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Dog model of therapeutic ketosis induced by oral administration of *R,S*-1,3-butanediol diacetoacetate

Michelle A. Puchowicz, Chris L. Smith, Catherine Bomont, John Koshy, France David, and Henri Brunengraber

Department of Nutrition, Case Western Reserve University, Cleveland, OH USA

A high-fat, almost carbohydrate-free diet is used in children with intractable epilepsy to help control seizures by inducing a permanent state of ketosis. Esters of ketone bodies have been previously studied for their potential as parenteral and enteral nutrients. We tested in conscious dogs whether ketosis could be induced by repeated ingestion of R,S-*1,3-butanediol diacetoacetate with or without carbohydrates. This ester is a water-soluble precursor of ketone bodies. Two constraints were imposed on this preclinical study: The rate of ester administration was limited to one half of the daily caloric requirement and to one half of the capacity of the liver to oxidize butanediol derived from ester hydrolysis. Under these conditions, the level of ketosis achieved in this dog model (0.8 mM) was lower than the level measured in children whose seizures were controlled by the ketogenic diet (1–3 mM). However, because humans may have a lower capacity for ketone body utilization than dogs, the doses of* R,S-*butanediol diacetoacetate used in the present study might induce higher average ketone body concentrations in humans than in dogs.* (J. Nutr. Biochem. 11:281–287, 2000)

Keywords: acetoacetate esters; 1,3-butanediol; ketone bodies; epilepsy; ketogenic diet

Introduction

Intractable epilepsy that is resistant to multiple drug therapy has been treated by a high-fat diet (also called ketogenic diet) in order to induce a permanent state of ketosis in the low millimolar range.[1,2] Therapeutic ketosis markedly decreases the frequency of seizures in a fraction of patients.[1,3,4] In some centers, the success rate of the diet therapy is reported as high as 75%.[1,3] The mechanisms by which ketone bodies inhibit seizure activity are not understood. It is not clear whether the active agent is *R*-3-hydroxybutyrate (*R*-BHB[a]),[1,2] acetoacetate (AcAc), or acetone, resulting from the spontaneous decomposition of acetoacetate.

Most of the calories of the ketogenic diet are supplied in the form of long-chain triacylglycerols, often in combination with medium-chain triacylglycerols.[1,2] The latter are often used to induce ketosis on the first day of treatment. Medium-chain fatty acids are only metabolized via β-oxidation, the products of which are mostly ketone bodies [with a small fraction of the carbon being oxidized to carbon dioxide (CO_2)]. In contrast, a sizable fraction of the long-chain fatty acids are re-esterified to storage lipids. Because ketogenesis from long-chain fatty acids is inhibited by carbohydrates,[5,6] the induction and maintenance of therapeutic ketosis requires a diet that is almost devoid of carbohydrates. A single ingestion of carbohydrates (such as a candy bar or medication containing carbohydrates) can result in a flare of seizure activity.[7]

Because ketone bodies are good fuels for peripheral tissues in nondiabetic subjects or animals,[5,6] we have recently synthesized compounds that allow delivery of large amounts of these compounds by the parenteral or enteral routes without sodium. One such compound is *R,S*-1,3-

Address correspondence to Dr. Henri Brunengraber, Department of Nutrition, Case Western Reserve University, Cleveland OH 44106 USA.
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[a]The physiologic 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.

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butanediol diacetoacetate, which is hydrolyzed by plasma and digestive esterases to *R,S*-1,3-butanediol (referred to henceforth as butanediol) and AcAc.[8] In the liver, *R,S*-1,3-butanediol is oxidized to *R,S*-BHB by alcohol dehydrogenase and aldehyde dehydrogenase.[9] *R*-BHB is the physiologic ketone body that is in equilibrium with AcAc via *R*-BHB dehydrogenase. *S*-β-hydroxybutyrate (*S*-BHB) in the free form is not a natural compound,[10] but is activated to mitochondrial *S*-BHB-coenzyme A (CoA), one of the final intermediates of β-oxidation.[11] The reducing equivalents generated in the liver by the conversion of *R,S*-1,3-butanediol to *R,S*-BHB are trapped in the reduction to *R*-BHB of the AcAc moieties of the ester.[a] This prevents the rise in the [NADH]/[NAD^+] ratio in liver cells, which could inhibit gluconeogenesis and result in hypoglycemia.[9]

Because the yield of conversion of *R,S*-butanediol diacetoacetate to ketone bodies is 100%, and because this conversion is catalyzed by processes that are not inhibited by carbohydrates, we hypothesized that this ester could replace part or all of the long-chain triacylglycerols in the traditional ketogenic diet. It would also allow for more carbohydrates in the modified ketogenic diet and make this diet more acceptable to children.

