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R,S-1,3-butanediol acetoacetate esters, potential alternates to lipid emulsions for total parenteral nutrition

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We present the preparation and characterization of totally and partially water-soluble forms of fat which could replace emulsions of long-chain triacylglycerols for total parenteral nutrition. R,S-1,3-butanediol acetoacetate monoesters and diester represent pH-neutral, sodium-free, diffusible precursors of ketone bodies. The latter are water-soluble forms of fat that are well used by peripheral tissues except in prolonged starvation and diabetic ketoacidosis. The esters are rapidly hydrolyzed by plasma and tissue esterases. R,S-1,3-butanediol liberated is oxidized in liver to R,S-β-hydroxybutyrate. Reducing equivalents generated during this oxidation are trapped in the conversion of acetoacetate to R-β-hydroxybutyrate. So both the carbon and the hydrogen of the esters are exported from the liver to peripheral tissues in the form of R- + S-β-hydroxybutyrate. Thus, contrary to what occurs after administration of ethanol or R,S-1,3-butanediol alone, administration of the R,S-1,3-butanediol acetoacetate esters does not lead to major shifts in the liver's [NADH]/[NAD$^+$] ratio. Such shifts are responsible for the toxic effects of ethanol on the liver. It is therefore likely that long-term administration of the R,S-1,3-butanediol acetoacetate esters will not lead to liver toxicity. (J. Nutr. Biochem. 6:111–118, 1995.)

Keywords: acetoacetate esters; 1,3-butanediol; ketone bodies; total parenteral nutrition

Introduction

Concentrated fat emulsions used for parenteral nutrition have undesirable side effects like catheter obstruction, hyperlipemia, thrombopathy, fat overload syndrome, and fat embolism. There is a need for developing water-soluble energy substrates for long-term intravenous feeding. Ketone bodies, that is *R*-3-hydroxybutyrate[(1)] (*R*-BHB)[(2)] and acetoacetate (AcAc) are water-soluble equivalents of long-chain fatty acids. They are good fuels for peripheral tissues, except during prolonged starvation and diabetic ketoacidosis.[1,2] Thus, ketone bodies could, in principle, be used for intravenous nutrition in lieu of lipid emulsions. In spite of being strong acids, ketone bodies are ultimately oxidized to CO_2, a weak and volatile acid. However, ketone bodies cannot be administered in acid form that would cause peripheral vein irritation. Infusion of sodium salts of ketone bodies would result in dangerous sodium overload. Beylot et al.[3] administered *R*-BHB as an equimolar mixture of free acid, sodium salt, lysine salt, and arginine salt to minimize the sodium load. Other investigators infused large amounts of the arginine salt in anesthetized dogs.[4] It is not clear whether massive infusions of individual salts of basic aminoacids might interfere with the transport of other amino acids across the blood-brain barrier, perturbing the production of some neurotransmitters. Also, such infusions might be harmful to patients with hepatic or renal pathologies.

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Received April 28, 1994; accepted September 22, 1994.

[1]The physiological enantiomer *R*-BHB is designated as D-BHB in the recent clinical literature. In early publications and in the Merck Index, it is called *L*-BHB. *S*-BHB is the unnatural enantiomer.

[2]The abbreviations are: AcAc, acetoacetate; BD, *R,S*-1,3-butanediol; BD(AcAc), *R,S*-1,3-butanediol monoacetoacetate; BD(AcAc)$_2$, *R,S*-1,3-butanediol diacetoacetate; BHB, β-hydroxybutyrate; GC-MS, gas chromatography-mass spectrometry; TBDMS, *t*-butyldimethylsilyl;

Nutritional Biochemistry 6:111-118, 1995

655 Avenue of the Americas, New York, NY 10010

0955-2863/95/$10.00
SSDI 0955-2863(94)00011-A

Birkhahn et al.[5,6] synthesized the monoester of glycerol and AcAc (monoacetoacetin) which is hydrolyzed in plasma and tissues to glycerol and AcAc. This ester was the first practical form for administering large amounts of ketone bodies without sodium load. Maiz et al.[7] showed that monoacetoacetin can be safely administered to normal and burned rats. In addition they reported that monoacetoacetin exerts a nitrogen sparing effect in burned rats.

One could supply ketone bodies in the form of a nonionized water-soluble precursor such as *R,S*-1,3-butanediol (BD). This compound is metabolized in liver to *R,S*-BHB via alcohol and aldehyde dehydrogenase.[8,9] *S*-BHB derived from *S*-BD is not a natural compound,[10] but is well used by mammalian cells.[11–14] We have[12] identified the pathway of *S*-BHB metabolism in liver: mitochondrial activation to *S*-BHB-CoA, an intermediate of fatty acid β-oxidation, then conversion to physiological ketone bodies, lipids, and CO_2. We have also described the metabolism of *R*- and *S*-BD in the liver.[14]

In spite of the above, *R,S*-BD is not suitable as a peripheral intravenous nutrient because it is a small molecule, with a low caloric density per osmol. In addition the rapid oxidation of BD in liver, like that of ethanol, markedly increases the [NADH]/[NAD^+] ratio.[9,15] Thus administration of BD alone might induce alcoholic hypoglycemia especially in fasting subjects.

To increase the caloric density of BD and to prevent the increase in the hepatic [NADH]/[NAD^+] ratio, we synthesized the mono- and diester of BD and AcAc, namely *R,S*-1,3-butanediol monoacetoacetate (BD-AcAc) and *R,S*-1,3-butanediol diacetoacetate (BD$(AcAc)_2$). We reasoned that the esters[3] should be easily hydrolyzed by plasma and tissue nonspecific esterases. In addition, the AcAc moiety of these esters should trap reducing equivalents generated in the liver during the oxidation of BD to BHB, thus preventing an increase in the [NADH]/[NAD^+] ratio and alcoholic hypoglycemia. The present report concentrates on the preparation of the esters and on their hydrolysis in vitro and in vivo.

