_4 .

Esters of Phenylbutyrate. Dihydroxyacetone-di-PB, glycerol-tri-PB, ribose-tetra-PB, glucose-penta-PB, and sorbitol-hexa-PB were prepared by reacting the polyol/sugar with excess phenylbutyryl chloride in the presence of pyridine and catalytic amounts of *N,N*-dimethylaminopyridine. Products were purified by flash column chromatography on silica. To prepare glycerol-mono-PB, isopropylidene glycerol was reacted with phenylbutyryl chloride as above, and the isopropylidene group was removed by mild acidic hydrolysis in water. The structure and purity of all products were confirmed by ^1H and ^{13}C NMR. The structure of glycerol-mono-PB was confirmed by acetylation with acetic anhydride, followed by GC-MS analysis of the derivative.

Sample Preparation. For the determination of the free acids concentration (PA, PHB, PKB, P/C, and PB) in perfusate, samples (0.1 ml) were spiked with 0.17 μmol of [$^2\text{H}_2$]PA, [$^2\text{H}_2$]PB, and *R,S*-[$^2\text{H}_5$]PHB before deproteinization with 20 μl of saturated sulfosalicylic acid. The slurries were saturated with NaCl, acidified with one drop of 6 M HCl, and extracted three times with 5 ml of diethyl ether. For the assay of conjugates of PB and PA, 0.1-ml aliquots of final liver perfusate or of human urine were spiked with internal standards and treated with 1.0 ml HCl (6 N) at 90°C overnight to hydrolyze the conjugates. Glucuronides of PB and PA were identified by the amount of these compounds released after incubation of perfusate and urine samples with β -glucuronidase in 0.2 M ammonium acetate buffer, pH 5.0, overnight at 37°C.

Bile samples were analyzed for free and total (conjugated + free) PB and its metabolites. In the first series of assays, 0.05-ml samples of bile were spiked with internal standards, acidified to pH 2 to 2.5, and extracted three times with diethyl ether. In the second series of assays, samples spiked with internal standards were hydrolyzed with 0.3 ml of NaOH (6 N) at 90°C for 3 h before acidification and extraction.

Phenylacetyl-glycine was extracted in acid and derivatized with methanol/HCl. For the assay of phenylacetone and 1-phenyl-2-propanol, urine and perfusate samples were spiked with the structural analog 1-phenyl-[$^2\text{H}_5$]ethanol and then treated with NaB^2H_4 to reduce phenylacetone to monodeuterated 1-phenyl-2-propanol. The labeled and unlabeled 1-phenyl-2-propanol were assayed as TMS derivatives.

For the assays in urine, 0.1-ml samples were spiked with 0.15 μmol [$^2\text{H}_2$]PHB, acidified to pH 1 to 2 with HCl, saturated with NaCl, and extracted three times with 3 ml of diethyl ether. The combined extracts were dried with Na_2SO_4 and evaporated before reacting the residues with 70 μl of TMS at 60°C for 20 min.

For the chiral assay of PHB enantiomers (Powers et al., 1994), 0.1-ml samples were spiked with *R,S*-[$^2\text{H}_3$]PHB, and either deproteinized with 50 μl

of saturated sulfosalicylic acid (if containing proteins) or acidified to pH 1 to 2 with HCl (for urine). Then, the slurries or solutions were saturated with NaCl and extracted three times with 3 ml of diethyl ether. The combined extracts were dried with Na_2SO_4 and evaporated before reacting the residues with 0.15 ml of methanol/HCl for 1 h at 65°C, to derivatize the carboxyl groups of the PHB enantiomers. After cooling, 1 ml of water was added to the mixture and the hydroxyacid methyl ester was extracted with diethyl ether (three times in 3 ml). After complete evaporation of the combined ether extract, *S*-2-phenylbutyryl chloride benzene solution (0.1 ml, 0.5 M) and 0.05 ml of aqueous 12 N NaOH were added. After vortexing, the mixture was incubated for 1 h on a slow shaker at room temperature. The derivatives were extracted with ether (three times in 3 ml) and 1 ml of water. The combined ether phase was dried with Na_2SO_4 and evaporated completely. The residue was dissolved in 0.1 ml of ethyl acetate, and 1 μl was injected into the GC-MS.

