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Disposition of D-[U-^{14}C]Tagatose in the Rat

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The purpose of this experiment was to determine the disposition of D-tagatose, under development as a low-calorie sweetener, in conventional and germ-free male rats. One group of conventional rats was fed a diet containing D-tagatose (100 g/kg) mixed with the non-purified diet (900 g/kg) for 28 days. Then, [U-^{14}C]-labeled D-tagatose was administered as a single dose (approximately 220–380 kBq) to 4 of these adapted rats, as well as to 15 conventional and germ-free rats with no prior exposure (i.e., unadapted) to D-tagatose. Eleven of the 19 dosed animals (4 adapted conventional, 3 unadapted conventional and 2 unadapted germ-free, all dosed orally, plus 2 unadapted conventional dosed intravenously) were placed in metabolism chambers and samples of CO_2, urine, and feces taken at regular intervals. At termination, a complete material balance was obtained based on the recovery of ^{14}C. Over the 6-h digestive period, D-tagatose was metabolized to release 39.9 and 13.9% of the oral dose as CO_2 in the adapted conventional rats and in the unadapted germ-free rats, respectively. Total releases approximated 68 and 22%, respectively. The difference in CO_2 evolution is ascribed to microbial fermentation of D-tagatose in the gut of the conventional rats. The role of adaptation was confirmed by finding 93% less D-tagatose in the feces of the adapted conventional rat than in the feces of the unadapted conventional rat. The intestinal absorption of D-tagatose in the rat is estimated to be 20%. The results demonstrate that D-tagatose is metabolized primarily by microorganisms in the gut of the rat, with an upper limit between 15 and 20% of oral dose metabolized by the host. © 1999 Academic Press

Key Words: **D-tagatose; fermentation; short-chain fatty acids; rats.**

INTRODUCTION

D-Tagatose is a naturally occurring, sweet (Zehner, 1988, Levin *et al.,* 1995) ketohexose related to D-galactose and is the C-4 epimer of D-fructose. Because of its sucrose-like taste, its zero net energy content, as measured by the energy balance method of Livesey and Brown (1996), its physical properties, and its browning on baking (Szepesi, 1996), D-tagatose holds promise as a sucrose substitute. An economical process to synthesize D-tagatose from lactose has been developed (Beadle *et al.,* 1991 1992).

In animals, radiolabel (disposition) techniques have been used to study the metabolism of fermented carbohydrate-bulking agents such as erythritol (Van Ommen *et al.,* 1996), polydextrose (Figdor and Rennhard, 1981; Juhr and Franke, 1992), "Neosugar," a mixture of fructoöligosaccharides (Tokunaga *et al.,* 1989), sorbitol (Figdor *et al.,* 1987), L-sorbose (Würsch *et al.,* 1979), and maltitol (Rennhard and Bianchine, 1976). It is well known that the major products of carbohydrate fermentation are the short-chain fatty acids (SCFA)[2] acetate, propionate, and butyrate, as well as lactate, carbon dioxide (CO_2), hydrogen, and methane (Miller and Wolin, 1979).

The present study was conducted to determine the absorption, distribution, and elimination of D-[U-^{14}C]tagatose in unadapted conventional (CV) and germ-free (GF) male rats, and CV male rats adapted to consumption of D-tagatose at 100 g/kg of diet for 28 days, using radioisotopic and chromatographic techniques. Chromatographic techniques were used to gain some indications of the metabolic pathways.

MATERIALS AND METHODS

Two batches of D-[U-^{14}C]tagatose were prepared synthetically by Biospherics Incorporated. The first batch, used to dose rats with no previous exposure (i.e., unadapted) to D-tagatose, had a radiochemical purity of 94.2% and a specific activity of 1.64 kBq/mg. The second batch was used to dose rats adapted to D-tagatose, and had a radiochemical purity of 98.8% and a specific activity of 1.03 kBq/mg. Unlabeled D-tagatose (Sigma Chemical Company, St. Louis, MO) used to dilute both batches of the labeled D-tagatose had a chemical purity of 99.5%.

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[2] Abbreviations used: CO_2, carbon dioxide; CV, conventional; GF, germ-free; iv, intravenously; SCFA, short-chain fatty acids.

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TABLE 1
Summary of Treatment Groups Used in the Study[a]

Treatment	Rat model	*n*
Orally dosed with D-[U-^{14}C]tagatose, with no prior exposure (unadapted) to D-tagatose	CV	3
Orally dosed with D-[U-^{14}C]tagatose, adapted to D-tagatose at 100 g/kg of diet[b] for 28 days	CV	4
Intravenously (iv) dosed with D-[U-^{14}C]tagatose, unadapted to D-tagatose	CV	2
Orally dosed with D-[U-^{14}C]tagatose, unadapted to D-tagatose	GF	2
Orally dosed with D-[U-^{14}C]tagatose, unadapted to D-tagatose, killed at various time intervals to obtain blood and cecum contents	CV	8

[a] Abbreviations used: CV, conventional; GF, germ-free; iv, intravenously.
[b] Diet contained D-tagatose (100 g/kg) mixed with the nonpurified diet (900 g/kg).

Male CV rats (n = 23, 44–46 days old) of the Sprague–Dawley CD outbred strain were purchased from Charles River Breeding Laboratories, Inc. (Kingston, NY). The rats were examined for good health on receipt and placed in plastic box-type cages (one animal per cage) with raised wire floors in an environmentally controlled animal room (temperature 20–23°C, humidity 18–59%, and a 12-h light:dark cycle). The rats were given free access to Certified Rodent Chow 5002 (pelleted form, Purina Mills, Richmond, IN),[3] and tap water acidified to pH 2.5 with hydrochloric acid to control microbial growth. The rats were acclimated to these laboratory conditions for at least 6 days.

Before study initiation, the protocol was reviewed and approved by an animal ethical treatment committee consisting of Biospherics Incorporated's senior research scientists and an outside consultant.

Table 1 summarizes the various treatment groups used in the study. Prior to being dosed with radiolabeled D-tagatose, 10 of the CV rats were randomly selected and allowed free access to a diet containing D-tagatose (100 g/kg) mixed with the nonpurified diet (900 g/kg) for 28 days. The day before dosing with radiolabeled D-tagatose, the remaining 13 CV rats unadapted to D-tagatose were randomly assigned one of three possible treatments—oral dosing (n = 3), intravenous (iv) dosing (n = 2), or oral dosing with slaughter at predetermined intervals (n = 8) using a random numbers table. The bulk unlabeled D-tagatose (95.5% purity) was prepared synthetically by Biospherics Incorporated. Beginning 2 days before exposure to D-tagatose in the diet, and during the entire treatment period, food and water consumption and body weights were monitored daily. Fecal consistency was observed and reported as "normal" or "soft." Soft stools included soft formed, soft unformed, and liquid stools. On a daily basis, feces and spilled food were removed from the cages, and cage floors were wiped with paper towels to absorb urine and spilled water.