Methods and materials

Chemicals

Diketene, ethyl acetoacetate, t-butyl-acetoacetate, and *R,S*-1,3-butanediol were purchased from Aldrich (Milwaukee, WI, USA) and Sigma (St. Louis, MO, USA). Biochemicals and enzymes were supplied by Boehringer Mannheim (Indianapolis, IN, USA). N-methyl-N-(*t*-butyldimethylsilyl)-trifluoroacetamide was obtained from Regis Chemical Co. (Morton Grove, IL, USA). Internal standard of *R,S*-[1,1,3-$^2H_3$]-1,3-butanediol was prepared[13] by reduction of ethyl AcAc with $LiAl^2H_4$ (MSD Isotopes, Dorval, Québec, Canada).

Synthesis of 1,3-butanediol acetoacetates

Procedure 1. Add 1 mol of BD and 0.5 ml of triethylamine to a three-necked round bottom flask equipped with a mechanical stirrer, flushed with N_2, and cooled with ice + water. Over 2.5 hr, add 1.1 mol of diketene freshly distilled under vacuum (42°C, 22 mm Hg). The reaction mix is further stirred at 0°C for 2.5 hr, then at room temperature for 15 hr. The reaction is monitored (1) by proton NMR, following diketene disappearance, and (2) by thin layer chromatography on silica gel (IB2-F, Baker-Flex) developed with ethyl acetate:hexane (60:40) and revealed with 1 M ammonium molybdate/1 M ceric ammonium sulfate in 1.8 M H_2SO_4. The pale yellow raw product contains about 80% of BD (AcAc), 20% of BD $(AcAc)_2$ and traces of BD. One can favor the synthesis of diester by reacting 2.2 mol of diketene with 1 mol of BD. Then the raw product contains >95% of diester. ^{13}C-NMR analysis shows two monoesters[4]: 25% of 1-methyl,3-hydroxypropyl-acetoacetate (secondary alcohol esterified) and 75% of 3-hydroxybutyl-acetoacetate (primary alcohol esterified, *Figure 1*). This was confirmed by gas chromatography-mass spectrometry (see below).

The esters are purified by flash chromatography on a 600 g of silica gel column (6 × 16 cm). The raw product (100 g) is dissolved in 150 ml of ethyl acetate and slowly loaded onto the column, which is then developed with increasingly polar solutions of ethyl acetate:hexane: 1 L of 60:40, 2 L of 65:35 and 2 L of 70:30. Compounds elute in the order diester, monoesters, and traces of BD. The total yield in esters is 85%. The solvent is

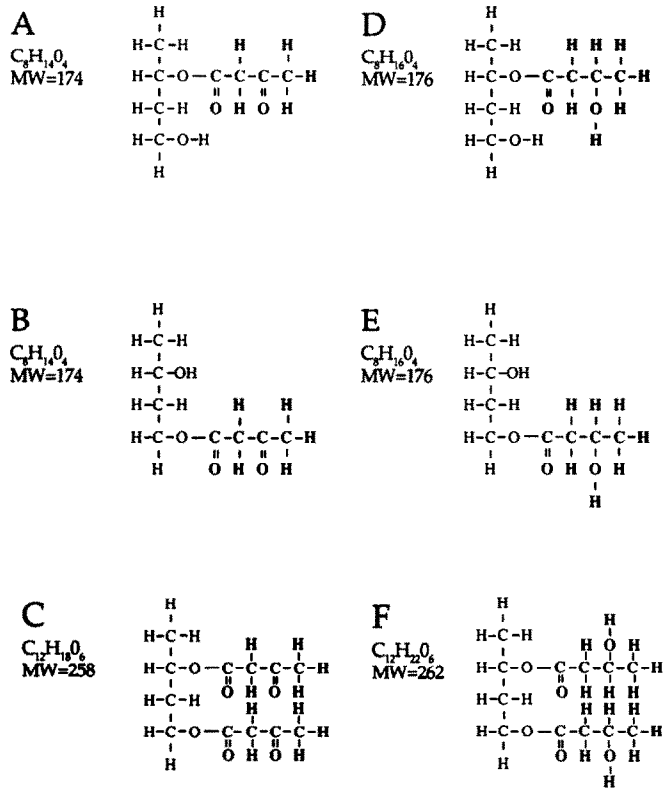

Figure 1 Formulas of *R,S*-1,3-butanediol monoacetoacetates (A,B), *R,S*-1,3-butanediol diacetoacetate (C), *R,S*-1,3-butanediol mono-β-hydroxybutyrates (D,E), and *R,S*-1,3-butanediol di-β-hydroxybutyrate (F) esters. For each ester, the global formula and the molecular weight are indicated in parentheses.

[3]Unless otherwise indicated, the word "esters" refers to the *R,S*-1,3-butanediol acetoacetate esters.

[4]Since *R,S*-1,3-butanediol monoacetoacetate always contains a mixture of the two types of monoesters, the word "monoesters" is used as a plural throughout the report.

evaporated first on a rotary evaporator, then under vacuum pump at 40°C to constant weight.

The purity of the esters was checked by HPLC, with refractive index detector, on a Biorad HPX-87 Aminex column (30 × 0.78 cm), developed with 0.8 mL/min of 0.01 N H_2SO_4. Compounds elute in the order traces of BD, monoesters, and diester.

The structures of the monoesters and diester, including the corresponding enol forms, were confirmed by proton NMR, ^{13}C-NMR, and infrared spectra (data not shown). Mass spectrometric characterization of the esters is presented in the Results section. A mixture of internal standards of *R,S*-[$^2H_3$]BD-AcAc and *R,S*-[$^2H_3$]BD-$(AcAc)_2$ was synthesized from *R,S*-[1,1,3-$^2H_3$]-1,3-butanediol and diketene. All lots of *R,S*-1,3-butanediol acetoacetate and β-hydroxybutyrate esters used in experiments reported here were prepared from diketene.