GC-MS Methods. All of the metabolites, except phenylacetyl-glycine, were analyzed as their TMS derivatives on a Hewlett-Packard 5890 gas chromatograph equipped with a ZB-5 capillary column (60 m \times 0.25 mm i.d., 0.5 mm film thickness; Hewlett Packard, Palo Alto, CA) and coupled to a 5989A mass selective detector. Samples (0.2–1 μl) were injected with a split ratio 20 to 50:1. The carrier gas was helium (1 ml/min) and nominal initial pressure was 20.61 psi. The injector port temperature was at 270°C, the transfer line at 305°C, the source temperature at 200°C and quadrupole at 150°C. The column temperature program was: start at 100°C, hold for 1 min, increase by 8°C/min to 236°C, increase by 35°C/min to 310°C, 6 min at 310°C. After automatic calibration, the mass spectrometer was operated under ammonia-positive ionization mode. Appropriate ion sets were monitored with a dwell time of 25 to 35 ms/ion, at m/z 226/233 (PA/[$^2\text{H}_2$]PA), 254/259 (PB/[$^2\text{H}_2$]PB), 325/330 (PIIB/[$^2\text{H}_5$]PIIB), and 252 (P/C). Note that PKB and phenylacetone had been reduced with NaB^2H_4 to monodeuterated *R,S*-3-hydroxy-4-phenylbutyrate (monitored at m/z 326/330) and 1-phenyl-2-propanol (monitored at m/z 200/210 and/or 217/227). Also, since 1-phenyl-2-propanol was assayed with a standard of 1-phenyl[$^2\text{H}_5$]ethanol, ions monitored for this assay were 200/209 or 217/226.

For the analysis of chiral PIIB derivatives, the GC injector temperature was set at 280°C. The column (DB-5, 60 m \times 0.25 mm i.d., 0.5 mm film thickness; Hewlett Packard) program was modified to the initial 150°C for 2 min, increased by 15°C/min to 230°C, 25 min at 230°C, increased by 35°C to 290°C, and held 10 min. Ions monitored were 1) 358 ($M + 18$, i.e., $M + \text{NH}_4^+$) for analytes and 2) 363 ($M + 18 + 5$, i.e., $M + 5 + \text{NH}_4^+$) for [$^2\text{H}_2$]PHB. The mass spectrometer was operated under ammonia-positive ionization and was tuned automatically.

Phenylacetyl-glycine was analyzed as its methyl ester derivative using an OV-225 column (29 m \times 0.32 mm i.d., 1 μm film thickness; Quadrex Corporation, Woodbridge, CT). This column yielded better resolution of *N*-phenylacetyl-glycine methyl ester with no peak tailing. Samples (0.2–1 μl) were injected with a split ratio 20:1. The carrier gas was helium (constant flow: 1.2 ml/min). The injector port temperature was at 220°C, the transfer line at 240°C, the source temperature at 200°C, and quadrupole at 106°C. The column temperature program was: start at 90°C, hold for 1 min, increase by 10°C/min to 240°C, 15 min at 240°C. After automatic calibration, the mass spectrometer was operated under ammonia-positive ionization mode (pressure adjusted to optimize peak areas). Ions monitored were 1) 208 ($M + 1$, i.e., $M + \text{H}^+$) and 225 ($M + 18$, i.e., $M + \text{NH}_4^+$) for the analyte and 2) 215 ($M + 7 + 1$, $M + 7 + \text{H}^+$) and 232 ($M + 7 + 18$, $M + 7 + \text{NH}_4^+$) for *N*-[$^2\text{H}_7$]PA-glycine with a dwell time of 25 ms/ion.

Areas under each chromatogram were determined by interactive computer integration, and corrected for naturally occurring heavy isotopes and light isotopic impurities in the synthesized labeled internal standards.