Germ-free male rats (n = 3; 127 days old) of the same strain as the CV rats were obtained from Lobund Laboratory at the University of Notre Dame (Notre Dame, IN). These rats were shipped by overnight carrier in plastic bottles containing sterile food (Diet L-485, 5% mouse/rat, autoclavable, Teklad, Madison, WI). To prevent microbial contamination, the GF rats were kept in their containers until dosing, which occurred on the day of arrival. The GF rats were not acclimated to laboratory conditions because of the difficulty maintaining a germ-free environment for the typical acclimation period.

The average weights of the unadapted orally and iv dosed CV rats were 244.6 and 223.2 g, respectively. These rats were each dosed with approximately 220–380 kBq of the [U-^{14}C]-labeled D-tagatose. Two of the three GF rats were orally dosed with approximately 370 kBq. The GF rats were not weighed until immediately before being killed, at which time they weighed an average of 305.8 g. The technician wore a dust–mist respirator, surgical gloves, and disposable coveralls when dosing the GF rats to minimize the risk of contamination. During dosing, each rat was exposed to ambient air for approximately 2 min. After 27 days on the D-tagatose diet, 4 of the 10 adapted CV rats (mean wt, 409.3 g) were randomly selected and placed in the metabolism chambers 17 h before dosing with radiolabeled D-tagatose (approximately 370 kBq).

Approximately 16 h prior to dosing, 5 of the 13 unadapted CV rats were placed in individual glass metabolism chambers. During this acclimation period, background samples of urine, feces, and expired CO_2 for ^{14}C counting were collected (see below). Food (granular nonpurified diet) was not withheld during the acclimation period nor during the experimental period when samples were collected. Germ-free rats were allowed free access to sterilized Diet L-485 and sterilized tap water acidified to pH 2.5 with hydrochloric acid during the experimental period. The two selected GF rats were not acclimated to the metabolism chambers prior to dosing. The chambers were disinfected by spraying with a 0.5% (v/v) solution of Colcide (Shaldra Inc., Bethesda, MD), and the glass surfaces were swabbed with sterile gauze. The third GF rat was not dosed with D-tagatose, but was used as a control to collect background ^{14}C data for CO_2, urine, and feces. This rat was housed in a disinfected glass metabolism chamber. Two of the five unadapted CV rats in the

[3] Proximate composition: 201 g/kg protein, 45 g/kg fat, 46 g/kg crude fiber, and 16.9 MJ/kg gross energy.

metabolism chambers were dosed iv, and three were dosed orally. The dosed animals were immediately returned to their metabolism chambers designed for the separate collection of urine, feces, and expired CO_2. The urine and feces reception vessels were maintained in dry ice after collection of the radioactive excretions to prevent microbial decomposition. Room air was pulled through the system using the laboratory vacuum line. The air was passed over anhydrous calcium sulfate to remove moisture, over Ascarite (Thomas Scientific, Swedesboro, NJ) to remove CO_2, and through a 0.3-μm membrane microbial filter before entering the animal chamber. Expired CO_2 was trapped as it exited the animal chamber in four gas-washing bottles each containing 250 ml of either aqueous 2 M potassium hydroxide or 2.5 M sodium hydroxide.

Oral dosing was carried out by drawing up a known volume of sterile aqueous solution of radiolabeled D-tagatose of known concentration and specific activity in a disposable plastic syringe attached to a gavage needle, weighing the full syringe and needle, expressing the volume into the stomach of the rat, and reweighing the syringe with needle. The weight difference in the syringe allowed calculation of the administered dose. Intravenous dosing was accomplished by drawing up a known volume of sterile aqueous solution of radiolabeled D-tagatose of known concentration and specific activity in a disposable plastic syringe, weighing the full syringe, inserting the needle into a tail vein, administering the dose over approximately a 1-min period, and reweighing the syringe. As with oral dosing, knowing the weight difference in the syringe allowed calculation of the dose.

After dosing, the sample collection periods in the metabolism chambers were 72 h for both the adapted and unadapted orally dosed CV rats, 48 h for the unadapted iv dosed CV rats, and 24 h for the orally dosed GF rats. Samples of caustic solutions of trapped $^{14}CO_2$ were collected at 2, 4, 6, 8, 12, and 24 h after dosing (all rats), 36 and 48 h after dosing (orally and iv dosed CV rats), and 60 and 72 h after dosing (orally dosed CV rats). The caustic solutions were replaced at 6, 12, 24, 36, 48, and 60 h after dosing. Separate urine and feces samples were collected at 6, 12, 24, 48, and 72 h after dosing. At termination of the sample collection period, chamber surfaces were rinsed with a known amount of deionized water. Collected samples of urine and feces were stored at approximately −20°C, and CO_2 solutions and chamber rinsings were stored at 2°C until analysis.

Blood samples (approximately 5 ml) and cecum contents were collected from the eight remaining unadapted CV rats at 0.5, 2, 4, 8, 12, 24, 36, and 48 h after oral dosing with radiolabeled D-tagatose (approximately 370 kBq). These animals were housed in plastic box-type cages. At each time interval, one rat was killed by decapitation and blood was collected by exsanguination. For the metabolism chamber studies, at termination, all animals were killed and blood was collected in the same way. The total weight of blood was estimated as 6.1% of body weight (Ringler and Dabich, 1979). Blood samples were collected in nonheparinized test tubes. Serum was obtained by centrifugation for 20 min at 2000 rpm. Serum and red cells were weighed and frozen at approximately −20°C. Selected tissues were removed, rinsed with saline solution, blotted, weighed, and frozen in liquid nitrogen. The remaining carcasses were also frozen in liquid nitrogen. The carcasses and tissues were then held frozen at approximately −20°C until analyzed. Blood, ceca with contents, intestinal tracts with contents, kidneys, and livers were collected from the GF rats, as well as from the unadapted iv and orally dosed CV rats which had been kept in the metabolism chambers. In the experiments on adapted rats, brain, epididymal fat pads, and perirenal fat pads were also collected.

An aliquot (0.1 ml) of each sample of caustic solution of $^{14}CO_2$ was measured into a glass scintillation vial and diluted with deionized water to 1.0 ml. To this was added 10 ml of Maxifluor scintillator (J. T. Baker Chemical Co., Phillipsburg, NJ). Aliquots of urine and chamber rinsings (1.0 ml) were added directly to 10 ml of Maxifluor. Feces samples were weighed and homogenized in a minimum amount of deionized water. Aliquots of the resulting slurries were weighed and combusted in a biological materials oxidizer (R. J. Harvey Instrument Corp., Hillsdale, NJ). The resulting $^{14}CO_2$ was trapped in R. J. Harvey Carbon 14 Cocktail, and the sample was counted directly. Samples of blood, tissues, cecal and intestinal tract contents, and carcass homogenates were weighed and combusted as described for feces. Carcass homogenates were prepared by placing the carcass in a 3.8-liter stainless-steel blender, adding ice, tissue rinsings, and tap water (each approximately equal to the carcass weight), blending, and weighing the mixture.

