Ascorbic acid catabolism by gut microflora: studies in germ-free and conventional guinea pigs

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The role of gut microflora in ascorbic acid catabolism was investigated in both conventional and germ-free guinea pigs. In vitro studies demonstrated extensive degradation of the vitamin by fresh feces, cecal, and colonic contents of conventional guinea pigs. Direct injection of [1-^{14}C]ascorbic acid into the cecum of conventional guinea pigs in vivo yielded a 70% recovery of the label as respiratory $^{14}CO_2$ within 6 hr compared with only 5% recovery following injection into the virtually sterile peritoneum in a comparable group of conventional guinea pigs. Thus, ascorbic acid not absorbed prior to reaching the lower gastrointestinal tract stands to be extensively decarboxylated by microflora in the cecum. In a companion study of germ-free guinea pigs, 10% of an administered dose of [1-^{14}C]ascorbic acid was expired as $^{14}CO_2$ within 36 hr post-injection following intraperitoneal injection compared with 16% recovery in a matched group of conventional animals injected at the same site. Results of this series of studies suggest that hepatic decarboxylation and gut microflora, in tandem, contribute to ascorbic acid decarboxylation in this species.

Keywords: ascorbic acid decarboxylation; germ-free guinea pig; gut microflora

Introduction

Both humans and guinea pigs require exogenous sources of vitamin C (ascorbic acid) to prevent the signs and symptoms of scurvy. The germ-free guinea pig is an important animal model for investigating the interaction between ascorbic acid nutriture and gastrointestinal microflora. The major metabolite of the radiolabeled vitamin, [1-^{14}C]ascorbic acid, in the guinea pig is $^{14}CO_2$.[1] Most studies in humans,[2-4] although not all, [5,6] show urine, meanwhile, as the sole excretory route of tracer doses of [1-^{14}C]ascorbic acid. Aside from this difference in catabolic pathway, the ascorbate absorption,[7,8] tissue distribution,[9] and body pool size,[10-14] expressed in mg/kg, appear similar in the two species.

Numerous studies have shown that ascorbic acid is rapidly decomposed in vitro by both mixed cultures and specific strains of enteric bacteria.[15-17] Thus, the intestinal tract could be a potentially important site for decarboxylation of the vitamin and conceivably compromise vitamin C bioavailability. Levenson et al.[18] demonstrated a two-fold increase in mean survival time (before onset of scurvy) of guinea pigs reared on an ascorbic acid-free diet under germ-free conditions compared to isolator reared litter-mates on the same diet but "conventionalized" by contamination with cecal contents. The authors attributed this difference to the catabolism of endogenous ascorbic acid by intestinal microflora in the "conventionalized" animal. Reid[19] has demonstrated that in the guinea pig large amounts of intraperitoneally administered ascorbic acid is secreted into both the relatively sterile, upper gastrointestinal tract, and the enteric flora inhabited, lower tract. Phillips et al.[20] reported that two groups of newborn guinea pigs, one reared under germ-free and one raised under conventional conditions, but fed the same diet, developed symptoms of scurvy at the same rate. Following treatment with ascorbic acid, conventional animals took three times as long to recover. This difference in response to ascorbic acid supplementation between conventional and germ-free animals further supports the hypothesis that gut microflora play an important role in catabolism of the vitamin. To further affirm this hypothesis, we present a series of experiments conducted in conventional ani-

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mals and the first documented in vivo study using radiolabeled ascorbic acid in germ-free and age-matched conventional guinea pigs to assess the magnitude and site of ascorbic acid decarboxylation influence by enteric microflora.

Materials and methods

Conventional guinea pig studies

Animals and diets. Hartley strain guinea pigs (Charles River Breeding Laboratory, Wilmington, MA) were used in all experiments. Animals were housed in either plastic or stainless steel cages in a 12-hr, light-dark controlled room and fed either a powdered vitamin C-deficient diet formulated according to Krehl* (Teklad Mills, Madison, WI) to which 200 mg ascorbic acid per kg were added, or a commercial laboratory ration (Charles River Guinea Pig Diet).†

[1-^{14}C]ascorbic acid, specific activity of 8.44 mCi/mmol (New England Nuclear, Boston, MA) was 91% to 95% pure, as determined by thin-layer chromatography.[21] The isotope was dissolved in deionized water immediately before aspiration and the exact dosage was determined by weighing syringes before and after injections. Radioactivity was measured in a Beckman LS-7000 Liquid Scintillation Counter (Beckman Instruments, Inc., Fullerton, CA). Quenching was corrected by the sample channels ratio method.