Procedure 2. Recently, diketene has been reclassified as a chemical that must be used at its site of preparation. Therefore, it can no longer be shipped to US laboratories. Fortunately, an alternate procedure for the preparation of AcAc esters was recently published by Witzeman and Nottingam.[16] This procedure involves transacetoacetylation between an alcohol and t-butyl-acetoacetate. We applied this much simpler procedure to the synthesis of the BD-AcAc esters (procedure A in Ref. 16). In a round-bottom flask equipped with a dry N_2 inlet, a Vigreux column and a distillation condenser, pour 1 mol of t-butyl-acetoacetate, 1 mol of BD, and 130 ml of xylene. Heat under a slow stream of N_2 and collect t-butanol which distills with a small amount of xylene between 75 and 90°C. When the expected amount of t-butanol has been recovered, remove xylene on a rotary evaporator at 60°C and distill the remains of the reaction mixture under vacuum. The head of the distillation carries any leftover BD. Then the mixture of esters distills at about 150°C under 0.25 mm Hg. When the initial reagent ratios t-butyl-acetoacetate/BD are 1/1 or 2/1, the ratios monoesters/diester in the distilled product is 75/25 and 5/95, respectively.

Synthesis of R,S-*1,3-butanediol*-R,S-*3-hydroxybutyrates*

To an aqueous solution of BD-(AcAc) esters kept at 0°C, one adds a 2 fold molar excess of solid $NaBH_4$. After 1 min of vigorous stirring, excess $NaBH_4$ is destroyed with concentrated HCl. Rapid acidification prevents hydrolysis of the BD(BHB) esters by the alkalinity of excess $NaBH_4$. The BD-BHB esters are extracted with ether and purified by chromatography on silica gel, as described for the BD-AcAc esters.

Heats of combustion

Heats of combustion of compounds were calculated by first evaluating their heat of formation with a computer software from the National Institute of Standards and Technology.[17] This software uses equations and bond energies taken from Ref. 18. From the structural formula of the compound drawn on the computer screen, the software calculates heats of formation of groups. It includes corrections[18] for the nonadditivity of heats of formation bonds in specific groups. Then, using the stoichiometric equation of combustion of the compound to $CO_2 + H_2O$, the heats of formation of CO_2 gas (−94.05 kcal/mol) and liquid water (−68.32 kcal/mol) are deducted from the heat of formation. For example, for BD-$(AcAc)_2$ ($C_{12}H_{18}O_6$, formula weight 258, *Figure 1*, compound C), the heat of formation is −285.1 kcal/mol. The oxidation of 1 mol of diester yields 12 mol of CO_2 + 9 mol of H_2O. So the heat of combustion is 12(−94.05) + 9(−68.32) − (−285.1) = −1458.4 kcal/mol. A final small correction (about 1%) must be made to the latter number to take into account the heat of vaporization of the ester. For compounds of this kind, the heat of vaporization is 1.0 kcal/g of atom of carbon and oxygen,[19] or for $C_{12}H_{18}O_6$ the correction term is 1(12 + 6) or 18 kcal/mol. Thus, the heat of combustion of the diester is −1458.4 + 18 = 1440.4 kcal/mol or 5.58 kcal/g.

Animal experiments

Two suckling pigs (4.5 and 4.0 kg), anesthetized with halothane and fitted with carotid artery and controlateral jugular vein catheters, were infused for 2 hr with 25 mmol/hr of either BD (15.3 kcal/hr) or BD-AcAc ester mix (81% monoesters/19% diester; 27.9 kcal/hr), dissolved in 75 mM NaCl. These rates of infusion, equimolar in BD, correspond to 108 and 215% of the hourly energy requirements of the animals. The latter were calculated by the formula 110× (kg of body weight)$^{0.75}$/24.

A 15 kg mongrel dog was anesthetized with sodium pentobarbital dissolved in saline[5] and fitted with femoral artery and controlateral femoral vein catheters. A bolus of 6.5 g of ester mix (75% monoesters/25% diester) dissolved in 20 ml of saline was injected over 2 min. One milliliter of arterial blood samples were taken at various times, vortexed with 0.05 ml of saturated sulfosalicylic acid and quick-frozen until analysis.

Analytical procedures

To prevent hydrolysis of the esters after blood sampling, 1 mL blood samples were injected into tubes containing 50 μL of saturated sulfosalicylic acid. After vortexing, the slurry was spiked with internal standards of *R,S*-[1,1,3-$^2H_3$]BD, *R,S*-[$^2H_3$]BD-AcAc, and *R,S*-[$^2H_3$]BD-$(AcAc)_2$, revortexed, and centrifuged. The acidic supernatants were saturated with NaCl and extracted with 3 × 5 ml ethyl ether. The combined extract, dried with Na_2SO_4 and evaporated to about 50 μL, was reacted overnight at 50°C with N-methyl-N-(*t*-butyldimethylsilyl)-trifluoroacetamide before injection into the GC-MS.

GC-MS analyses were performed on a Hewlett-Packard 5988A GC-MS (Montreal, Quebec, Canada) and on a Hewlett Packard 5989 GC-MS (Cleveland, OH USA). The gas chromatographs were equipped with HP-5 fused silica columns (25 m × 0.2 mm, 0.2 or 0.33 μm film thickness, Hewlett-Packard). Operating conditions: helium flowrate, 0.7 mL/min; split ratio, 1:10; injection port temperature, 270°C. Set column temperature at 150°C for 5 min, increase by 5°C/min for 5 min, increase by 15°C/min until 275°C; bake column at 250°C for 3 min between samples.