NMR Spectroscopy. Proton NMR spectroscopy was performed at 400 MHz on a Bruker Avance (Bruker, Newark, DE) DMX 400 wide-bore spectrometer using a 5-mm inverse probe. Full-strength urine samples were obtained by lyophilizing 5 ml of urine to dryness. The residue was dissolved in 0.5 ml of $^2\text{H}_2\text{O}$, and the solution was introduced into a 5-mm NMR tube. An external standard made of a sealed capillary containing a solution of trimethylsilylpropionic acid in $^2\text{H}_2\text{O}$ was introduced into the NMR tube and used as chemical shift reference. Standard acquisition conditions were as follows for one-dimensional spectra: 45° pulse, 8-s repetition time, water saturation during the relaxation delay, sweep width (SW) 6775 Hz, 64K data points (TD), and

32 scans of data collection. Two-dimensional correlation spectroscopy (COSY) spectra were obtained with the following conditions for the second dimension: SW 3500 Hz, TD 2K, 16 scans, and for the first dimension, 512 increments of 278 μ s zerofilled to 1K. A nonshifted sinebell window was applied in both dimensions, and magnitude spectra were calculated. Two-dimensional $^1\text{H}/^{13}\text{C}$ correlations via double insensitive nuclei enhanced by polarization transfer (HSQC) were performed in the phase-sensitive mode (TPPI; time-proportional receiver phase incrementation) using gradients for coherence selection and carbon decoupling during acquisition. The following conditions were used in the second dimension: SW 3200 Hz, TD 2K, 128 scans, and in the first dimension: SW 12 kHz, 256 increments of 20.7 μ s zerofilled to 512. A shifted sinebell window was applied in both dimensions.

Proton-decoupled carbon spectra of the concentrated urine samples were obtained at 100.62 MHz in a 5-mm dual probe. Acquisition conditions were as follows: 20° pulse, repetition time 1.3 s. SW 25 kHz, TD 64K, 40,000 scans. The free induction decays were zerofilled to 128K, and a Lorentz to Gauss transformation (LB = -1 Hz, GB = 0.1) was applied before Fourier transformation.

Clinical Investigation. The protocol was reviewed and approved by the Institutional Review Board of University Hospitals of Cleveland. All subjects were free of any chronic or acute illness. Women had a negative pregnancy test and were not breastfeeding. Seven subjects (three men, four women; 31.7 ± 5.0 years; 171.3 ± 3.4 cm; 79.5 ± 5.9 kg) received detailed information on the purpose of the investigation and signed an informed consent form. After an overnight fast, the subjects were admitted to the Clinical Research Center at 7:30 AM. They remained fasting until the completion of the study. An intravenous line was installed in the forearm with a saline infusion (20 ml/h) and a short blood sampling catheter was inserted into a superficial vein of the contralateral hand. The hand was placed in a heating box at 60°C for sampling of arterialized venous blood. At 8:00 AM, after baseline blood and urine sampling, each subject ingested 0.36 mmol/kg (5 g/75 kg) Na-PB. This dose corresponds to 11 to 17% of the doses commonly used in the treatment of patients with inborn errors of urea synthesis ($0.4\text{--}0.6 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$). Water intake was adjusted to induce a diuresis of at least 100 ml/30 min. Urine samples were collected at 30-min intervals for the first 3 h after PB ingestion, and then every hour until 8 h. Urine samples were quickly frozen and stored at -80°C until analysis.

Organ Perfusion Experiments. Livers from fed male Sprague-Dawley rats kept on standard rat chow (200–230 g) were perfused (Brunengraber et al., 1975) with recirculating Krebs-Ringer-bicarbonate buffer containing 4% bovine serum albumin (fraction V, fatty acid poor; Intergen, Purchase, NY) and 10 mM glucose. The bile duct was cannulated with PE 10 tubing (BD Biosciences, San Jose, CA) for bile collection. Throughout the 2-h experiment, sodium taurocholate (38 μ mol/h) was infused into the perfusion reservoir to stimulate bile flow (Robins and Brunengraber, 1982). After 30 min of equilibration, a calculated amount of either PB, *R,S*-PHB, or PKB was added to the perfusate to set an initial concentration of 5 mM. The perfusion continued until 120 min. The pH of the perfusate was monitored and kept at 7.3 to 7.4 by adding 0.3 M NaOH. Samples of bile and perfusate were collected at regular intervals. For the assay of PKB, perfusate samples (2 ml) were treated immediately with 0.2 ml of 0.1 M NaB^2H_4 in 0.1 mM NaOH to convert unstable PKB to stable monodeuterated *R,S*-PHB. Bile samples were collected every 30 min. At the end of the experiment, the livers were quick-frozen with aluminum tongs precooled in liquid nitrogen.