Protocols. Experiment 1. We attempted in-vivo site-specific comparison of the degree of catabolism of intraperitoneally versus intracecally-administered [1-^{14}C]ascorbic acid. Adult male guinea pigs (fed the Charles River Guinea Pig Diet) were injected with 8–10 μCi of [1-^{14}C]ascorbic acid. The isotope was injected either directly into the cecum following surgical incision, intraperitoneally (ip), following sham operation, or ip, in non-operated controls. Animals were placed immediately into individual glass metabolism chambers, and respiratory carbon dioxide was collected continuously for 60 hr. Carbon dioxide was trapped in 2.5 N NaOH and collected at 3-hr intervals. Samples were counted in Bray's solution[22,23] containing 4% thixotropic gel (Cab-O-Sil; Packard Instrument Company, Downers Grove, IL).

Experiment 2. We attempted to identify the specific classes of enteric bacteria present in the feces of our conventional guinea pigs and determine their capacity, if any, to degrade ascorbic acid. Adult guinea pigs were fed sterilized Charles River Guinea Pig Diet ad libitum. Fresh fecal samples were collected on sterile bedding between 0800 and 1030, cultured, gram stained and examined microscopically. The degree of ascorbic acid decomposition in fresh feces was determined by the method of Kendall and Chinn.[15] Fecal samples (500 mg) were added to duplicate flasks of sterilized nutrient broth. One of these samples was autoclaved for 15 min at 123° C to assess chemical decomposition of the vitamin. Freshly prepared ascorbic acid (final concentration of 11 mmol/L broth) was dispensed into each flask through a millipore filter (0.22 μM; Millipore Corp., Bedford, MA). Cultures were incubated at 37° C on a shaker bath for 91 hr. Aliquots were removed and assayed for total ascorbic acid by the 2,4-dinitrophenylhydrazine method.[24]

Germ-free guinea pig studies

We attempted, through comparison of a group of young, adult conventional guinea pigs with a cohort group of germ-free animals, to quantify the relative contributions of hepatic mechanisms and gut microflora to decarboxylate ascorbic acid.

Gnotobiotic equipment and procedures. Germ-free studies were conducted in a laminated vinyl film isolator (Germ-free Laboratories, Miami, FL). The ventilation system in the isolator provided independent, positive-pressure microfiltration. Three all-glass metabolism chambers were assembled within the isolator. The carbon dioxide traps, as well as drying towers, were mounted outside the isolator to ease sample collections. Constant pressure was maintained within this microfiltration subsystem by in-line manostats between vacuum pumps and chambers.

All supplies needed for the studies were sterilized either in a cyclic high vacuum autoclave or with ethylene oxide (heat- and moisture-sensitive items), and loaded into the isolator via the exit-entry port. The isolator was then sterilized chemically with 4% peracetic acid and allowed to aerate for 4 days. Paper strips embedded with *Bacillus sterothermophilus* spores (Space Strips, Scientific Products, Evanston, IL), placed in the isolator during sterilization, were cultured at 55° C for 7 days to verify sterility. Transfer of supplies and samples during the study occurred through the sterile lock with an ethylene oxide trap.

Maintenance of sterility during the germ-free study was confirmed by the technique of Wagner[25] in an independent laboratory (Dr. J. Gilmartin, Veterinary Medicine, Cornell University, Ithaca, NY). Oral and anal orifices of the animals and surfaces of metabolic and plastic housing cages were swabbed with moist cotton applicators. Samples were incubated on blood agar, trypticase soy agar, and thioglycolate broth aerobically at 27° C, 37° C, and 55° C and anaerobically at 37° C. Trypticase plates were also passed into the isolator and inoculated with swabs in situ. Samples taken aseptically from cecal contents of germ-free and conventional guinea pigs at sacrifice were cultured in the same manner.

*Krehl, W.A. Laboratory Manual at Yale Nutritional Laboratory, New Haven, CT. Percent Composition: ground rolled oats, 40.0; wheat bran, 15.0; alfalfa leaf meal, 8.0; whole milk powder, 20.0; casein, 10.0; cottonseed oil, 5.0; sodium chloride, 0.5; calcium carbonate, 1.0; magnesium sulfate, 0.5.

†Charles River Guinea Pig Diet. Ingredients: Alfalfa meal, wheat middlings, soybean meal, ground oats, ground corn, ground wheat, animal fat, wheat germ meal, liver meal, linseed meal and brewer's yeast.