The mass spectrometers were operated in positive chemical ionization mode: ammonia pressure, 0.5 to 2 × 10^{-4} torr, electron energy, 250 eV. Ions monitored and elution times: m/z 319, 322 for BD (11.6 min); 289, 292, 403, 406 for the monoesters (15.5 min); 271, 274 for the diester (26.8 min).

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[5]The commercial solutions of sodium pentobarbital used for animal anesthesia (for example, Nembutal® from Abbott Labs) contain 10% of ethanol + 40% of propylene glycol. Their use leads to high body fluid concentrations of the alcohols that interfere with many metabolic processes. In this case we did not want the alcohols from the anesthetic to interfere with hepatic oxidation of *R,S*-1,3-butanediol derived from the injected esters.

Standards and samples were analyzed in duplicate. Areas were determined by interactive computer integration and corrected for naturally occurring heavy isotopes and light isotopic impurities as described previously.[20]

Concentrations of AcAc[21] and glucose were assayed enzymatically. The concentrations of *R*-BHB and *S*-BHB were measured by an isotope dilution chiral GC-MS assay that yields distinct peaks for *R*- and *S*-BHB.[22] Briefly, plasma samples are spiked with an internal standard of R,S-[$^2H_6$]BHB and deproteinized with saturated sulfosalicylic acid. The acid extract is saturated with NaCl and extracted three times with ether. The evaporated extract containing *R*-BHB, *S*-BHB, and R,S-[$^2H_6$]BHB is reacted first with 2-butanol/HCl (to derivatize the carboxyls) then with chiral S(+)-2-phenylbutyryl chloride (to derivatize the hydroxyls). The derivatives are assayed by positive chemical ionization GC-MS. Standard curves are linear in the range 25 to 800 nmol of each enantiomer.

The kinetics of esterase activity in human and dog plasma toward a 75%/25% mixture of mono- and diester was measured at pH 7.4 by a coupled assay with *R*-BHB dehydrogenase. Initial concentration of NADH was 0.15 mM. The assay was started by addition of plasma to the cuvette. Spontaneous hydrolysis of the esters in the blank cuvettes was negligible. Rates of hydrolysis were proportional to plasma volume. The assay was set up on a manual spectrophotometer and then transferred to a Cobas-Fara (Roche, Basel, Switzerland) centrifuge analyzer.

Results

The *R,S*-1,3-butanediol acetoacetate esters are colorless, oily liquids with a faint fruity smell. Their refractive indices are 1.4462 (mono) and 1.4541 (di). They can be distilled under reduced pressure. The monoesters are fully water-soluble. The solubility of the diester in water is 5% (w/v) but it is somewhat increased in the presence of the monoester. Caloric equivalents were calculated as described in the Methods: BD, 6.73 kcal/g; BD(AcAc), 5.91 kcal/g; BD(AcAc)$_2$, 5.58 kcal/g; *R,S*-1,3-butanediol mono-*R,S*-β-hydroxybutyrate, 6.06 kcal/g; *R,S*-1,3-butanediol di-*R,S*-β-hydroxybutyrate, 5.84 kcal/g. These values are higher than those of acetoacetic acid (4.2 kcal/g) and *R*-β-hydroxybutyric acid (4.7 kcal/g).

HPLC analysis of the BD-AcAc and BD-BHB esters allows separation of each pair of monoesters from the corresponding diester. The two monoesters of each type are not resolved by this HPLC procedure. HPLC fractions, treated with pig liver esterase at pH 7.4, were rechromatographed. The ester peaks disappeared and were replaced by peaks corresponding to BD and AcAc or BHB (data not shown). Relative areas of the latter peaks confirmed the identity of the original monoesters and diester fractions.

The spontaneous hydrolysis of BD-AcAc esters was tested in aqueous solutions. Solutions of esters in water remain at pH 5.5 for at least a week at 4°C. Spontaneous hydrolysis becomes significant at pH > 8.0 as determined by AcAc production. After autoclaving BD-(AcAc)$_2$ for 3 hr at 120°C, under N_2, GC-MS and enzymatic analyses did not reveal any degradation or hydrolysis. Deproteinization of plasma or blood can be conducted with perchloric acid or saturated sulfosalicylic acid without loss of ester. As a precaution against hydrolysis, perchloric acid extracts are neutralized to only pH 5.0.

The concentrations of stock solutions of BD-AcAc and BD-BHB esters can be determined by incubation with pig liver esterase, followed by enzymatic assay of AcAc[21] and GC-MS assay of BD.[13] The production of AcAc from the hydrolysis of BD-AcAc esters can be monitored continuously. Note that the enzymatic assay of BHB, with *R*-BHB dehydrogenase, is specific for the *R*- isomer. Thus, the actual concentration of racemic *R,S*-BHB is twice that assayed enzymatically.

Figure 2 shows the GC-MS chromatogram, in total ion current, of a mixture of BD-AcAc monoesters and diester. The quadruplet, eluting around 16 min, corresponds to the