HPLC was used to identify and quantify the products of metabolism. Samples of blood and cecum contents from some of the unadapted CV rats killed at predetermined time intervals, and urine and feces from both an unadapted and an adapted CV rat dosed orally with radiolabeled D-tagatose, were analyzed. Before HPLC analysis, serum samples were thawed and passed through 0.2-μm filters. The samples were then transferred to Minicent-10 or Ultracent-10 ultrafilters (Bio-Rad, Hercules, CA), and centrifuged for 3–6 h at 2000–3000 rpm. Ten- or 20-μl aliquots of the samples (depending on availability of sample) were added to Maxifluor and counted with the liquid scintillation counter. Urine samples were thawed and passed through 0.2- or 0.45-μm filters. Soxhlet extractions were performed on the cecum contents from the three unadapted CV rats killed 2, 4, and 8 h after dosing. Aliquots of cecum contents were weighed into cellulose

extraction thimbles and extracted for approximately 16 h with 100 ml of organics-free deionized water.

After the extractions were completed, the extracts were allowed to cool to ambient temperature. Volumes were measured, and then aliquots of the extracts were passed through 0.2-μm filters. Aliquots (100 μl) of the filtrates were counted for ^{14}C activity to determine the extraction efficiency (102.4–104.4% of the expected radioactivity was recovered). Soxhlet extractions were also performed on the 0- to 24-h feces samples from an unadapted CV rat and the 12- to 24-h feces sample from an adapted CV rat. Aliquots of the feces samples were weighed into cellulose extraction thimbles and extracted for approximately 15.5 h with 100 ml of organics-free deionized water. The water used to extract the 12- to 24-h sample from the adapted rat was adjusted to pH 8.5 with sodium bicarbonate to prevent loss of short-chain fatty acids. Sample preparation for the feces extracts was the same as that for the cecum contents extracts. The extraction efficiency ranged from 66.5 to 96.4% as determined by liquid scintillation counting.

An orbital shaker was used to extract the 0- to 6- and 6- to 12-h feces samples produced by the adapted rat. Feces samples were weighed into wide-mouth plastic bottles and organics-free deionized water added at a weight 10 times that of the sample. Bottles were placed on the orbital shaker at 350–400 rpm for 7–8 h and then were centrifuged at 8000 rpm for 20 min. The supernatant was removed and the volume was measured. Aliquots were passed through 0.45-μm filters, and 100-μl samples were counted for ^{14}C activity. It was found that 44.0–93.2% of the expected radioactivity was extracted. The remainder of the ^{14}C activity in the feces was presumed to be present as biomass, and therefore not easily extracted (Hobbs, 1988). The solids remaining in the extraction thimble or as sediment following centrifugation, as well as the thimble itself, were sampled and combusted with the biological materials oxidizer. For the 6- to 12-h sample from the adapted rat, 41.0% of the total radioactivity was recovered in the sediment. For the 12- to 24-h sample from the same rat, 16.6% of the total ^{14}C activity was present in the solids, and 22.2% in the extraction thimble. Considering all sources, the ^{14}C balances for these samples ranged from 85.0 to 104.8%. The ^{14}C label in the extracts was concentrated with a rotary evaporator until activity was approximately 17 Bq/100 μl.

Before samples were analyzed by HPLC, standards were prepared from stock D-[U-^{14}C]tagatose and sodium [2-^{14}C]acetate (Amersham Corp., Arlington Heights, IL) by dilution with organics-free deionized water. An aliquot of the stock D-[U-^{14}C]tagatose solution was serially diluted to produce standards of approximately 60, 17, and 2 Bq/20 μl. Twenty microliters of each standard was dispensed in duplicate into 10 ml of Maxifluor and then counted with the liquid scintillation counter to determine the activity injected onto the HPLC column. As with D-tagatose, an aliquot of the sodium [2-^{14}C]acetate solution was serially diluted, resulting in standards of approximately 5 and 1 Bq/20 μl. As described above, the standards were counted to verify the activity. The standards were analyzed by HPLC using a Bio-Rad Aminex HPX-87H organic acid analysis column (length 30.0 cm $\times$ i.d. 7.8 mm). Injection volume was 20 μl. The mobile phase was 0.005 M sulfuric acid in organics-free deionized water (degassed). The reservoir, column, and detector were maintained at ambient temperature. The detector was a Ramona-5-LS flowthrough monitor for ^{14}C (Raytest USA Inc., Wilmington, DE) with a range setting of 15–750 cps, an integration time of 5 or 6 s, a flow cell volume of 2700 μl, and a 1:4 eluate:liquid scintillator ratio. The pump was a Gilson Model 302 (Gilson Medical Electronics, Inc., Middleton, WI) with a flow rate of 0.45 ml/min.

The retention times for D-tagatose and sodium acetate were 14.4 $\pm$ 1.2 and 23.1 $\pm$ 1.0 min, respectively. Chart speed was 2 mm/min and the sample run time was 40 min. The integrator was a Shimadzu Corporation Model C-R3A (Kyoto, Japan). Instrument response over the range of the standards proved to be linear. Standards of sodium [2-^{14}C]lactate (Amersham), sodium [1-^{14}C]propionate (Amersham), and sodium [1-^{14}C]butyrate (ICN Biomedicals Inc., Costa Mesa, CA) were also prepared. These were injected onto the HPLC system to obtain reference retention times. A standard of pyruvic acid (thought to be a possible metabolite of D-tagatose) was also prepared and injected onto the HPLC column. Since this standard was not ^{14}C-labeled, a Shodex Model RI SE-51 refractive index detector (Showa Denko K.K., Tokyo, Japan) was used in place of the Ramona-5-LS detector.

Injection volumes were 100 μl for serum, as well as fecal and cecum contents extracts, and either 20 or 100 μl for urine samples. The range setting of the Ramona-5-LS detector was varied depending on each sample's ^{14}C activity. All other conditions were the same as for the ^{14}C-labeled standards.

Mean values and SD for the variables measured in each treatment group (where n = 3 or 4) were calculated. Statistical tests were not utilized in this study because the treatment groups were not intended to be directly compared. The study was performed to determine the disposition of D-tagatose.

RESULTS

All the animals' health and behavior appeared to be normal following dosing and throughout the study. Table 2 presents observations of soft stool during the 27-day adaptation phase in rats fed D-tagatose at 100 g/kg of diet. After the fifth day of adaptation, very few

TABLE 2

Soft Stool Observations during 27-Day Adaptation Study in Male Conventional Sprague–Dawley Rats[a] Fed D-Tagatose at 100 g/kg of Diet[b,c]

	Day in life						
	1	2	3	4	5	6	7–27
Number of observations of soft stools[d]	10	9	3	5	4	1	6

[a] $n = 10$.

[b] Diet contained D-tagatose (100 g/kg) mixed with the nonpurified diet (900 g/kg).

[c] Each animal was housed individually and given free access to tap water acidified to pH 2.5 with hydrochloric acid, and the D-tagatose diet.