Animals, experimental conditions and diet. Germ-free guinea pigs were obtained by Caesarian section from conventional Hartley dams (Charles River Breeding Laboratory, Wilmington, MA). At 15 days of age, the germ-free guinea pigs were transported to our facility, under sterile conditions. They were transferred into the germ-free isolator, weighed, and housed in individual plastic cages.

The conventional group of guinea pigs, obtained from the same laboratory, were age- and strain-matched to the germ-free animals. Dams and pups were transported to our laboratory immediately following normal delivery, and the pups were separated from the dams. To simulate the housing environment of the germ-free guinea pigs, the control pups were housed in individual plastic cages in a peracetic acid-sprayed isolator open to air. Autoclaved wood shavings served as bedding for both groups of animals.

Feeding of these control pups, through age 15 days, followed the same procedure as the germ-free guinea pigs at the Charles River Breeding Laboratory. Both groups of guinea pigs were fed a sterilized, milk-base formula containing 1% ascorbic acid, by dropper, three times daily, plus a sterilized guinea pig pelleted diet fed ad libitum. Both types of food were formulated and prepared by Charles River Breeding Laboratory. Freshly sterilized water was provided daily. The pelleted diet was analyzed for total ascorbic acid[26] before and after autoclaving and contained 20 and 0.2 mmol/kg diet of ascorbate, respectively.

Ascorbic acid metabolism. In vivo study. Immediately before use the [1-^{14}C]ascorbic acid was dissolved in distilled water in a sterile glass container and dispensed through a 0.22 μm millipore filter into sterile ampules. Three germ-free and four conventional guinea pigs were injected intraperitoneally with four to nine μCi of [1-^{14}C]ascorbic acid and placed immediately into individual metabolism chambers for continuous collection of respiratory carbon dioxide and urine. Carbon dioxide was trapped in 2.5 or 5 N NaOH, collected at 3-hour to 6-hour intervals, respectively. At the termination of the collection periods, in both the conventional and germ-free guinea pigs, urine and feces were collected. Aliquots of urine, feces, and NaOH samples were counted as previously described.

The semilogarithmic plots of cumulative ^{14}C retention over time were analyzed initially by graphical peeling to determine the excretory rate constants. The data were further subjected to nonlinear analysis using the method of Gauss-Newton.[27] The total sum of the squares of differences between the computer-fitted and experimental curves was less than 5×10^{-4}. The half-life of ascorbic acid (t ½) in the slow and rapid expiratory CO_2 phases was calculated by the equation, t ½ = 0.693/k, where k is the excretory rate constant.

At the end of excreta collection periods, guinea pigs were sacrificed and blood obtained by cardiac puncture, and bile aspirated from the gallbladder. Liver, heart, adrenal glands, spleen, and cecum were excised, placed on ice, and weighed. Plasma, liver, adrenal, and spleen samples were extracted with 5% trichloroacetic acid and analyzed for ascorbic acid.[24]

Plasma and bile samples were counted in Aqueous Counting Scintillant (ACS; Amersham Corporation, Arlington Heights, IL). Tissue samples (35–100 mg) were solubilized in Soluene 100 (Packard Instrument Company, Downers Grove, IL) and counted in toluene scintillant.

The amount of ^{14}C remaining in the animals was estimated at the time of sacrifice to permit calculation of total isotope recovery. The remaining carcass, after removal of skin and fur, was homogenized in 300 mL of ice-cold 3% metaphosphoric acid using a Polytron homogenizer fitted with a PT 35 head (Evanston, IL, USA). Samples of the homogenate, skin, and fur were combusted for radioactivity determinations and total recovery calculated. Total ^{14}C recovery was consistent between the groups.

In vitro metabolism. We sought to confirm the bacterial status of each group, to assess the influence of chemical degradation of cecal contents in both groups of animals, and to document the negligible cecal ascorbate decarboxylation in the germ-free guinea pigs. Autoclaved, labeled ascorbic acid-fortified broth alone, and broth inoculated with fresh (microflora present) or autoclaved (microflora destroyed) conventional and germ-free cecal contents were compared for ascorbic acid degradation with assays taken at 2, 24, and 48 hr after sacrifice.

Plasma cortisol. Duplicate 20 μL samples of guinea pig plasma were diluted with 180 μL of double distilled water and extracted with 3 mL of nanograde methylene chloride (Mallinkrodt, Inc., Jersey City, NJ). Samples were assayed for cortisol following the method of Butler and Des Bordes,[28] except for an antibody dilution of 1:2,000. Reagents used in this assay were [1,2,6,7-^{3}H]cortisol, specific activity 100 Ci/mmol (New England Nuclear, Boston, MA), nonradioactive cortisol (Sigma Chemical Co., St. Louis, MO) and cortisol antiserum (Endocrine Sciences, Tarzana, CA). Recovery of cortisol in the extraction procedure averaged 90%. Final cortisol values were not corrected for incomplete extraction.