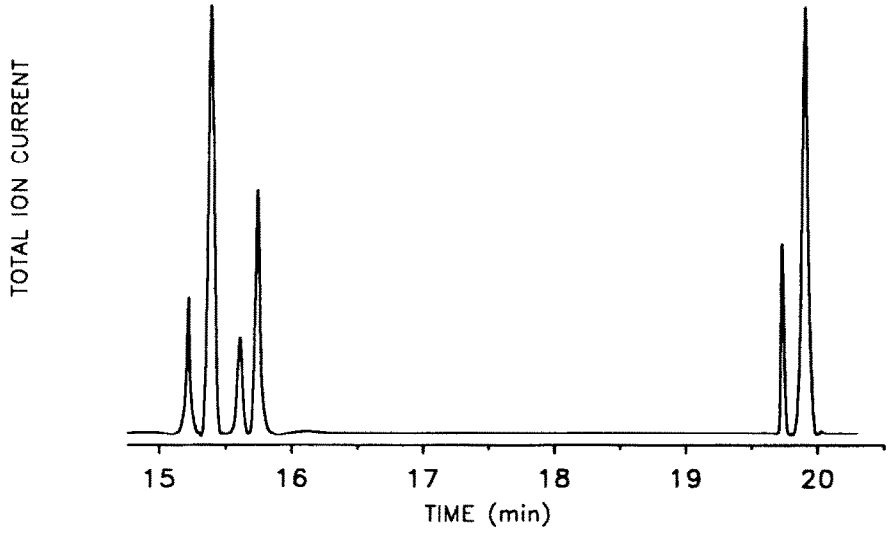

Figure 2 Gas chromatographic-mass spectrometric analysis of a mixture of *R,S*-1,3-butanediol monoacetoacetate and diacetoacetate. Total ion current trace. The quadruplet on the left side corresponds to the mixture of monoesters in Z and E forms. The doublet on the right corresponds to the diester.

bis-TBDMS derivatives of the two monoesters, each in Z and E enol forms. Under the conditions or derivatization, the AcAc groups of the esters are in enol configurations. This is also the case when free AcAc is derivatized with TBDMS.[23] The four peaks of the monoesters quadruplet yielded identical mass spectra under ammonia chemical ionization for the unlabeled and the [$^2H_3$]monoesters, respectively. The quasi-molecular $[M + H]^+$ ion at m/z 403 shifts to m/z 406 in the spectrum of the monoesters internal standards. Another main ion at m/z 289 shifts to 292 in the spectrum of the deuterated monoesters. This $[M - 114 + H^+]$ ion appears to derive from the loss of the t-butyl groups from the two TBDMS radicals.

When the monoesters are reacted overnight at 50°C with N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide, the di-TBDMS is the only derivative formed. However, when the derivatization is conducted either overnight at room temperature or for 10 min at 50°C, the quadruplet is preceded by a large peak, eluting at 13.5 min. This peak corresponds to the mono-TBDMS derivative of the monoesters. The quasi-molecular $[M + H^+]$ ion at m/z 289 shifts to 292 in the spectrum of the deuterated monoesters. This peak includes presumably unresolved components resulting from (1) the two types of monoesters, (2) the two types of TBDMS derivative, and (3) the E and Z enol forms of the AcAc group (when the AcAc group is derivatized). When the derivatization mixture is kept for a few days at room temperature, the mono-TBDMS peak decreases progressively in size reflecting the formation of the di-TBDMS derivative.

The doublet (*Figure 2*), eluting around 20 min corresponds to the bis-TBDMS derivatives of the diester. Although the diester could yield 4 peaks because of the Z and E enol forms of each AcAc group, only 2 peaks were observed. A mixture of internal standards of *R,S*-[$^2H_3$]BD-AcAc and *R,S*-[$^2H_3$]BD-$(AcAc)_2$ gave the same total ion chromatographic profile as the unlabeled esters (data not shown). The mass spectra of the diester peaks contains a very small peak at m/z 487 (shifted to 490 in the standard) corresponding to the $[M + H]^+$ ion. A more suitable peak for quantification occurs at m/z 271 (shifted to 274 in the standard). This $[M\text{-}215]^+$ peak corresponds presumably to the loss of an AcAc-TBDMS radical from the quasi-molecular ion. Standard curves of BD-AcAc and BD-$(AcAc)_2$, assayed by isotope dilution GC-MS, are linear in the 0 to 5 mM range (*Figure 3*). Using splitless injection, ester concentrations as low as 5 μM can be detected by the GC-MS assay.

Since most in vivo experiments to be reported elsewhere[6,7] were conducted with a mixture of BD-AcAc monoesters and diester (75%/25%), this mixture was used to assay the esterase activity of human and dog plasma by a coupled spectrophotometric assay with *R*-BHB dehydrogenase at pH 7.40 and 38°C (*Figure 4*). *Km* and V_{max} were 4 mM and 0.13 U/mL in dog plasma, and 2.9 mM and 0.20 U/mL in human plasma, respectively. Since the assay was conducted with a mixture of mono and diester, rates are expressed as (μmol AcAc released)/min/mL.

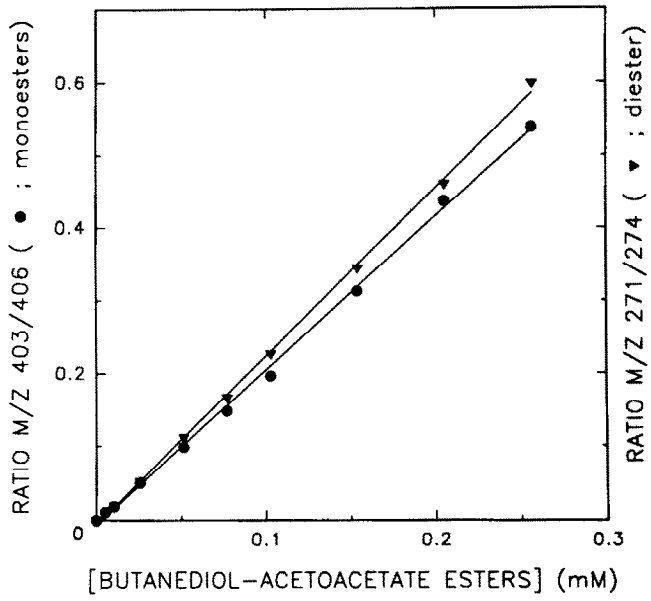

Figure 3 Standard curves of *R,S*-1,3-butanediol acetoacetate esters. Isotope dilution gas chromatography-mass spectrometric assays of monoesters (●), diester (▼).

To test the capacity of the body's esterase activity toward the BD-AcAc esters, an anesthetized piglet was infused for 2 hr with 25 mmol/hr of ester mix (81% mono/19% di) at a rate corresponding to 2.15 times the animal's hourly caloric requirement in the conscious state. During the infusion, the total plasma concentration of nonhydrolyzed esters (assayed enzymatically) plateaued at only 0.5 mM, and decreased to zero as soon as the infusion of esters was interrupted (*Figure 5A*).