The goal of the present study was to test whether stable ketosis could be induced in an animal model by repeated ingestion of *R,S*-1,3-butanediol diacetoacetate with or without carbohydrates. Given the preclinical context of this investigation, we imposed two constraints on the protocol. First, we administered the ester at a rate that corresponded to approximately one half of the capacity of dog liver to oxidize the butanediol moiety of the ester via alcohol dehydrogenase.[12] This would prevent excessive accumulation of butanediol. Second, in the hope that a diet based on *R,S*-butanediol diacetoacetate could contain approximately 25% of the calories as carbohydrates, we limited the rate of *R,S*-butanediol diacetoacetate administration to approximately 50% of the daily caloric requirement (DCR).

Materials and methods

Chemicals

R,S-1,3-Butanediol and general chemicals were purchased from Aldrich and Sigma Chemical Co. (St. Louis, MO USA). Biochemicals and enzymes were supplied by Boehringer Mannheim, (Indianapolis, IN USA). *R,S*-[1,1,3-$^2H_3$]-1,3-Butanediol and *R,S*-3-hydroxy-[2,2,3,4,4,4-$^2H_6$]butyrate internal standards were prepared as described by Desrochers et al.[13] and Mamer.[14] [$^2H_8$]Isopropanol, which becomes [$^2H_7$]isopropanol when dissolved in water, was from Cambridge Isotopes Lab (Andover, MA USA) and NaB^2H_4 was purchased from Merck Frost (Montreal, Quebec Canada). Citrotein enteral supplement (composition by weight: 25% egg white protein, 22.5% sucrose, 50.5% maltodextrin, 2% soybean oil), was from Sandoz Nutrition Corporation (Minneapolis, MN USA). *R,S*-Butanediol diacetoacetate was prepared from *t*-butyl acetoacetate and *R,S*-1,3-butanediol.[8]

Animal experiments

All animal protocols were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University. Twelve mongrel dogs (18–25 kg) selected for their tame temperament were trained for 2 weeks to be handled and to stand on a cart for increasing periods of time. On the day of the experiment, after an overnight fast each conscious dog had a permanent catheter inserted in a front leg vein for blood sampling. After baseline blood sampling (t = 0), six of the dogs swallowed two gelatin capsules containing *R,S*-butanediol diacetoacetate in a total amount corresponding to 2% of their DCR [calculated as (110 kcal)(body weight in kg)$^{0.75}$]. The caloric density of the ester is 5.6 kcal/g.[8] The administration of *R,S*-butanediol diacetoacetate was repeated at 60, 120, 180, 240, and 300 min. The second group of six dogs was treated as above, but in addition to each dose of *R,S*-butanediol diacetoacetate, they received 3.5% of their DCR as Citrotein. Thus, over 6 hr, all dogs received 12% of their DCR as *R,S*-butanediol diacetoacetate, and half the dogs received 21% of their DCR as Citrotein. The caloric density of Citrotein is 3.83 kcal/g. Blood was drawn into heparinized tubes at 15- to 30-min intervals for assays of *R*-BHB, *S*-BHB, AcAc, butanediol, glucose, and in some samples, acetone, general blood chemistry, and insulin. The total volume of blood sampled was 75 mL over 6 hr.