Statistical treatment. The two-tailed student's t test[29] was used to determine the significance of the difference between mean values of the conventional and germ-free animals (in vivo and in vitro studies). The significance of difference between the mean values within groups (in vitro studies) was evaluated by the t test for related samples.[29] Values of $P < 0.05$ were considered statistically significant.

Results

Conventional guinea pig studies

Site of ascorbate decarboxylation. *Figure 1* presents results of $^{14}CO_2$ retention following different routes of [1-^{14}C]ascorbic acid administration (Experiment 1). Within 6 hr after injection of [1-^{14}C]ascorbic acid di-

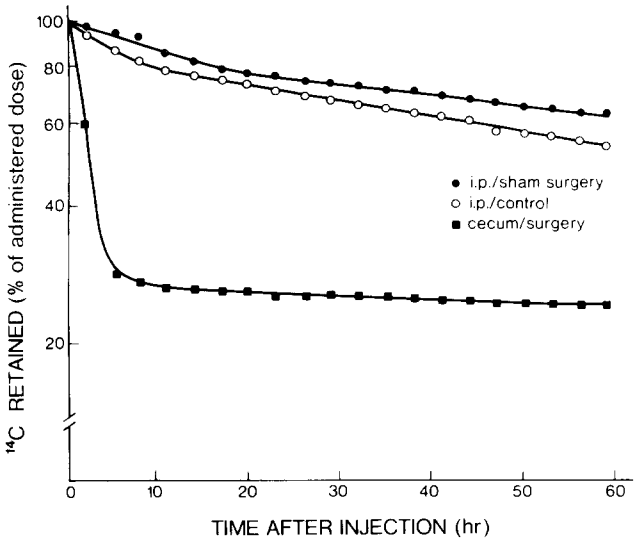

Figure 1 Percent of [1-^{14}C]ascorbic acid retained following different routes of administration.

$$\frac{\text{Administered doses} - \text{expired}^{14}CO_2}{\text{administered dose}} \times 100$$

rectly into the cecum, nearly 70% of the dose was recovered as $^{14}CO_2$. In contrast, only 5% to 8% of the dose was recovered in the same period from sham-operated and control animals injected intraperitoneally. Under aerobic conditions, gram-negative rods from the genera *Proteus*, family *Enterobacteriaceae*, predominated (Experiment 2) in the fecal specimens from conventional guinea pigs. In addition, gram-positive rods (sporeformers and nonsporeformers) and gram-positive cocci were identified in the dam and pup smears, respectively. However, in an anaerobic atmosphere, gram-staining positive rods predominated in the dam, while gram-negative rods were most common in the pup. Gram-negative *Coccobacillus* were also present in the dam.

Based on cell morphology, gram-staining reactions, and oxygen requirements, this study verified the families *Lactobacillaceae*, *Enterobacteriaceae*, *Bacillaceae*, (genera: *Clostridium*) and *Neisseriaceae* (genera: *Veillonella*) in the dam. In the pup, the families *Lactobacillaceae* and *Enterobacteriaceae* predominated. Near complete degradation of ascorbic acid, greater than 99%, was obtained in incubations containing feces from a conventional adult guinea pig (57 μmol/L remaining compared with 7,329 μmol/L broth in autoclaved specimens).

Germ-free studies

In vitro studies. Cecal ascorbate catabolism. *Table 1* presents results of in vitro studies comparing ascorbic acid degradation and vitamin decarboxylation by fresh (microflora, if present) and autoclaved (microflora destroyed) conventional and germ-free cecal contents. Progressive disappearance of ascorbic acid (30% remaining at 48 hr) and marked $^{14}CO_2$ production (73% greater than autoclaved controls) was corroborated by our findings in earlier experiments with fresh cecal inoculum from conventional guinea pigs (Experiment 2). However, cecal contents from germ-free guinea pigs did not destroy ascorbic acid, as determined by the disappearance of unlabeled ascorbic acid from the incubation mixture and by the recovery of $^{14}CO_2$ from [1-^{14}C]ascorbic acid (data not presented). The quantities of ascorbic acid were identical in fresh and autoclaved incubation mixtures containing cecal contents from germ-free guinea pigs.