In the same experiment, the diol concentration increased linearly until the end of the infusion, then it decreased linearly. Thus, the massive administration of BD-AcAc esters overwhelmed the capacity of the pig to eliminate BD released by the esterases. There are three mechanisms for BD elimination, i.e., oxidation in the liver, oxidation in the stomach, and urinary excretion. In another study,[7] conducted on anesthetized dogs infused for 4 hr with the BD-AcAc esters at 90% of the caloric requirement, we showed that the urinary clearance of BD is equal to the urinary flow. Thus, plasma and urine concentrations of BD are equal. The fraction of the infusion of BD (as BD-AcAc esters) that was excreted in urine was less than 2%. A similar percent of urinary excretion of a load of BD was reported in the dog by Münst et al.[24] The only known pathway for BD metabolism is by oxidation via alcohol dehydrogenase. Most of this activity is present in the liver. However, some alcohol dehydrogenase activity is present in the gastric mucosa of

[6]Desrochers, S., Dubreuil, P., Brunet, J., Jetté, M., David, F., Landau, B.R., and Brunengraber, H. (1993) Metabolism of *R,S*-1,3-butanediol acetoacetate, parenteral nutrients, in pigs. *Am. J. Physiol.* (in press)

[7]Ciraolo, S.T., Previs, S.F., Agarwal, K.C., David, F., Koshy, J., Lucas, D.T., Tamaro, A.C., Stevens, M.P., Tserng, K.-Y., Halperin, M.L., and Brunengraber, H. Model of extreme hypoglycemia and hypolactatemia in dogs made ketotic with 1,3-butanediol acetoacetate. *Am. J. Physiol.* (submitted)

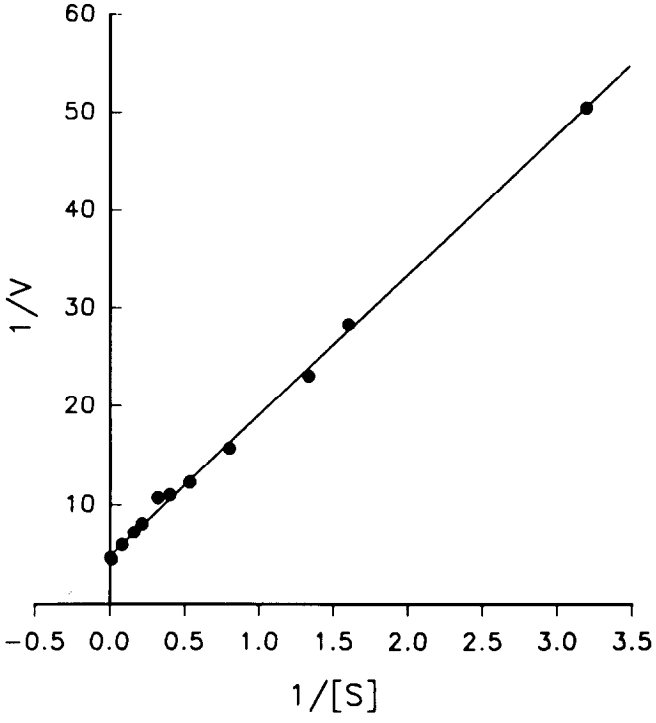

Figure 4 Kinetics of human plasma esterases toward *R,S*-1,3-butanediol acetoacetate esters. Lineweaver–Burk plot of the esterase assay coupled with excess *R*-β-hydroxybutyrate dehydrogenase. Units of activity are calculated as (μmol AcAc released)/min/mL. *Km* = 2.9 mM; V_{max} = 0.20 U/mL.

humans.[25] After oral ingestion of ethanol, the first-pass metabolism has been ascribed to oxidation by gastric alcohol dehydrogenase.[26] In the present study, where BD-AcAc esters were given intravenously, it is unlikely that a sizeable fraction of BD was oxidized in the stomach, given the relative weights of the gastric mucosa and of the liver. Therefore, in the present study, we ascribe practically all the elimination of BD to oxidation in the liver. The rate of decrease of BD concentration corresponds to an activity of liver alcohol dehydrogenase of 2.78 μmol/min/g (based on the measured weight of the liver). In a second piglet infused with an equimolar amount of BD, we observed a similar profile of plasma diol concentration (*Figure 5B*) and calculated a similar activity of liver alcohol dehydrogenase (3.31 μmol/min/g).

In the piglet infused with BD (*Figure 5B*), the concentrations of *R*-BHB and AcAc remained very low (0.3 and 0.12 mM, respectively). *S*-BHB was not assayed. Thus, physiological ketone bodies were used as fast as they were generated from the oxidation of the *R*- moiety of BD. Note that some physiological ketone bodies also derive from the metabolism of *S*-BHB derived from *S*-1,3-butanediol.[12,14]

In the piglet infused with BD-AcAc esters (*Figure 5A*), The concentration of physiological ketone bodies increased linearly. Note that the rate of infusion of ketone body equivalents (*R*-1,3-butanediol + AcAc) was more than twice that infused in *Figure 5B* (as BD alone). Here, the capacity of ketone body utilization was saturated.