Rat in Vivo Experiments. We tested the bioavailability of two PB esters as a means to deliver large amounts of PB without the corresponding sodium load. Overnight-fasted rats (330–400 g) were divided into six groups (5–7 rats per group) for the testing of three different PB preparations: Na-PB, glycerol-mono-PB, glycerol-tri-PB, ribose-tetra-PB, glucose-penta-PB, and sorbitol-hexa-PB. Each rat received one stomach gavage of the sodium salt or ester in an amount that delivered 2.15 mmol PB/kg. The weighed dose for each rat was mixed with 3 ml of Tween and administered to the rats through a stomach gavage needle.

Whole blood samples (100–200 μ l) were taken at -5, 15, 30, 60, 150, 240, 330, 420, and 480 min from a small incision in a tail vein. Blood was collected in heparinized microcapillary tubes and centrifuged. The plasma (50–100 μ l) was transferred to an Eppendorf tube and quick frozen.

Results

Human Study. In our previous study, we had identified PBGN in the plasma and urine of normal adults who had ingested a small dose of PB. We now report the data of additional analyses conducted on the same samples of human urine. First, we subjected to NMR analysis two samples of urine produced by each subject before and 2 h after ingestion of PB. The NMR spectrum of the second sample, but not of the first, was highly suggestive of the presence of a product of hydroxylation of the side chain of PB. In the COSY spectrum of the lyophilized urine dissolved in D_2O (after PB ingestion), we identified a proton at 4.25 ppm coupled with two CH_2 's. The first CH_2 has protons at 2.87 and 2.70 ppm; the second has nearly identical protons at 2.4 ppm. A chemical shift at 4.25 ppm is likely corresponding to a proton coupled to the OH group. Therefore, the COSY spectrum revealed the presence of a metabolite having $-\text{CH}_2\text{--CHOH--CH}_2\text{--}$ moiety. In the HSQC spectrum these proton signals correlated with the following carbons: CH at 70 ppm overlapping with other CH carbohydrate carbons, CH_2 at 44.6 ppm and CH_2 at 42.5 ppm. Lastly, in the aromatic region, signals at 129.6, 128.7, and 126.6 ppm corresponded to a monosubstituted phenyl group having the same intensity per carbon as the signals of the $-\text{CH}_2\text{--CHOH--CH}_2\text{--}$ group. We therefore concluded that β -hydroxy-PB (PHB) was present in the urine of patients treated with PB. PHB would be a very likely metabolite since it would be formed via partial β -oxidation of PB to PHB-CoA (presumably the *S*-enantiomer), which would be hydrolyzed to free PHB.

To further confirm the identity of the urinary metabolite detected by NMR, we synthesized unlabeled and R,S - $[\text{}^2\text{H}_5]$ PHB. The di-TMS derivative of synthetic *R,S*-PHB was analyzed by GC-MS in parallel with an extract of human urine (after PB ingestion) that had been reacted with TMS. In the sample derived from urine, we found a peak at the same retention time and with the same mass spectra (electron ionization and NH_3 -positive chemical ionization) as the standard of *R,S*-PHB. In addition, the NMR spectrum of synthetic PHB had the same chemical shifts as the material identified in human urine. This confirmed the identity of PHB in human urine but did not yield information about its chirality. The chirality of excreted PHB yields information on the mechanism of its formation (see below).

For the chromatographic separation of PHB enantiomers from the synthetic racemate, we tried various chiral hydroxyl derivatization reagents before selecting the combination of 1) methylation of the carboxyl group, and 2) reaction of the hydroxyl group with *S*-2-phenylbutyryl chloride (Powers et al., 1994). The expected derivatives of *R*- and *S*-PHB were well separated, and their order of elution was confirmed using a sample of *R*-PHB that we had synthesized. *R*-PHB elutes ahead of the *S*-derivative (Fig. 2).