[d] Included soft formed, soft unformed, and liquid stools.

observations of soft stool were noted. Overall, the rats tolerated D-tagatose.

Table 3 presents the ^{14}C recovery from expired CO_2 after the single dose of [U-^{14}C]-labeled D-tagatose to seven unadapted and four adapted rats. The total recovery of ^{14}C as $^{14}CO_2$ averaged 49.4% of the dose for the unadapted orally dosed CV rats over 72 h, 36.6% for the unadapted iv dosed CV rats over 48 h, 21.8% for the unadapted orally dosed GF rats over 24 h, and 67.9% for the adapted CV rats over 72 h. From Fig. 1, it is apparent that $^{14}CO_2$ evolution for the CV unadapted orally dosed rats was rapid up to 12 h after dosing and then began to plateau. The $^{14}CO_2$ evolution for both the adapted CV and unadapted GF orally dosed rats was rapid up to 8 h after dosing and then began to plateau.

In Fig. 2, ^{14}C activities in both the cecum contents and feces of unadapted CV orally dosed rats are plotted over time. The total ^{14}C activity (sum of values in both plots) peaked at 8 h after dosing and declined steadily thereafter. The rate of $^{14}CO_2$ formation (% of dose/h) for 18 h after an oral dose of radiolabeled D-tagatose for unadapted CV and GF rats, and adapted CV rats is depicted in Fig. 3. The rate of $^{14}CO_2$ formation peaked at 7 h after dosing for the unadapted CV rats and decreased gradually after that time. For the unadapted GF rats and the adapted CV rats, the rates of $^{14}CO_2$ formation peaked at 3 h after dosing and declined sharply after that time.

Total radioactivity of blood samples and component analyte fractions from six unadapted CV orally dosed rats was determined as a function of time and is presented in Table 4. ^{14}C-labeled D-tagatose and its SCFA and lactate metabolites accounted for a low percentage of total radioactivity in the blood, especially at 8 h after dosing and later. At 0.5 and 4 h after dosing, these ^{14}C-labeled compounds accounted for ~30% of total whole blood ^{14}C (Table 4), and represented only 0.1% of dose. At 2, 8, 12, and 24 hr after dosing, ^{14}C-labeled compounds accounted for 10% or less of total whole blood radioactivity. The remainder of the radioactivity is due primarily to $^{14}CO_2$. Of the SCFA, acetate was predominant, followed by butyrate and propionate. Figure 4 illustrates the concentration of the major ^{14}C-labeled compounds in serum for unadapted orally dosed CV rats during the first 24 h after dosing. The concentration of D-tagatose, SCFA, and lactate peaked at 4 h after dosing.

Since the blood revealed the presence of two major types of intermediate metabolites, lactate and SCFA, we decided to examine the urine of the unadapted orally dosed CV rats. HPLC analysis (Table 5) indicated the presence of ^{14}C-labeled D-tagatose (3.2% of dose) and SCFA salts such as acetate, propionate, and butyrate (~0.2% of dose), lactate (~0.1% of dose), and pyruvate (less than 0.1% of dose). The predominant SCFA in the urine was propionate, followed by acetate and butyrate.

A large quantity of ^{14}C label (42.6% of dose) was excreted in the urine of the unadapted iv dosed CV rats over 48 h (Table 3) with most (>90%) being cleared by the kidneys within 6 h after dose (data not shown). Little difference was noted between the adapted and unadapted orally dosed CV rats, both in total ^{14}C recovered, and in the types and amounts of labeled compounds (Table 5). Apparently, little if any host adaptation occurred with respect to absorption of D-tagatose. Over the first 24 h after dosing, D-tagatose was the major ^{14}C-labeled compound in the urine of both adapted and unadapted CV rats, accounting for 88% of the ^{14}C label in the urine of the adapted rat, and 68% of the ^{14}C label in the urine of the unadapted rat (from Table 5). No other labeled compounds were found in significant quantities. For the adapted rat, propionate was the predominant SCFA, followed by butyrate and acetate.

In an effort to identify possible ^{14}C-labeled compounds transported from the intestine to the blood, we examined the cecum contents of three unadapted CV rats killed at 2, 4, and 8 h after oral dosing. The cecum contents held a maximum of 29.6% of the ^{14}C dosed at 8 hr (Fig. 2). The major ^{14}C-labeled compound was D-tagatose (21–24% of dose, Table 4), with some SCFA, lactate, and propionate detected as well (Table 4). ^{14}C-labeled acetate was the predominant SCFA in the cecum contents, followed by butyrate, and propionate. HPLC analysis of feces from an unadapted orally dosed CV rat confirmed these results (Table 5). The rat's feces showed the presence of D-tagatose (25.7% of dose) as well as lactate (0.2% of dose), pyruvate (1.2% of dose), and SCFA (0.9% of dose), the last again being predominantly acetate, followed by butyrate and propionate.

Averages of 28.7% (unadapted CV orally dosed rats), 4.8% (CV iv dosed rats), 4.2% (GF orally dosed rats), and 11.4% (adapted CV orally dosed rats) of the ^{14}C dose were excreted in the feces (Table 3). The major ^{14}C-labeled compound detected in the feces of both the

TABLE 3
Metabolism of Radiolabeled D-Tagatose in Conventional and Germ-Free Male Sprague–Dawley Rats[a]

	Conventional unadapted rats, orally dosed	Conventional unadapted rats, intravenously dosed	Germ-free unadapted rats, orally dosed	Conventional adapted rats, orally dosed
n	3	2	2	4
Dose (mg D-tagatose/kg body wt)	806	620	762	876
	Percentage of ^{14}C dose recovered[b]			
CO_2				
(0–2 h)	3.1 ± 0.4	16.0	2.5	5.4 ± 1.5
(0–6 h)	16.2 ± 1.1	27.5	13.9	39.9 ± 5.7
(0–12 h)	38.3 ± 5.6	33.2	20.0	57.2 ± 4.0
(0–24 h)	46.5 ± 9.2	35.2	21.8	61.3 ± 3.9
(0–48 h)	48.5 ± 9.6	36.6	—	64.6 ± 4.3
(0–72 h)	49.4 ± 9.8	—	—	67.9 ± 2.7
Urine				
(0–24 h)	5.5 ± 0.6	41.9	3.8	4.9 ± 0.6
(0–48 h)	5.7 ± 0.6	42.6	—	5.1 ± 0.5
(0–72 h)	5.8 ± 0.6	—	—	5.2 ± 0.5
Feces				
(0–24 h)	26.8 ± 10.9	4.4	4.2	9.0 ± 2.4
(0–48 h)	28.5 ± 10.1	4.8	—	11.0 ± 1.5
(0–72 h)	28.7 ± 10.0	—	—	11.4 ± 1.4
Select tissues	1.3 ± 0.1[c,d]	1.2[c,e]	1.4[c,f]	2.5 ± 0.9[d,g]
Cecum contents and intestinal tract with contents	0.5 ± 0.1[d]	0.5[e]	59.2[f]	0.6 ± 0.2[d]
Remaining carcass	7.5 ± 0.8[d]	6.2[e]	4.8[f]	7.6 ± 1.2[d]
Chamber deionized water rinsings	0.1 ± 0.0	0.8	0.6	0.1 ± 0.0
Total	93.3 ± 1.1[d]	92.6[e]	96.0[f]	95.3 ± 2.0[d]