Analytical procedures

Neutralized perchloric acid extracts of plasma were assayed for *R*-BHB and glucose by standard enzymatic assays on a Cobas Fara centrifuge analyzer (Roche Diagnostic, Nutley, NJ USA). For the assays of *R,S*-butanediol, *R*+*S*-BHB,[b] AcAc, and acetone, 1 mL samples of plasma were spiked with internal standards of *R,S*-[$^2H_3$]butanediol (1.0 μmol), *R,S*-[$^2H_6$]BHB (0.5 μmol), and [$^2H_7$]isopropanol (0.5 μmol) before being treated with NaB^2H_4. Treatment with NaB^2H_4 stabilizes AcAc by converting it to M+1 *R,S*-BHB[15] and converts acetone to less volatile M+1 [2-^{2}H]isopropanol.[16] After strong vortexing the samples were frozen and stored at −80°C until analysis. After thawing, the samples were deproteinized and the excess NaB^2H_4 was destroyed by treatment with 0.02 mL of saturated sulfosalicylic acid and 0.02 mL of 6N HCl before extraction, derivatization, and gas chromatography (GC)-mass spectrometry (MS) assays.[15,16] The concentration of *S*-BHB was calculated[13] as the difference between the GC-MS assay of *R*+*S*-BHB and the enzymatic assay of *R*-BHB. AcAc was also assayed by GC-MS.[9,15] The concentration of acetone in plasma was assayed by GC-MS as previously described.[16] Heparinized whole blood samples were assayed for standard clinical chemistry parameters (CHEM19) by the clinical laboratory of Mt. Sinai Medical Center (Cleveland, OH USA).

Results

In one orientation experiment, a 17.5-kg dog received a single oral bolus of *R,S*-butanediol diacetoacetate (32 mmol) corresponding to 5% of its DCR. Plasma concentrations of butanediol and AcAc, the immediate products of *R,S*-butanediol diacetoacetate hydrolysis, peaked in the first sample taken at 15 min (*Figure 1*). This reflects the rapid absorption and hydrolysis of the ester. At 15 min the AcAc concentration was less than twice the butanediol concentration, as would have been the case for a simple absorption plus hydrolysis of diester. We previously showed that,

[a]Unless otherwise indicated, the word "ester" refers to *R,S*-1,3-butanediol diacetoacetate.

[b]Note that the metabolism of *R,S*-1,3-butanediol diacetoacetate results in unequal concentrations of *R*-BHB and *S*-BHB in body fluids.[7,8]