In vivo studies. *Isotope excretion and ascorbic acid half-life.* Cumulative $^{14}CO_2$ excretion data following [1-^{14}C]ascorbic acid intraperitoneal administration to conventional and germ-free guinea pigs are presented in *Figure 2*. The ^{14}C recovery as respiratory CO_2 was significantly greater in conventional guinea pigs at each 3-hr interval. Germ-free guinea pigs excreted 10% of the [1-^{14}C]ascorbic acid dose as $^{14}CO_2$ within 36 hr, compared to 16% by conventional guinea pigs. Fecal recovery of ^{14}C in both the germ-free and conventional animals was comparable and negligible (< 1%).

Results of systemic ascorbic acid half-life calcula-

Table 1 Cecal degradation of unlabeled ascorbic acid[a]

Inoculum		Status	Cecal contents	Ascorbic acid remaining		
				2 hours	24 hours	48 hours
	(*n*)			mmols per liter broth		
Autoclaved broth	(3)	Control	None	7.4 ± 0.3	6.7 ± 0.4	6.0 ± 0.4
Cecal	(3)	Germ-free	Fresh	6.7 ± 0.4	6.8 ± 0.4	6.1 ± 0.3[c]
Cecal	(3)	Germ-free	Autoclaved	6.9 ± 0.5	6.6 ± 0.2	6.0 ± 0.2
Cecal	(4)	Conventional	Fresh	5.9 ± 1.2	5.2 ± 1.1	1.9 ± 1.1[b]
Cecal	(4)	Conventional	Autoclaved	7.5 ± 0.2	6.9 ± 0.5	6.4 ± 0.2

[a] Initial unlabeled ascorbate concentration was 11.4 mmol/L broth. Values represent mean ± SD.
[b] Significantly different from corresponding autoclaved control, ($P < 0.05$).
[c] Significantly different from corresponding germ-free specimen, ($P < 0.05$).

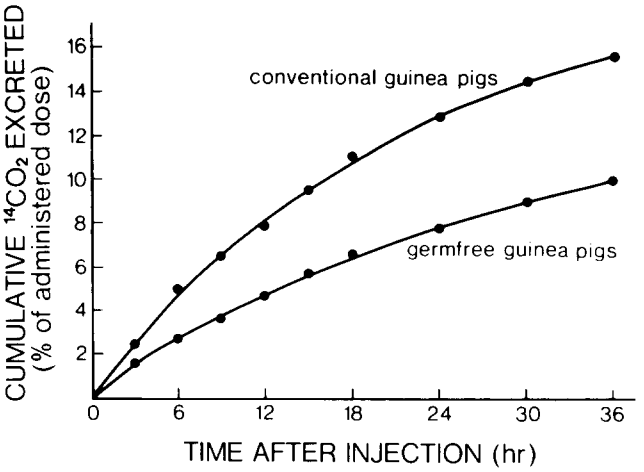

Figure 2 Cumulative excretion of $^{14}CO_2$ following an intraperitoneally injected dose of [1-^{14}C]ascorbic acid in germ-free and conventional guinea pigs.

Table 2 Ascorbic acid half-life (t ½) in conventional and germ-free guinea pigs, as determined from $^{14}CO_2$ excretion curves[a]

	Component	
	Rapid	Slow
	t ½	t ½
Guinea pig	hours	
GERM-FREE (n = 3)	5.9 ± 2.2	328 ± 35[b]
CONVENTIONAL (n = 4)	6.7 ± 2.0	234 ± 16

[a] Values represent mean ± SD.
[b] Significantly different from conventional animals, ($P < 0.05$).

tions from $^{14}CO_2$ excretion data for both groups of animals are given in *Table 2*. The half-life of the rapid component was similar in the two groups. However, the half-life of the slow component was significantly prolonged in germ-free guinea pigs (328 ± 35 hr versus 234 ± 16 hr in conventional guinea pigs, $P < 0.05$).

Growth and organ weights. The body weight of 15-day-old germ-free guinea pigs was considerably less than that of conventional guinea pigs (123 ± 4 g versus 196 ± 3 g, respectively), even though they appeared to consume food in equal, or even greater quantities than the control animals. Organ weights are presented in *Table 3*. The cecum and cecal contents were nearly three-fold heavier and accounted for a greater proportion of body weight in the germ-free guinea pigs as compared with the conventional controls (27% versus 8%, respectively). Spleen, heart, and liver of germ-free animals were significantly smaller, and the adrenal glands significantly larger, than corresponding tissues in conventional animals. In contrast to the decreased weights of other tissues, the adrenal glands of germ-free guinea pigs were significantly enlarged by 37% and plasma cortisol levels were three-fold higher, 2687 ± 72 versus 858 ± 17 nmol/L, ($P < 0.001$), compared with the conventional animals.