[a] Single doses of D-[U-^{14}-C]tagatose (approximately 220–380 kBq) were administered orally or intravenously after feeding nonpurified diet (unadapted), or diet containing 100 g/kg D-tagatose mixed with the nonpurified diet (900 g/kg) for 28 days (adapted). The rats were allowed free access to tap water acidified to pH 2.5 with hydrochloric acid and the diets, while monitored in individual metabolism chambers.
[b] Values are means ± SD, where n = 3 or 4. Where n = 2, values are means.
[c] Select tissues: blood, liver, and kidneys.
[d] Animals killed 72 h after dosing.
[e] Animals killed 48 h after dosing.
[f] Animals killed 24 h after dosing.
[g] Select tissues: blood, liver, brain, kidneys, epididymal, and perirenal fat pads.

adapted and the unadapted CV rats was D-tagatose (Table 5). In a single adapted rat, approximately 20% of the ^{14}C activity found in the feces in the first 24 h following dosing was present as D-tagatose, which also accounted for 84% of the ^{14}C in the feces of a single unadapted rat in the first 24 h (see Table 5). The feces of the adapted CV rat contained 93% less D-tagatose than the feces of the unadapted CV rat. Metabolites resulting from fermentation of D-tagatose such as lactate, pyruvate, and SCFA were more prevalent (in comparison with D-tagatose levels) in the adapted rat's feces than in the unadapted rat's feces. For the adapted rat, the predominant SCFA was acetate, followed by propionate and butyrate (compared with acetate, followed by butyrate and propionate, in the unadapted rat).

Average recoveries of 0.5% of the ^{14}C dose (both unadapted orally dosed and iv dosed CV rats) and 59.2% (orally dosed GF rats) were found in the combined cecum contents plus intestinal tract. The remaining amount of ^{14}C was widely distributed in the tissues. Table 3 presents the ^{14}C recovery in select tissues and remaining carcass of rats after dosing with [U-^{14}C]-labeled D-tagatose. Recoveries of 8.8% (unadapted orally dosed CV rats), 7.4% (iv dosed CV rats), 6.2% (orally dosed GF rats), and 10.1% (adapted orally dosed CV rats) were recovered from the combined select tissues plus carcasses.

Table 3 also presents the total recovery from all sources following the administration of [U-^{14}C]-labeled D-tagatose to the rats. An average of 93.3 ± 1.1% of the administered dose was accounted for in the unadapted

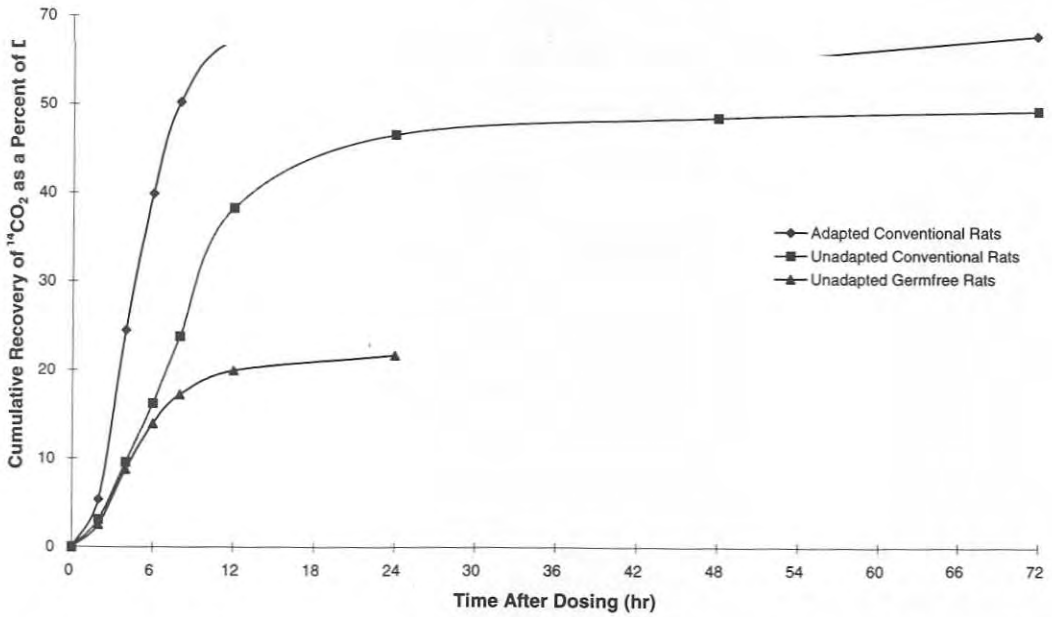

FIG. 1. Cumulative recovery of $^{14}CO_2$ (as a percentage of dose) after an oral dose of approximately 370 kBq of D-[U-^{14}C]-labeled tagatose given to conventional male Sprague–Dawley rats (n = 3) with no prior exposure to D-tagatose (unadapted), conventional male Sprague–Dawley rats (n = 4) adapted to D-tagatose (100 g/kg diet) for 28 days, and unadapted germ-free male Sprague–Dawley rats (n = 2). The $^{14}CO_2$ data are plotted over 72 h after dosing for the conventional rats, and over 24 h after dosing for the germ-free rats. Points are mean values.