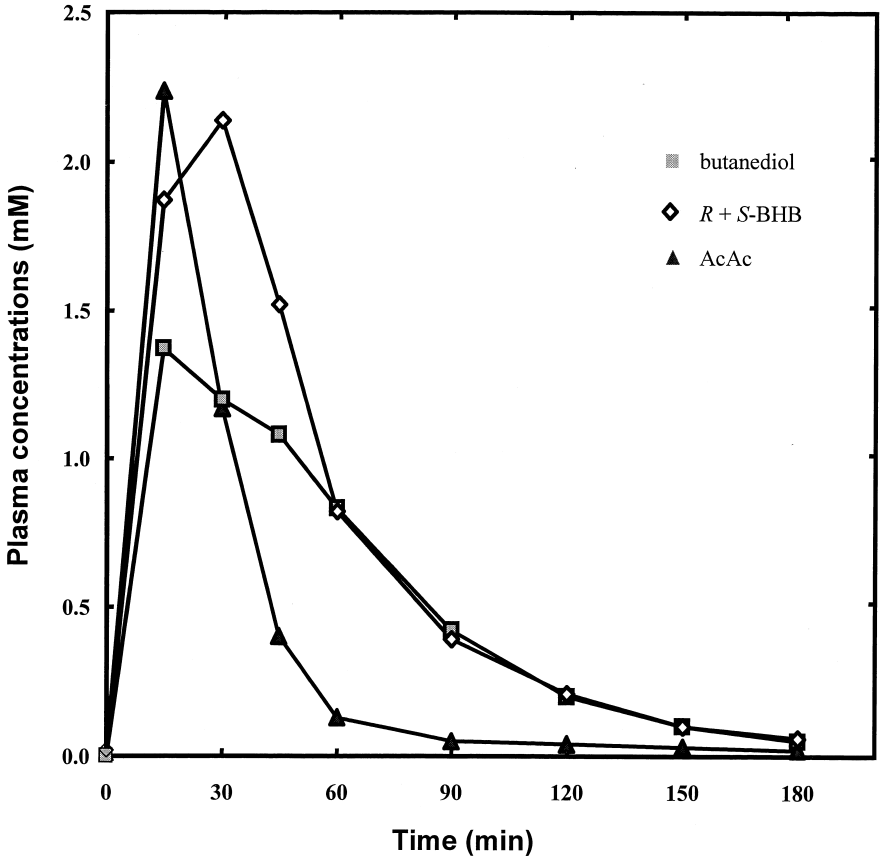

Figure 1 Profile of plasma ketone body and *R,S*-1,3-butanediol concentrations in a 17.5-kg dog following an oral bolus of *R,S*-butanediol diacetoacetate (5% of the daily caloric requirement). BHB, β-hydroxybutyrate; AcAc, acetoacetate.

following intravenous bolus injection, *R,S*-butanediol diacetoacetate is completely hydrolyzed within minutes and the initial AcAc concentration is approximately twice that of butanediol.[8] In the present experiment where the ester was administered orally, the *R* + *S*-BHB concentration at 15 min was greater than that of butanediol. This reflects the rapid metabolism of butanediol and AcAc derived from ester hydrolysis. From the rate of disappearance of plasma butanediol between 15 and 60 min, one can calculate a minimal rate of butanediol metabolism as 0.37 $\mu mol \cdot min^{-1} \cdot (g\ liver)^{-1}$ (based on a volume of distribution of two thirds of body weight and a liver weight of 2.1% of body weight,[13,17] respectively). This corresponds to approximately one third of the activity of liver alcohol dehydrogenase in dogs.[12] This rate may be somewhat underestimated if some of the butanediol derived from ester hydrolysis was still being absorbed from the gastrointestinal tract. Incomplete early absorption is also reflected by the spuriously high apparent volume of distribution of butanediol calculated by extrapolating the butanediol concentrations to zero time (114% of body weight).

By 180 min, the butanediol and ketone body concentrations had decreased to very low levels, almost to baseline for *R*-BHB and AcAc. Over the 3 hr of the experiment, the integrated concentrations of butanediol and total ketone bodies were 0.53 and 1.04 mM, respectively.

The orientation experiment revealed that ketosis induced by a single oral bolus of the ester is short-lived. Therefore, we planned experiments with repeated doses of ester. Two groups of six dogs received, over 300 min, six oral boluses of *R,S*-butanediol diacetoacetate, each one corresponding to 2% of the DCR. One half of the dogs received Citrotein (3.5% of the DCR) at the same times as and in addition to the *R,S*-butanediol diacetoacetate. We hypothesized that the supply of the carbohydrates and proteins of Citrotein would spare ketone body utilization in the same way that carbohydrates decrease fatty acid oxidation[18] and result in a higher average total ketone body concentration when compared with the dogs receiving only the ester. *Figures 2* and *3* show the profiles of individual ketone body concentrations and the butanediol concentrations. *S*-BHB arises only from the hepatic oxidation of the *S*- moiety of *R,S*-butanediol. *R*-BHB arises from two sources: the hepatic oxidation of the *R*-moiety of *R,S*-butanediol and the reduction of AcAc via *R*-BHB dehydrogenase in various tissues. *Figure 4A* compares the profiles of total ketone body concentrations (*R*-BHB + *S*-BHB + AcAc) achieved in the two groups. There were no significant differences in these total concentrations at any time. In addition, the integrated total ketone body concentrations [0.61 ± 0.05 mM (SE, $n = 6$) and 0.63 ± 0.03 mM ($n = 6$)] were not significantly different. Plasma acetone concentrations were 0.09 to 0.10 mM at zero time. At 360 min, they ranged from 0.16 to 0.31 mM in both groups. Thus, within the 6 hr of the experiment, there was little accumulation of acetone.