Chiral GC-MS analysis of the human urine samples (after PB ingestion) revealed that PHB is present as an enantiomeric mixture with 10% *R*-PHB and 90% *S*-PHB (Fig. 2). Figure 3 shows the time profile of (*R*+*S*)-PHB excretion in urine after an oral bolus of Na-PB. The excretion of (*R*+*S*)-PHB peaked at 120 to 240 min. Eight hours after ingestion of PB, the cumulative excretion of (*R*+*S*)-PHB (1.35 ± 0.13 mmol) amounted to $4.4 \pm 0.56\%$ of the PB dose.

Small amounts of phenylacetone and 1-phenyl-2-propanol were identified in the urine samples (Table 1). Treatment of urine samples with β -glucuronidase increased their PB + PA content. The total amount of PB + PA released by β -glucuronidase amounted to $2.4 \pm 0.3\%$ of the PB dose.

Perfused Rat Liver Study. The metabolism of PB was studied in perfused rat livers by the addition of 5 mM PB to the recirculating perfusate. The time profile of the PB concentration was curvilinear

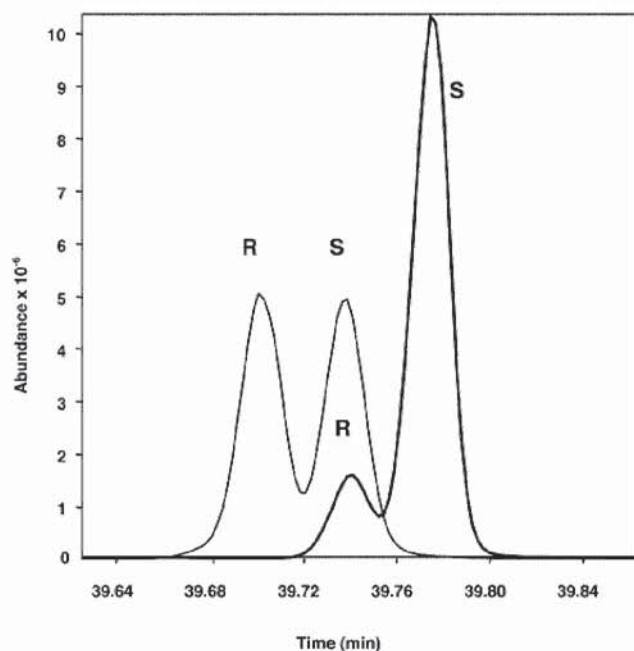


Fig. 2. Chiral GC-MS assay of *R*- and *S*-PHB excreted in one sample of human urine after oral ingestion of 0.36 mmol/kg Na-PB (bold trace).

The sample was spiked with *R,S*-[²H₅]PHB, the enantiomer profile of which is shown by the thin trace.

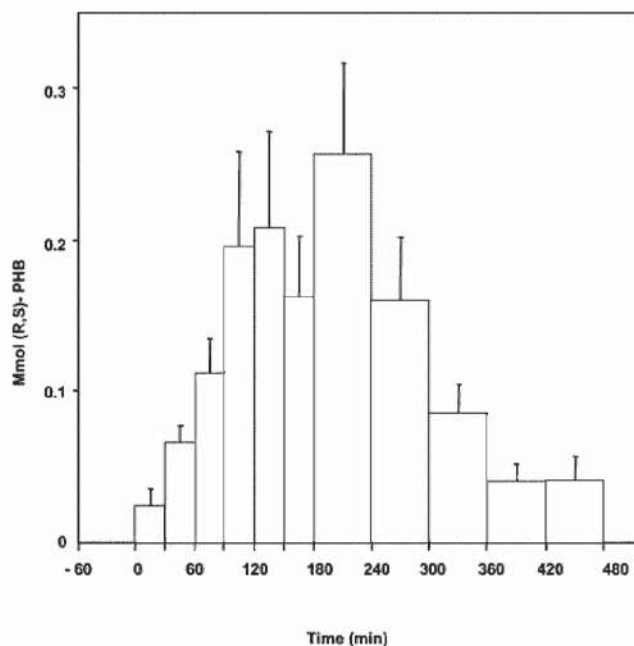


Fig. 3. Time course of (*R,S*)-PHB urinary excretion in normal humans after oral ingestion of 0.36 mmol/kg Na-PB (mean + S.E.M.; *n* = 7).