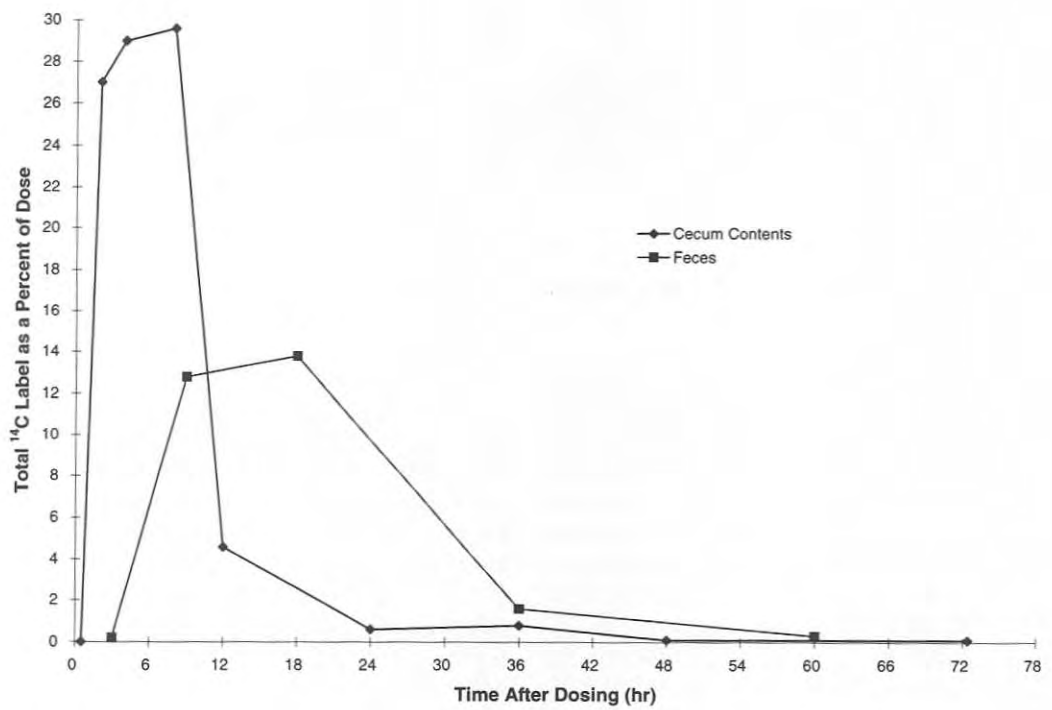

FIG. 2. Total ^{14}C label (as a percent of dose) in cecum contents and feces of conventional male Sprague–Dawley rats with no prior exposure to D-tagatose for 72 h after an oral dose of approximately 370 kBq of [U-^{14}-C]-labeled D-tagatose. The data representing ^{14}C label in cecum contents were obtained from nine individual rats killed at 0.5, 2, 4, 8, 12, 24, 36, 48, and 72 h after dosing, all but the 72-h sample being from the rats kept in plastic box-type cages. The points for the feces data are mean values (n = 3) at 3, 9, 18, 36, and 60 h after dosing, derived from feces collected at 6, 12, 24, 48, and 72 h from the rats kept in the metabolism chambers.

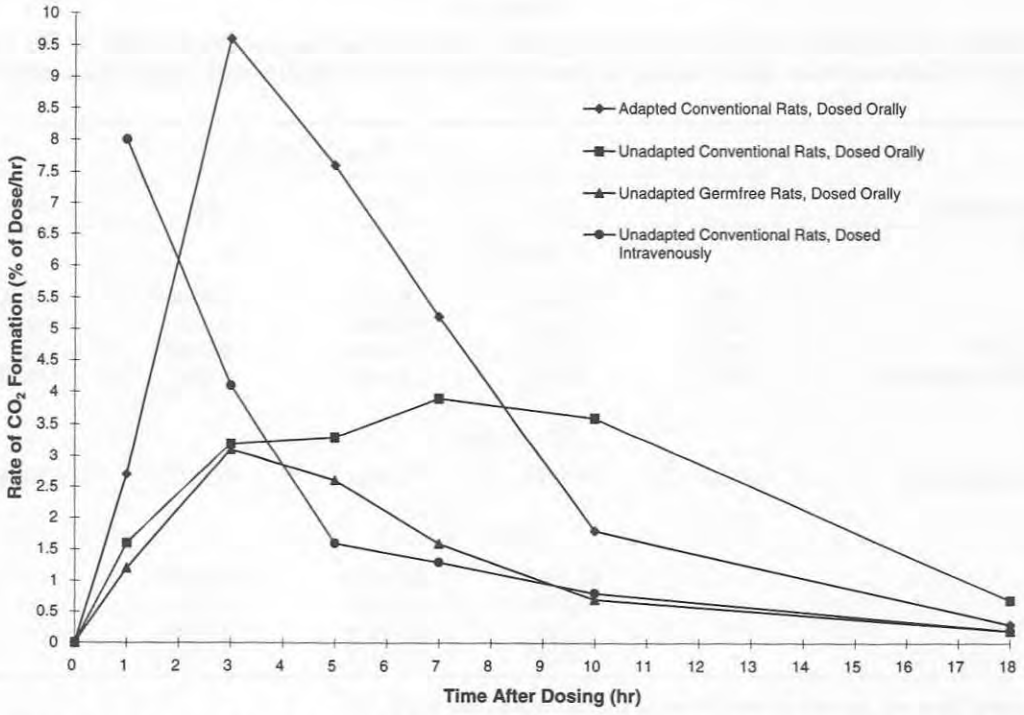

FIG. 3. Rate of $^{14}CO_2$ formation (% of dose/h) over 18 h after an oral dose of approximately 370 kBq of [U-^{14}C]-labeled D-tagatose given to conventional male Sprague–Dawley rats (n = 3) with no prior exposure to D-tagatose (unadapted), conventional male Sprague–Dawley rats (n = 4) adapted to D-tagatose (100 g/kg diet) for 28 days, and unadapted germ-free male Sprague–Dawley rats (n = 2), and after an intravenous dose of approximately 220 kBq of [U-^{14}C]-labeled D-tagatose given to unadapted conventional male Sprague–Dawley rats (n = 2). Points are mean values for 1, 3, 5, 7, 10, and 18 h, derived from samples collected at 2, 4, 6, 8, 12, and 24 h.

orally dosed CV rats, 92.6 ± 1.9% in the iv dosed CV rats, 96.0 ± 0.4% in the orally dosed GF rats, and 95.3 ± 2.0% in the adapted orally dosed CV rats, indicating excellent material balances. ^{14}C-labeled methane is not trapped in the caustic solutions and, therefore, if generated, would have escaped undetected.

DISCUSSION

The first 6 h after oral dosing is generally regarded as the time frame during which absorption and metabolism of absorbed nutrients by mammalian enzymes occurs in CV rats (Juhr and Franke, 1992). In our study, during this time period, the $^{14}CO_2$ evolution data indicated that D-tagatose had been metabolized to release 39.9 and 13.9% of the oral dose as $^{14}CO_2$ in the adapted CV rats and in the unadapted GF rats, respectively (Table 3). The difference in $^{14}CO_2$ evolution is ascribed to microbial fermentation of D-tagatose in the gut of the CV rats. The amount of $^{14}CO_2$ recovered in the first 6 h after oral dosing in the GF rats (13.9% of dose) is a valid estimate of CO_2 resulting from digestion in the CV rat (Juhr and Franke, 1992). This amount represented 63.8% of the total $^{14}CO_2$ of the GF rats over the 24-h sample collection period (13.9%/21.8%). In a similar manner, 63.8% of the ^{14}C label found in the select tissues and remaining carcass of the GF rats (6.2% × 0.638 = 4.0%) represents the ^{14}C label deposited in tissues and carcass of the CV rats due to digestion (Juhr and Franke, 1992). Likewise, 63.8% of the ^{14}C label found in the urine of the GF rats (3.8% × 0.638 = 2.4%) represents the ^{14}C label in urine resulting from digestion. The urine of the GF rats was not analyzed for tagatose, but based on the analysis of the CV rats' urine, it was assumed that most of the ^{14}C label was present as tagatose. The sum of the amounts in $^{14}CO_2$, tissues and carcass, and urine (13.9 + 4.0 + 2.4) equals 20.3% of the oral ^{14}C-labeled D-tagatose dose. This total provides an estimate of the intestinal absorption of D-tagatose in the CV rat. The sum of the amounts in $^{14}CO_2$, tissues, and carcass equals 17.9% of the oral ^{14}C-labeled D-tagatose dose. This sum is an estimate of the amount of D-tagatose metabolized in the CV rat.