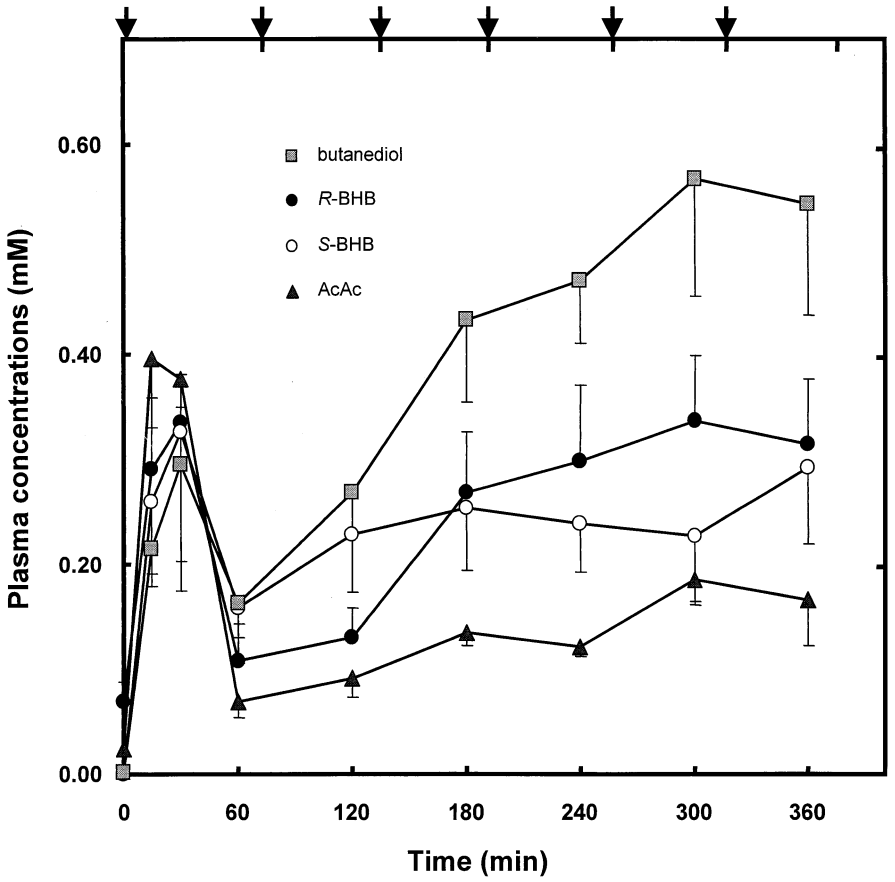

Figure 2 Profile of plasma ketone body, *R,S*-1,3-butanediol, and glucose concentrations in dogs during multiple oral administrations of *R,S*-butanediol diacetoacetate (2% of the daily caloric requirement for each bolus) at the times indicated by the bold arrows on top of the figure. Data are presented as mean ± SE (n = 6). BHB, β-hydroxybutyrate; AcAc, acetoacetate.

Figure 4B shows the plasma glucose concentrations in the two groups of dogs. There are no statistical differences between the two groups at any time. In addition, the areas under the curves are not statistically different (1,949 ± 40.6 vs. 1,898 ± 37.0 mM/min). As was shown earlier in pigs,[9] ingestion of *R,S*-butanediol diacetoacetate did not result in hypoglycemia. Although Citrotein contains carbohydrates, glucose concentration did not increase in the dogs ingesting Citrotein in addition to *R,S*-butanediol diacetoacetate (*Figure 4B*). Note that although carbohydrates account for 73% of the Citrotein calories, two thirds of the carbohydrate calories are in the form of maltodextrin, which is presumably digested more slowly than is sucrose. In addition, the total amount of carbohydrates given in each bolus of Citrotein would correspond to 13.3 g of glucose for an adult human on a 2,000 kcal/day diet. These considerations probably explain the flat profile of glucose concentration in the dogs ingesting Citrotein.

Standard clinical chemistry analyses were within normal ranges at 0 and 360 min (not shown). The animals did not show any sign of distress during or postexperiment.

Discussion

The present study was conceived to test in an animal model whether a diet containing one half of the DCR as *R,S*-butanediol diacetoacetate could achieve a stable level of ketosis in the 1 to 3 mM range. This range is considered optimal for the treatment of intractable epilepsy by the classical ketogenic diet.[1,2,4] When designing a protocol using *R,S*-butanediol diacetoacetate, we had to consider potential hypoglycemia and potential high levels of plasma butanediol resulting from the hydrolysis of the ester.