Tissue ascorbate concentrations, radioactivity uptake, and total ascorbate content *Tissue Concentrations.* Plasma and tissue ascorbic acid values are given in *Table 4*. Ascorbic acid appeared to be distributed more disproportionately among tissues in germ-free guinea pigs than in those of the conventional animals. Plasma and liver ascorbic acid concentrations were elevated 200% and 150% in germ-free compared with conventional animals, respectively, while adrenal concentrations were depressed nearly 50% in the germ-free group. The splenic concentrations were similar between the groups.

Total Ascorbate Pool Distribution. To quantify the vitamin C organ distribution in germ-free and conventional animals, the total quantity of organ ascorbate was calculated as the tissue concentration multiplied by the organ weight (in grams), (*Table 4*). Because of the inverse relationship of the adrenal (i.e., larger in the germ-free animal), and liver size (smaller in the germ-free animal) and differing ascorbate concentrations between the two animal groups, the total ascorbate pool at these sites was not significantly different between the groups. However, the splenic ascorbate pool was significantly greater in the conventional than in the germ-free animals due to the larger parenchymal size in the former group.

Radioactivity. The time-rate of ^{14}C radioactivity distribution among the tissues is presented in *Table 5*. At both 49 hr and 72 hr following injection of [1-^{14}C]ascorbic acid, plasma and liver radioactivity in germ-free guinea pigs was four-fold and two-fold greater, respectively, than in conventional animals, while adrenal radioactivity was depressed. Bile radioactivity coincided with hepatic tissue accumulation, but was 18-fold greater in germ-free than in conventional animals at 49 hr post-injection and remained elevated at 72 hr. Splenic radioactivity was similar in the two groups at both points in time. Differences between the groups in plasma, liver, and splenic radioactivity paralleled those observed for unlabeled tissue ascorbate concentrations (*Table 4*).

Radioactivity was measured in the total urine sample collected for 36–60 hr after injection of [1-^{14}C] ascorbic acid. Samples for the two groups were matched according to collection times. Urinary excretion of the isotope tended to be greater in germ-free than in conventional guinea pigs (6.8% ± 1.7% and 4.0% ± 0.3% of the injected dose, respectively).

In summary, germ-free guinea pigs excreted one-third less labeled-ascorbate as respiratory CO_2 and showed enhanced ^{14}C tissue uptake and elevated tissue ascorbate concentrations in plasma, liver, and bile compared with their conventional counterparts. Although the total hepatic and adrenal ascorbate pools were similar between the germ-free and conventional animals, the total splenic ascorbic content was dramatically depressed in the germ-free group.

Table 3 Organ weights of germ-free and conventional guinea pigs[a]

Tissue		Conventional (n = 4)		Germ-free (n = 3)	
		Weight	% Body weight[b]	Weight	% Body weight
Spleen	(mg)	309.4 ± 26.9	0.16 ± 0.01	74.2 ± 14.6[c]	0.06 ± 0.01[c]
Adrenal	(mg)	85.0 ± 3.8	0.05 ± 0.01	115.5 ± 12.3[c]	0.09 ± 0.01[c]
Heart	(g)	1.1 ± 0.1	0.55 ± 0.03	0.4 ± 0.1[c]	0.31 ± 0.01[c]
Liver	(g)	9.3 ± 0.6	4.87 ± 0.20	3.5 ± 0.4[c]	2.46 ± 0.32[c]
Cecum	(g)	12.6 ± 1.4	7.46 ± 1.8	33.6 ± 3.8[d]	26.75 ± 5.4

[a] Values represent mean ± SD.

[b] Percent Body Weight = $\frac{\text{Tissue Weight}}{\text{Body Weight}} \times 100$.

[c] Significantly different from conventional guinea pigs, $P < 0.025$.

[d] Significantly different from conventional guinea pigs. $P < 0.01$

Table 4 Plasma, adrenal, liver, and spleen concentrations and total ascorbate content in the conventional and germ-free guinea pigs[a]

		Conventional (n = 4)	Germ-free (n = 3)
Plasma	μmol/L	64.7 ± 6.8	21.6 ± 1.7
Adrenal	mmol/kg	3.3 ± 0.7	5.9 ± 0.7[b]
	μmol/total tissue	0.4 ± 0.1	0.5 ± 0.1
Liver	mmol/kg	2.2 ± 0.2	0.8 ± 0.2
	μmol/total tissue	7.7 ± 1.3	7.5 ± 2.3
Spleen	mmol/kg	2.6 ± 0.5	2.3 ± 0.5
	μmol/total tissue	0.2 ± 0.1	0.7 ± 0.3

[a] Values represent mean ± SD.