Further support for passive absorption of 12 to 20% of some hexoses is provided by studies with rats dosed with radiolabeled L-fructose or L-gulose (Levin *et al.*, 1995). These two L-sugars are metabolized to a very limited extent (less than 5%) by the rat. Rats dosed intravenously yielded nearly complete recoveries (>90%) of ^{14}C label in either urine. However, orally dosed rats not adapted to either sugar excreted feces containing approximately 40% of the ^{14}C label, urine containing about 20% of the dose, with the expired

TABLE 4

Recovery of Major ^{14}C-Labeled Compounds (D-Tagatose and Metabolites) as Percentage of Dose in Serum and Ceca of Unadapted Conventional Male Sprague–Dawley Rats Dosed Orally with Approximately 370 kBq D-[U-^{14}C]Tagatose[a,b]

^{14}C-labeled compound	Time (h) after dose					
	0.5	2.0	4.0	8.0	12.0	24.0
			Serum			
D-Tagatose	0.0762	0.0460	0.1324	0.0300	0.0082	0.0057
Lactate	0.0066	0.0049	0.0092	0.0030	ND[c]	0.0012
Short-chain fatty acids	0.0052	0.0014	0.0052	0.0012	ND	ND
Total (as ^{14}C-labeled compounds)	0.0880	0.0523	0.1468	0.0342	0.0082	0.0069
			Whole blood			
Total ^{14}C-label in whole blood	0.2563	0.5210	0.4641	0.6032	0.9918	0.4002
			Ceca			
D-Tagatose	—	21.0961	23.9588	22.0513	—	—
Lactate	—	0.5390	0.2451	0.5547	—	—
Short-chain fatty acids	—	0.7247	0.4654	1.3060	—	—
Pyruvate	—	0.3349	0.6177	1.5762	—	—

[a] Data were obtained from six individual rats killed at the indicated time intervals.
[b] Rats had no prior exposure to D-tagatose.
[c] ND, none detected.

$^{14}CO_2$ containing about 35%. Therefore, in the unadapted, orally dosed rats, at least 35% of the dose must have been fermented by microorganisms in the intestines. Thus, the 20% of dose in urine must have represented nearly all of the amount absorbed across the intestine of the unadapted rat. Approximately 60% of the ^{14}C label from the L-sugars orally administered to adapted rats was recovered in their expired CO_2, with 12% in urine and 13% in feces. These data show that approximations of absorption based on unadapted or germfree rats overestimate true absorption. The explanation may lie in additional absorption of hexoses in the cecum and large intestine (Juhr and Franke, 1992).

In Fig. 3, the rates of $^{14}CO_2$ formation for unadapted orally dosed CV and GF rats are compared up to 18 h after dosing. At 1, 3, and 5 hr, the rates of $^{14}CO_2$ formation agreed closely. The rates of $^{14}CO_2$ formation at 7, 10, and 18 h were much lower for the GF rats than for the unadapted CV rats. We thought it possible that some minor contamination of airborne microorganisms might have occurred during the few minutes required to dose the GF rats that could have led to $^{14}CO_2$ evolution in the intestine and its absorption into the blood. We therefore checked the rates of disappearance of ^{14}C as $^{14}CO_2$ from the GF and unadapted iv dosed CV rats and found them to be virtually identical (Fig. 3). Thus, microbial contamination had not occurred. However, the GF data provide evidence that some of the $^{14}CO_2$

TABLE 5

Recovery of Major ^{14}C-Labeled Compounds (D-Tagatose and Metabolites) as Percentage of Dose in Urine and Feces of Male Conventional Sprague–Dawley Rats Dosed Orally with Approximately 370 kBq D-[U-^{14}C]Tagatose[a]

^{14}C-labeled compound	Urine, adapted rat[b]	Urine, unadapted rat[c]	Feces, adapted rat	Feces, unadapted rat
D-Tagatose	4.4004	3.1782	1.8175	25.7122
Lactate	0.0418	0.0778	0.2582	0.2540
Short-chain fatty acids	0.0631	0.1794	0.5058	1.1177
Pyruvate	0.0214	0.0360	0.5226	1.1959
Total	4.5267	3.4714	3.1041	28.2798
Total ^{14}C-label in urine or feces	5.0	4.7	9.0	30.5

[a] 0–24 h after dose.
[b] Rat was fed a diet containing D-tagatose (100 g/kg) mixed with the nonpurified diet (900 g/kg) for 28 days.
[c] Rat had no prior exposure to D-tagatose.

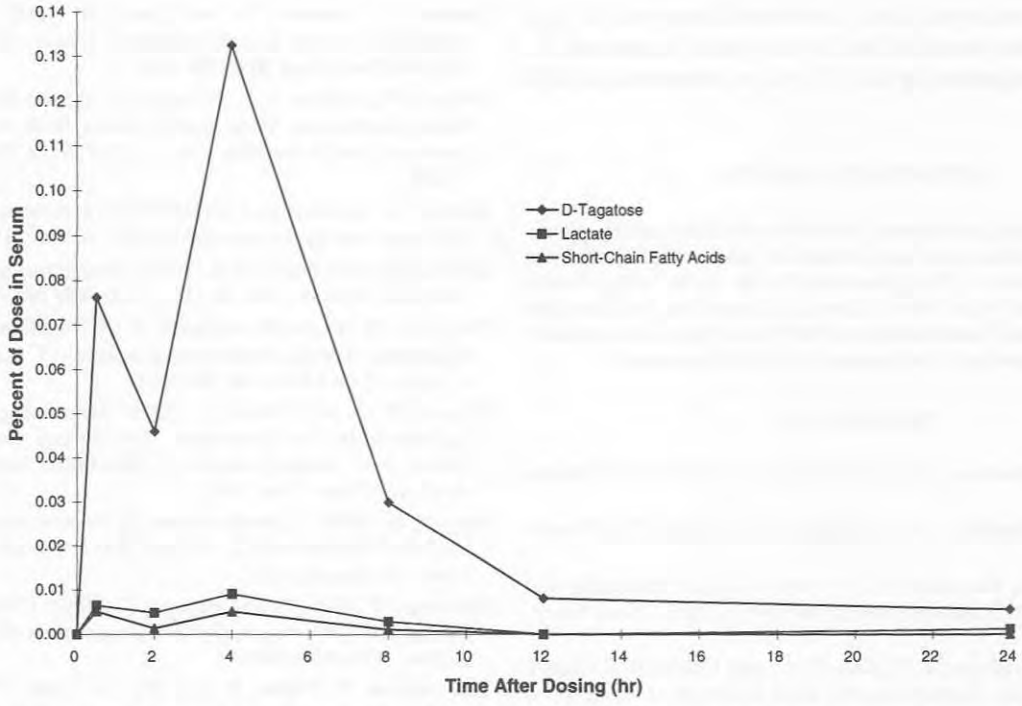

FIG. 4. Major ^{14}C-labeled compounds (as a percentage of dose) in serum of conventional male Sprague–Dawley rats, with no prior exposure to D-tagatose, for 24 h after an oral dose of approximately 370 kBq of [U-^{14}C]-labeled D-tagatose. The data were obtained from six individual rats killed at 0.5, 2, 4, 8, 12, and 24 h after dosing.