Figure 6 shows the data of a bolus injection of BD-AcAc

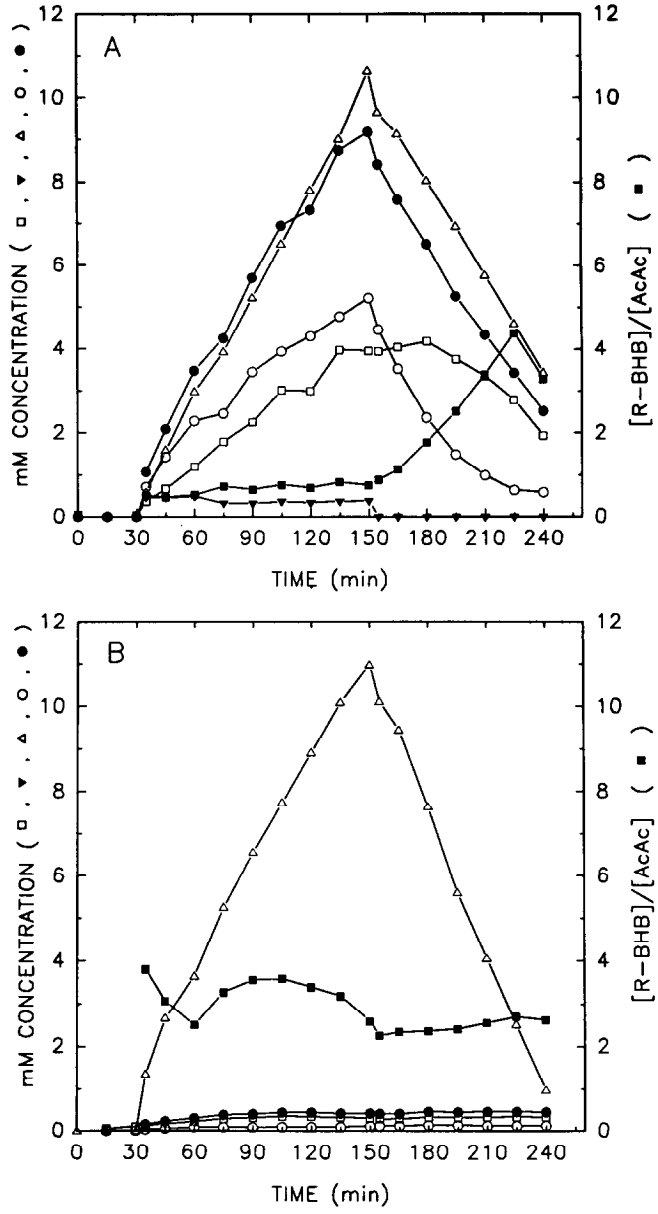

Figure 5 Infusions of *R,S*-1,3-butanediol acetoacetate esters (A) and of *R,S*-1,3-butanediol (B) to pigs. Two anesthetized piglets were infused for 2 hr (from 30 to 150 min) with 25 mmol/hr of an 81%/19% mixture of mono/diester (A) or of *R,S*-1,3-butanediol (B). The two infusions contained identical amounts of *R,S*-1,3-butanediol equivalents. Metabolite concentrations were measured in arterial blood: acetoacetate (○), *R*-β-hydroxybutyrate (□), *R*-β-hydroxybutyrate + acetoacetate (●), *R,S*-1,3-butanediol (△), total esters (▼), [*R*-BHB]/[AcAc] (■).

esters to an anesthetized dog. After the bolus was injected over 2 min, the concentration of nonhydrolyzed esters was measurable only for a few minutes after the bolus (see inset). The rapid ester hydrolysis is also reflected by the sharp increases in AcAc and BD concentrations. In this experiment, the concentrations of BD, *R*-, and *S*-BHB were assayed by GC-MS using chiral derivatives (see Methods). Throughout the experiment, the concentrations of *R*- and *S*-1,3-butanediol were identical (data not shown). This confirms previous observations of identical rates of uptake of

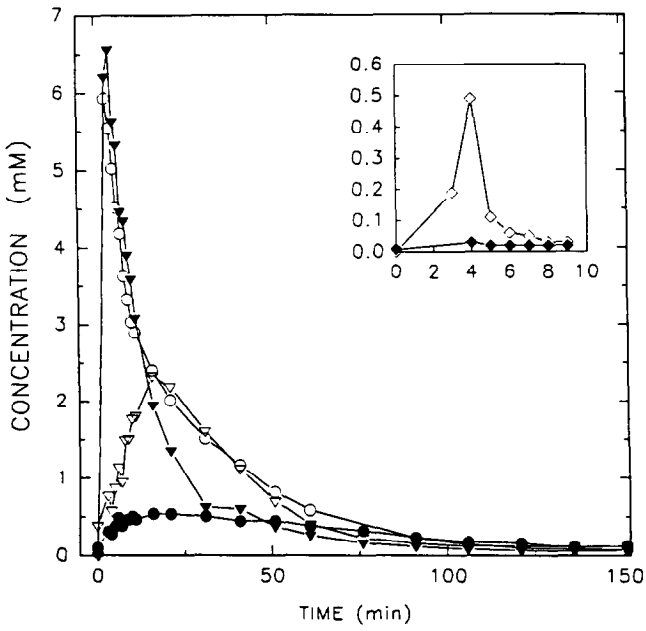

Figure 6 Bolus injection of *R,S*-1,3-butanediol acetoacetate esters to a dog. A 15.2 kg dog was injected with 6.5 g of a 75%/25% mixture of mono/diester. Metabolite concentrations were measured in arterial blood: acetoacetate (▼), *R*-β-hydroxybutyrate (▽), *S*-β-hydroxybutyrate (●), *R,S*-1,3-butanediol (○). The insert shows the concentrations of monoesters (◇) and diester (◆) just after the bolus injection.

R- and *S*-1,3-butanediol by perfused rat liver,[14] and by the dog in vivo.[22] The concentration of *R*-BHB was much greater than that of *S*-BHB. Note that although *R*- and *S*-BHB are formed in equal amounts from the hepatic oxidation of *R,S*-1,3-butanediol, additional *R*-BHB arises from (1) the reduction of AcAc derived from the hydrolysis of the esters, and (2) the metabolism of *S*-BHB.[12,14] This experiment confirms our previous finding that *S*-BHB is well used by dog tissues.[12–14]

Discussion

The *R,S*-1,3-butanediol acetoacetate esters represent fairly water-soluble forms of lipid substrates. The monoesters are fully water-soluble. The caloric density of the esters ranges from 5.6 to 6.0 Kcal/g, which is intermediate between carbohydrates and long-chain triacylglycerols. Because of their rapid conversion to ketone bodies, the esters are rapidly used for energy production rather than stored in lipid reserves as is the case for triacylglycerols.