[b] Significantly different from germ-free guinea pigs. $P < 0.05$.

Discussion

In a series of experiments conducted in conventional animals and germ-free guinea pigs, using radiolabeled ascorbic acid, we assessed the magnitude and site(s) of influence of enteric microflora on ascorbic acid decarboxylation. These preliminary studies in conventional animals revealed that the magnitude of ascorbate decarboxylation is dependent on the presence of gut microflora, showing enhanced in vivo and in vitro cecal and colonic decarboxylation (*Figure 1*, *Table 1*).

Our major findings suggest that both the gut microflora and endogenous hepatic decarboxylation mechanisms contribute to decarboxylation of the vitamin in conventional guinea pigs. The gut microflora accounts for the approximate 6% differential between the two animal groups in total decarboxylate excretion within 36 hr (*Tables 2–5*). Thus, the 60% greater $^{14}CO_2$ excretion observed in the conventional animals, compared with their germ-free counterparts is, overall, a significant contributor to the ascorbate decarboxylation differential between the two groups. These findings may explain the elevated uptake of radioactive ascorbate and enhanced ascorbate levels in plasma, liver, and bile in the germ-free animals.

Conventional guinea pig studies

The recovery of isotope as $^{14}CO_2$ following ip in conventional animals (*Figure 1*, Experiment 1) followed the same pattern previously reported by us[30] and others.[13,31] These results also demonstrate the rapid and extensive expiration of cecally decarboxylated ascorbate, which explains the low recovery (less than 2%) of $^{14}C_2$ radioactivity in guinea pig feces following administration of [1-^{14}C]ascorbic acid.[30] Furthermore, these findings could resolve the anomalously high rates of $^{14}CO_2$ expiration observed following ip administration of [1-^{14}C]ascorbic acid into the guinea pig as

Table 5 [1-^{14}C]Ascorbic acid tissue distribution in germ-free and conventional guinea pigs[a]

Time after dosing	Animal status	Animal no.	Plasma	Bile	Adrenal	Liver	Spleen
			% dose per liter		% dose per gram		
49 hours	Germ-free	1	198.5	552.4	235	228	NA
	Germ-free	3	156.1	417.9	241	428	261
	Conventional	6	33.3	18.9	500	121	227
	Conventional	7	53.6	35.9	637	82	229
72 hours	Germ-free	2	73.1	63.2	132	127	179
	Conventional	4	15.0	10.9	NA	50	103
	Conventional	5	22.3	24.4	390	67	280

[a] Values represent mean of 2–3 determinations.
NA denotes value not available.

observed by us and others.[14,31] In these instances, an extraordinarily high fraction of the dose was recovered as $^{14}CO_2$ in the first hours after administration, possibly due to communication of the injected isotope with cecal microflora and subsequent decarboxylation of the vitamin.

Farrar and Kent[32] first reported bacterial colonizations in the guinea pig, with *Lactobacillus, Bacillaceae,* and *Streptococcus* predominating over *Enterococcii,* the latter two of which have been shown to decompose ascorbic acid.[19,20] In our study we found similar bacterial colonizations and essentially complete degradation of ascorbic acid in incubations containing feces from both a conventional adult guinea pig and a 6-day old pup. These findings support earlier observations that ascorbic acid is readily metabolized in vitro by many species of enteric bacteria, [15-17], and further suggests colonization of ascorbate-degrading microflora within 6 days after birth in the guinea pig. Human feces also contain bacteria which decompose ascorbic acid.[17] However, only a few bacteria, and only those common to the oral cavity, are found in the human small intestine; colonic microflora are not present in areas as proximal as the upper jejunum.[33] This finding led to an investigation with young, adult germ-free guinea pigs to quantify the relative influences of both the hepatic decarboxylase enzyme system and gut microflora on ascorbate decarboxylation.

Germ-free studies

In the present study, germ-free guinea pigs excreted one-third less of the labeled vitamin as respiratory CO_2 than their conventional counterparts, thus, one-third more of the injected dose of ascorbic acid was retained (*Figure 2*), and presumably available for enzyme cofactor functions. Results of ascorbic acid half-life calculations from $^{14}CO_2$ excretion data for both groups of animals (*Table 2*) suggests a less rapidly catabolizing ascorbic acid pool in our germfree group. Although the site of this pool cannot be determined from the present study, incubation of cecal cultures from both groups of animals demonstrated no bacterial degradation of the vitamin in the germ-free animals (*Table 1*). These results imply that the absence of cecal bacteria may account for this difference in ascorbate decarboxylation.