evolution resulted from host metabolism of D-tagatose. The balance of the $^{14}CO_2$ evolution, constituting the major portion, resulted from fermentation of D-tagatose in the cecum. D-Tagatose is also being decomposed by the gut microflora in a fermentative process to form hydrogen and methane. The ^{14}C data do not give a direct measure of the production of methane and hydrogen. In humans, D-tagatose is known to be fermented in the gut by lactobacilli (Lenzner *et al.*, 1968).

Table 4 presents the time course of the appearance and persistence of labeled D-tagatose and its metabolites in serum, whole blood, and ceca of unadapted, orally dosed CV rats. The time persistence of the label in the serum indicates incomplete metabolism for the fraction absorbed.

The summation of ^{14}C recovery in CO_2 and feces was nearly the same for adapted (79.3%) and unadapted CV orally dosed rats (78.1%). This lends further support to the conclusion that the difference in $^{14}CO_2$ evolution between the adapted and unadapted CV rat is due to microbial adaptation to D-tagatose in the gut, causing increased fermentation of D-tagatose to produce CO_2 in the adapted rat. Digestion and absorption of carbohydrates continues in the large intestine of GF rats more than 6 h after oral dosing (Juhr and Franke, 1992). Thus, the amount of mammalian enzyme metabolism in GF rats exceeds that of CV rats. The measurement of expired $^{14}CO_2$ over the normal digestive period of 6 h provides an upper limit of 13.9% of dose, based on the GF rat data. This is an upper limit because $^{14}CO_2$ evolution from 6 to 24 h in the GF rats would be due to continued digestion and absorption of D-tagatose in the large intestine (as there is no competition from fermentation in GF rats, Juhr and Franke, 1992). The $^{14}CO_2$ results, while still showing little energy utilization, differ from Livesey and Brown's results. The work reported herein indicates that intestinal absorption of D-tagatose by the rat is approximately 20% of the dose. Host metabolism of D-tagatose has an upper limit between 15 and 20% of oral dose.

CONCLUSIONS

Most $^{14}CO_2$ evolution in the orally dosed CV rats appears to originate from fermentation in the gut. The small quantities of intermediate metabolites detected in blood and urine leads us to believe that a large part of the detected $^{14}CO_2$ (67.9% of oral dose in adapted CV rats) and (possibly) undetected ^{14}C-labeled methane is being formed in the gastrointestinal tract and either absorbed into the blood and expired in the lungs or eliminated as flatus and eructations. Of the 46.5% of dose evolved as $^{14}CO_2$ during the first 24 h after oral dosing of unadapted CV rats, at least 24.7% was calculated to originate in the gut (based on the difference in $^{14}CO_2$ evolution between orally dosed unadapted CV and GF rats). Since only small quantities of intermediates capable of being further metabolized were detected in the blood, this suggests that D-tagatose is metabolized primarily by microorganisms in

the gut, with an upper limit estimated between 15 and 20% of oral dose metabolized by the host. Intestinal absorption of D-tagatose by the CV rat is estimated as 20% of the dose.

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REFERENCES

Beadle, J. R., Saunders, J. P., and Wajda, T. J. (1991). U.S. Patent 5,002,612.

Beadle, J. R., Saunders, J. P., and Wajda, T. J. (1992). U.S. Patent 5,078,796.

Figdor, S. K., and Rennhard, H. H. (1981). Caloric utilization and disposition of [^{14}C] polydextrose in the rat. *J. Agric. Food Chem.* **29,** 1181–1189.

Figdor, S. K., Allingham, R. P., Kita, D. A., and Hobbs, D. C. (1987). Caloric utilization of sorbitol and isomalt in the rat. *J. Agric. Food Chem.* **35,** 996–1001.

Hobbs, D. C. (1988). Methodology in the measurement of caloric availability. In *Low Calorie Products* (G. G. Birch and M. G. Lindley, Eds.), pp. 245–267. Elsevier, London.

Juhr, N. C., and Franke, J. (1992). A method for estimating the available energy of incompletely digested carbohydrates in rats. *J. Nutr.* **122,** 1425–1433.

Lenzner, A., Lenzner, H., and Toom, M. (1968). Fermentation of D-tagatose by the human microflora lactobacilli. *Tartu Riikliku Ulikooli Toimetised* **215,** 250–254.

Levin, G. V., Zehner, L. R., Saunders, J. P., and Beadle, J. R. (1995). Sugar substitutes: Their energy values, bulk characteristics, and potential health benefits. *Am. J. Clin. Nutr.* **62**(Suppl.), 1161S–1168S.

Livesey, G., and Brown, J. C. (1996). D-Tagatose is a bulk sweetener with zero energy determined in rats. *J. Nutr.* **126,** 1601–1609.

Miller, T. L., and Wolin, M. J. (1979). Fermentations by saccharolytic intestinal bacteria. *Am. J. Clin. Nutr.* **32,** 164–172.

Rennhard, H. H., and Bianchine, J. R. (1976). Metabolism and caloric utilization of orally administered maltitol-^{14}C in rat, dog, and man. *J. Agric. Food Chem.* **24,** 287–291.

Ringler, D. H., and Dabich, L. (1979). Hematology and clinical biochemistry. In *The Laboratory Rat, Biology and Diseases* (H. J. Baker, J. R. Lindsey, and S. H. Weisbroth, Eds.), Vol. 1, p. 108. Academic Press, New York.

Szepesi, B. (1996). Carbohydrates. In *Present Knowledge in Nutrition* (E. E. Zeigler and L. J. Filer, Eds.), 7th Ed., pp. 33–43. ILSI Press, Washington, DC.

Tokunaga, T., Oku, T., and Hosoya, N. (1989). Utilization and excretion of a new sweetener, fructooligosaccharide (Neosugar), in rats. *J. Nutr.* **119,** 553–559.

Van Ommen, B., DeBie, B., and Bär, A. (1996). Disposition of ^{14}C-erythritol in germfree and conventional rats. *Regul. Toxicol. Pharmacol.* **24,** S198–S205.

Würsch, P., Welsch, C., and Arnaud, M. J. (1979). Metabolism of L-sorbose in the rat and the effect of the intestinal microflora on its utilization both in the rat and in the human. *Nutr. Metab.* **23,** 145–155.

Zehner, L. R. (1988). U.S. Patent 4,786,722.