(Fig. 4A). Plotting of the data under semilogarithmic coordinates yielded a linear relationship compatible with a first order kinetic process for PB uptake. The total uptake of PB amounted to $310 \pm 16 \mu\text{mol} \cdot (90 \text{ min}^{-1}) \cdot \text{liver}^{-1}$.

Figure 4B shows the time accumulation of metabolites derived from PB and released into the perfusate. Phenylacetylglutamine is a

TABLE 1

Recovery of PB and its metabolites in human urine (*n* = 7) after the oral ingestion of 0.36 mmol/kg Na-PB and production of PB metabolites by rat liver (*n* = 5) perfused with 5 mM PB

Metabolite	% of PB Uptake	
	Humans	Rats
Free PB	0.97 ± 0.23	
PB-β-glucuronide	1.29 ± 0.33	21.52 ± 4.32
Free PA	0.26 ± 0.06	7.64 ± 0.87
PA-glycine		7.68 ± 0.84
PA-β-glucuronide	1.11 ± 0.19	5.25 ± 0.88
PAGN ^a	32.6 ± 1.9	
PBGN ^a	21.5 ± 2.4	
PHB	4.4 ± 0.56	15.71 ± 1.63
PKB		4.54 ± 0.29
PiC		5.15 ± 0.59
Phenylacetone	0.11 ± 0.007	3.67 ± 0.23
1-Phenyl-2-propanol	0.01 ± 0.001	Trace amounts
Total bile met		2.97 ± 0.61
Total	62.4 ± 2.1	74.12 ± 5.58

^a Previously identified metabolites (Comte *et al.*, 2002).

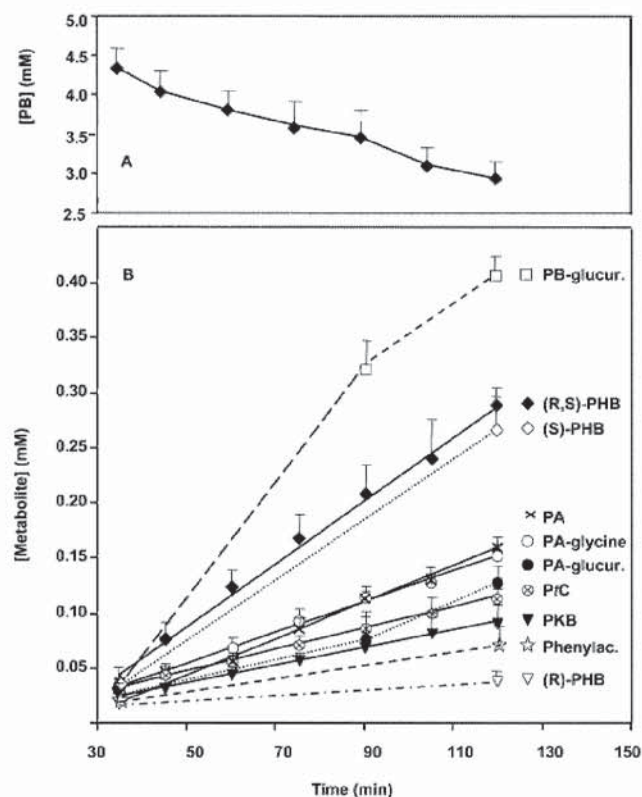


Fig. 4. Metabolism of PB in perfused rat livers.

A, uptake of PB from the recirculating perfusate. B, accumulation of PB metabolites in the perfusate (*n* = 5 for all compounds except for PKB, where *n* = 4).

known metabolite of PA in rats and dogs (Knoop, 1904; Ambrose and Sherwin, 1933; James *et al.*, 1972). About 16% of the uptake of PB was accounted for by the production of *R*- and *S*-PIIB, of which about 90% is the *S*-enantiomer. We also identified PiC, PKB, phenylacetone, and 1-phenyl-2-propanol, which, to our best review of the literature, have not been previously described as metabolites of PB. The identity of these metabolites was confirmed by GC-MS using standards we synthesized. Incubation of liver perfusate samples with

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