We conducted the experiments in dogs out of convenience, in spite of the notion that dogs seldom become markedly ketotic.[19] There is good evidence that dogs have a high capacity to utilize ketone bodies infused as such,[5,20,21] as butanediol precursors,[12,13] or as *R,S*-butanediol diacetoacetate.[8,22] The activity of 3-oxoacid CoA transferase in peripheral tissues of the dog is similar to that of other mammalian species.[23] Furthermore, pigs that are congenitally unable to synthesize ketone bodies because of a deficiency in liver mitochondrial HMG-CoA synthetase[24] have a high capacity to utilize ketone bodies derived from *R,S*-butanediol diacetoacetate.[8,9,22] Because the focus of the present study is on ketone body utilization, the low basal ketosis of fasted dogs was not a concern.

In the two groups of dogs given repeated doses of *R,S*-butanediol diacetoacetate at 2% of the DCR per hour (equivalent to 48% of the DCR per 24 hr), the integrated total ketone body concentrations in both groups were equal (0.6 mM). At the end of the experiment (t = 360 min), total

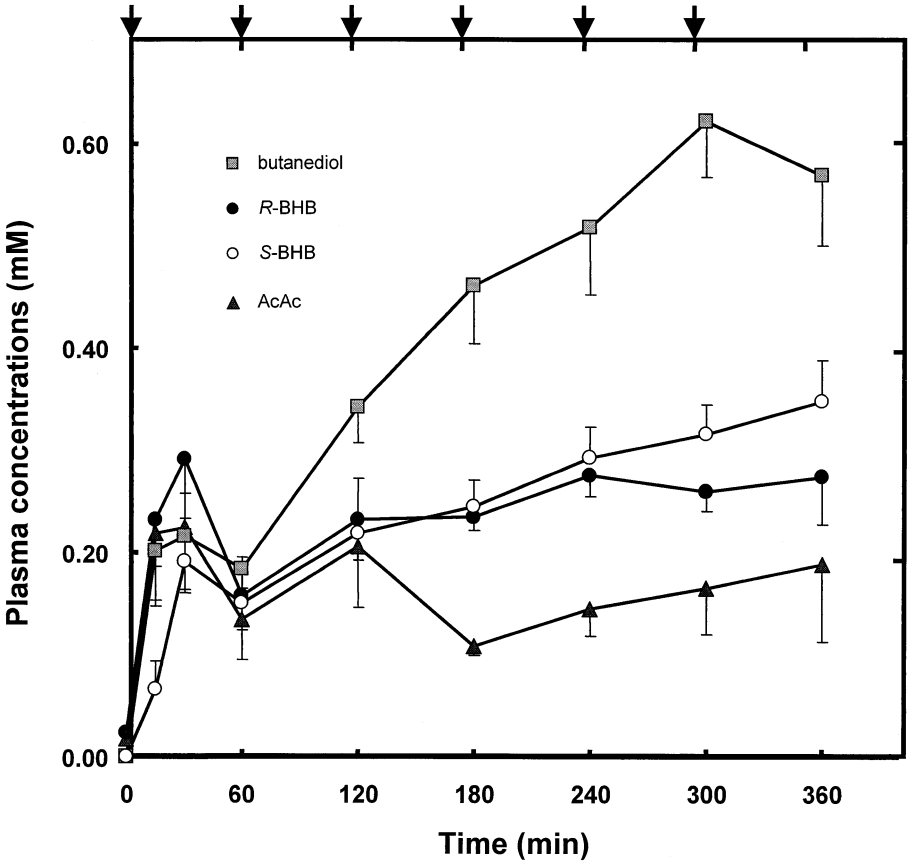

Figure 3 Profile of plasma ketone body, *R,S*-1,3-butanediol, and glucose concentrations in dogs during multiple oral administrations of *R,S*-butanediol diacetoacetate (2% of the daily caloric requirement for each bolus) and Citrotein (3.5% of the daily caloric requirement for each bolus) at the times indicated by the bold arrows on top of the figure. Data are presented as mean ± SE (n = 6). BHB, β-hydroxybutyrate; AcAc, acetoacetate.

ketone body concentrations were 0.77 and 0.80 mM in the dogs given the ester alone or the ester + Citrotein, respectively (*Figure 4A*). This level of ketosis would probably be qualified as insufficient for the treatment of epileptic children. Also of concern is the rapid decrease in total ketone body concentration during the hours following ingestion (*Figure 1*). One could possibly increase the level of ketosis by raising the fraction of calories administered as ester from 48% to 75% of the DCR. However, this would not allow the inclusion of 25% of the calories as carbohydrates as we had hoped. Indeed, in the dogs receiving Citrotein in addition to the ester, the total caloric ingestion corresponded to 132% of the DCR.