The *R,S*-1,3-butanediol-ketone body esters represent a convenient way to administer ketone bodies in a neutral form without sodium load. In general, ketone bodies are excellent fuels for peripheral tissues, except in diabetic ketoacidosis and in prolonged starvation. In experiments to be presented separately,[7] we infused the BD-AcAc esters to hypoglycemic dogs at 90% of their caloric requirements. The total concentration of plasma ketone bodies (including the unnatural *S*-BHB) was only 2 to 3 mM.

The animal experiments described in this report involved massive administration of the BD-AcAc esters. This was done deliberately to test the ketogenic potential of the esters and the esterase capacity of the animal's body fluids and tissues. Such high rates of infusion would never be used in total parenteral nutrition. For example in the anesthetized pig experiment shown in *Figure 5A*, the rate of ester infusion corresponded to 2.1 times the caloric requirement of the piglet in the conscious state. It took such a high rate of infusion to maintain only a 0.5 mM plasma concentration in nonhydrolyzed esters. As soon as the infusion of esters was interrupted, their plasma concentration decreased to zero.

In a 15 kg dog injected with a bolus of esters (40.7 mmol), low ester concentrations were detectable only for 3 min following the end of the 2 min bolus injection (*Figure 6*, insert). In this dog the plasma esterase activity toward the BD-AcAc esters was 0.13 μmol/min/mL. Thus, the bulk of the hydrolysis of the injected esters must have occurred in organ tissues. Sun and Birkhahn[8] assayed the esterase activity of rat organs toward glycerol monoacetoacetate and found the highest activity in liver (5.9 μmol/min/g). In an intact perfused rat liver,[9] we measured an esterase activity of 15.5 μmol/min/g toward BD-AcAc esters (120 times more than in plasma). Thus, it is likely that these esters are hydrolyzed mainly in tissues when they are administered as a large intravenous bolus. In the dog experiment (*Figure 6*), the rapid hydrolysis of the esters is confirmed by the sharp rises of AcAc and BD concentrations immediately after the bolus injection of esters. Comparison between the amount of ester injected (40.7 mmol) and the peak concentrations of BD (6 mM) and AcAc (6.5 mM) in the first sample confirms that (1) the bolus of ester was essentially completely hydrolyzed and (2) the products of hydrolysis were already distributed in a volume that was already greater than extracellular fluid (3 L). We showed earlier[13] that the final volume of distribution of BD in the dog is total body water (2/3 of 15 kg = 10 L).

Usually, the ratio [*R*-BHB]/[AcAc] reflects the [NADH]/[NAD^+] ratio in liver mitochondria, the main site of the *R*-BHB dehydrogenase activity. In the pig infused with BD (*Figure 5B*), this ratio is about 3.0. This high ratio reflects the accumulation of reducing equivalents generated in the oxidation of BD. In contrast, in the pig infused with BD-AcAc esters (*Figure 5A*), the ratio [*R*-BHB]/[AcAc] remained at 0.7 to 0.8 during the infusion of the esters. However, during infusion of BD-AcAc esters, the [*R*-BHB]/[AcAc] ratio is influenced by AcAc generated from ester hydrolysis in the extracellular fluid. The [*R*-BHB]/[AcAc] ratio in plasma at an oxidized level results from the balancing of two processes. First, the ratio is decreased by the production of AcAc via hydrolysis of the esters in liver, peripheral tissues and plasma. Second, the ratio is increased by the conversion of AcAc to *R*-BHB, which occurs mostly in liver mitochondria. This second process traps the reducing equivalents generated by the oxidation of BD by liver alcohol dehydrogenase and aldehyde dehydrogenase. Although we cannot infer the mitochondrial [NADH]/

[8]Sun, Q. and Birkhahn, R. (1993) Monoacetoacetin hydrolysis in the rat. *FASEB J.* **7**, p. A413 (abstract 2388)

[9]Desrochers, S. (1990) Métabolisme des isomères optiques du 1,3-butanediol et de leurs dérivés. Ph.D. thesis, University of Montreal.

[NAD^+] ratio in liver from the [*R*-BHB]/[AcAc] ratio in plasma, it is likely that the esters act as redox buffers which prevent hyperreduction of the NADH/NAD^+ couple in liver.

In conclusion, the data presented above confirm the rationale that led to the synthesis of the BD-AcAc esters. Esterification of BD with AcAc (1) increases the caloric density per osmol, (2) allows infusing ketone bodies without sodium counterion, and (3) provides a trap for the reducing equivalents generated in the liver by the oxidation of BD. Therefore, both the carbon and the hydrogen of the esters are exported from the liver in the form of β-hydroxybutyrate enantiomers which are used in peripheral tissues. Thus, contrary to what occurs with ethanol or BD alone, the BD-AcAc esters do not lead to major shifts in the liver's [NADH]/[NAD^+] ratios. Such shifts are responsible for the toxic effects of ethanol on the liver. It is therefore likely that long-term administration of the BD-AcAc esters will not lead to liver toxicity.

Acknowledgments

This work was supported by grants from the National Institutes of Health (DK35543), the Montreal Notre Dame Hospital Research Center, and the Cleveland Mt Sinai Medical Center. We thank Drs Christine Des Rosiers and Orval A. Mamer for their help with the interpretation of mass spectrometric data. We thank Dr Steven E. Stein from the National Institute of Standards and Technology for his help with the calculations of the heats of combustion.

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