It is somewhat surprising that the addition of Citrotein to the *R,S*-butanediol diacetoacetate did not increase ketosis by sparing the utilization of exogenous ketone bodies in peripheral tissues. Because ketone bodies represent a water-soluble form of fatty acids, the concept of the reverse glucose-fatty acid cycle[18] would lead one to predict that administration of large amounts of carbohydrates would slow ketone body utilization. This is indeed the case in normal rat hearts perfused with glucose and pyruvate.[25]

Our present and past studies on the metabolism of *R,S*-butanediol diacetoacetate did not identify all the fates of the carbon of this ester. Note that all the carbon from the ester passes by the ketone body pool before being oxidized to CO_2 or, possibly, incorporated into lipids.[8,9] In the present study, we assumed that most, if not all, of the carbon was oxidized to CO_2 by overnight-fasted dogs because the ester was the only substrate administered at 50% of the DCR. There is a possibility that some of the carbon was incorporated into lipids[26] when Citrotein was administered with the ester, resulting in a hypercaloric substrate supply. The fates of the ester carbon deserve further investigation; however, these fates have little bearing on the conclusions of the present study.

When considering increasing the fraction of the calories to be supplied as *R,S*-butanediol diacetoacetate, one needs to consider the capacity of the liver to oxidize the butanediol moiety of the ester. The capacity of liver alcohol dehydrogenase in the dog[8,12] is 1.2 to 1.4 $\mu mol \cdot min^{-1} \cdot (g\ liver)^{-1}$. In our experiments where *R,S*-butanediol diacetoacetate was given in multiple doses, the rate of administration of the butanediol moiety of the ester was equivalent to 0.56 $\mu mol \cdot min^{-1} \cdot (g\ liver)^{-1}$, taking into account that in dogs of this size the liver accounts for 2.1% of body weight.[17] Restricting the rate of *R,S*-butanediol diacetoacetate administration to one half of the capacity of the liver to oxidize the butanediol moiety was a constraint we imposed on the protocol, given its preclinical nature. Should epileptic children be treated with *R,S*-butanediol diacetoacetate to prevent seizures, it would not be advisable to administer this ester at a rate close to or greater than the capacity of liver alcohol dehydrogenase to oxidize butanediol derived from ester hydrolysis. This could lead to accumulation of butanediol to toxic levels.[8]

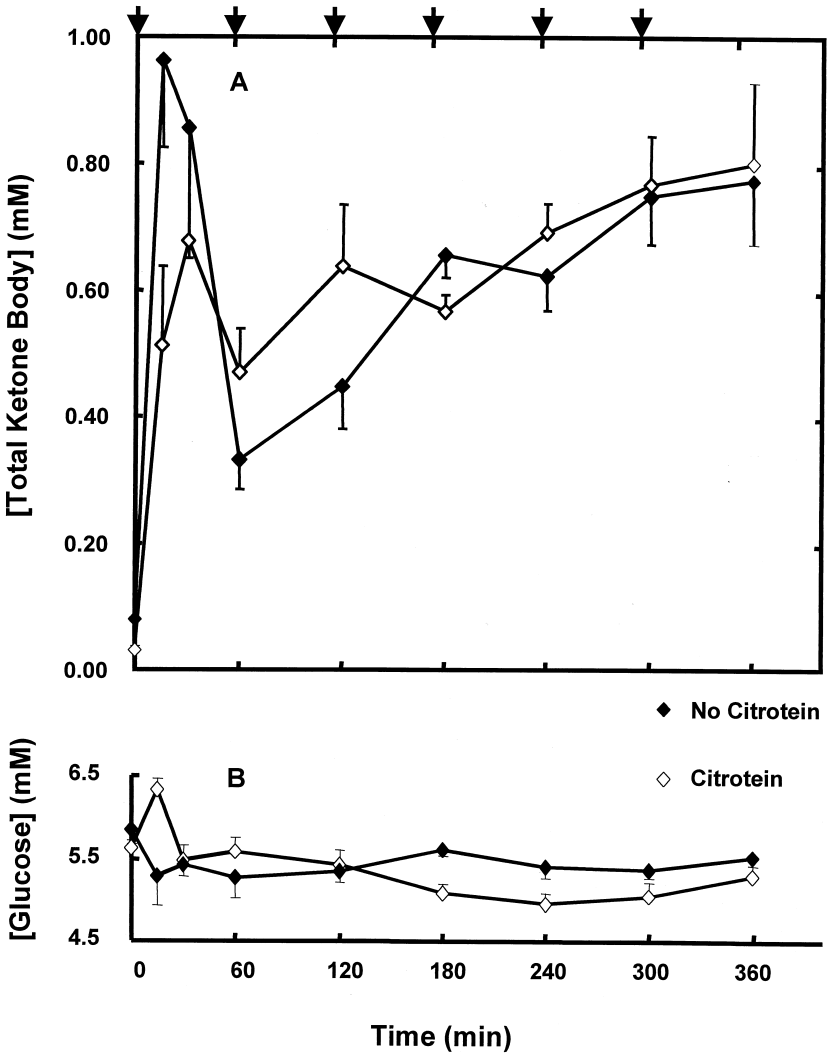

Figure 4 Comparison of total (A) ketone body and (B) glucose concentrations in dogs during multiple oral administrations of *R,S*-butanediol diacetoacetate (2% of the daily caloric requirement for each bolus) without or with Citrotein at the times indicated by the bold arrows on top of the figure. Data are presented as mean ± SE (n = 6).

There is good evidence that chronic administration of ethanol induces the activity of liver alcohol dehydrogenase[27] and thus the rate of ethanol oxidation to acetate. It is thus possible that chronic treatment of animals with *R,S*-butanediol diacetoacetate would increase liver alcohol dehydrogenase activity. Thus, one could consider increasing somewhat the contribution of the ester to the DCR. Because a normal protein supply of 1 g/kg corresponds to approximately 15% of the DCR, if one wanted to allow 20 to 25% of the DCR in the form of carbohydrates, then one could consider increasing the contribution of *R,S*-butanediol diacetoacetate from 50% to 60 to 65% of the DCR. However, this would not allow for any fat in the diet.

Birkhahn et al.[28] synthesized glycerol tri-*R,S*-3-hydroxybutyrate as a potential parenteral nutrient. The metabolism of this compound does not involve liver alcohol dehydrogenase, so it could conceivably be ingested by epileptic children at a rate greater than 50% of the DCR, perhaps 75%, as we suggested previously.[29] When using such a compound, the inhibition by glycerol[5,30,31] of ketogenesis from long-chain fatty acids would be of no concern because ketone bodies are released from this glycerol ester by simple hydrolysis. However, this would leave little room for including carbohydrates in the diet. In addition, this ester (like our *R,S*-1,3-butanediol diacetoacetate) would probably not maintain ketosis unless it were ingested at frequent intervals. This would not cover epileptic patients during the night.

In summary, two constraints were imposed on this preclinical study: The rate of ester administration was limited (1) to one half of the DCR and (2) to one half of the capacity of the liver to oxidize butanediol derived from ester hydrolysis. Under these conditions, the level of ketosis achieved in this dog model was lower than the level measured in children whose seizures were controlled by the ketogenic diet.[1–4] However, because humans may have a lower capacity for ketone body utilization than dogs,[20] the doses of *R,S*-butanediol diacetoacetate used in the present study might induce higher average ketone body concentrations in humans than in dogs. In addition, *R,S*-butanediol diacetoacetate is still a potential parenteral and enteral nutrient if administered at rates up to 50% of the alcohol dehydrogenase activity.[9] The nonsparing of the utilization of exogenous ketone bodies by dietary carbohydrates plus proteins deserves further investigation.

Acknowledgments

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