Plasma and hepatic tissues accumulated and concentrated ascorbic acid, with particular increases in the biliary system, again denoting reduced microbial degradation of the vitamin. Though differences in urinary excretion of labeled ascorbate were not statistically significant between the two groups, the slightly elevated excretion observed in the germ-free group was probably the result of greater availability of plasma ascorbate for clearance. Collectively, it appears that the germ-free state results in even greater tissue ascorbate levels, and, thus, an enhanced vitamin supply for tissue needs, and, potentially lowered dietary ascorbate requirements. These findings may explain the delayed onset of scorbutic symptoms in germ-free guinea pigs observed by Levenson et al.,[18] and the report of Phillips et al.[20] of a more rapid recovery from scorbutic symptoms in germ-free guinea pigs when supplemented with ascorbate.

Incubation and assay of the cecal contents of both animal groups clearly confirmed the presence of decarboxylation in the conventional animals and absence in the germ-free group (*Table 1*). However, the pivotal 6% differential in expired $^{14}CO_2$ between the two groups (at 36 hr post-injection, *Figure 1*) cannot necessarily be explained by the activity of gut microflora alone. Other key factors, including differences in body and tissue weights, and resultant ascorbate intakes, may account for the disparity in ascorbate levels, organ distributions and decarboxylation between the germ-free and conventional animals. Although mean tissue ascorbate concentrations in our conventional guinea pigs were similar to those in a comparable study group,[32] ascorbic acid distribution was unique to the germ-free group. Plasma, hepatic tissue, and biliary accumulations of the vitamin were much greater in the germ-free group, while adrenal ascorbate levels were 50% lower in the germ-free animals compared with their conventional counterparts. The ascorbic acid concentration in the spleen did not differ between the groups. In the present study, blood volume was not measured, but others[34] have demonstrated a significantly smaller plasma volume per unit of body weight in germ-free animals. A reduced plasma volume in germ-free guinea pigs could lead to hemoconcentration of the vitamin and thus explain the elevated plasma and hepatic ascorbate levels observed in these animals.

Interestingly, the splenic vitamin concentrations were similar between the groups contrasting with the differences observed in adrenal and hepatic concentrations. However, the total quantity of ascorbic acid, expressed as mg ascorbate per total tissue, was markedly reduced in the germ-free guinea pigs. This similarity in splenic concentrations, on the one hand, and, differences in hepatic and adrenal concentrations, on the other hand, may be due to the absence of immunologic challenge in the germ-free animals. The results raise interesting questions about tissue mechanisms controlling ascorbic acid transport and retention.

Classic studies in the guinea pig show adrenal tissue depletion of vitamin C, adrenal hypertrophy, and elevation of plasma cortisol levels in response to physiological stress.[35,36] In contrast to the decreased weights of other tissues, the adrenal glands of germ-free guinea pigs in our study were significantly enlarged by 37%, and plasma cortisol levels increased by 300%. Adrenal enlargement and elevated urinary corticoid levels have previously been reported in germ-free rats.[37] Although stress has been shown to enhance ascorbic acid decarboxylation in primates and humans,[38-40] we have previously shown that ACTH injections‡ and immobiliza-

‡Stallings, V.A. Effect of ACTH and epinephrine on ascorbic acid metabolism in guinea pigs fed three levels of ascorbic acid. (1975). M.S. Thesis, Cornell University.

tion stress§ diminish $^{14}CO_2$ expiration in conventional guinea pigs, but at a lower rate than observed here. It is plausible that the elevated plasma cortisol levels in our germ-free guinea pigs contributed to their depressed $^{14}CO_2$ expiration. However, the excretion of 10% of the [1-^{14}C]ascorbic acid dose as respiratory $^{14}CO_2$ in germ-free guinea pigs, even under these conditions, shows that endogenous decarboxylation of the vitamin still remains the major degradation pathway in the guinea pig.

The reduced expiration of $^{14}CO_2$ in our germ-free group may also reflect the fact that body weights at 15 days of age were considerably less than those of the conventional group. Others have demonstrated both similar weight differences between young germ-free and conventional guinea pigs,[20,41] and greater food consumption per unit body weight in germ-free guinea pigs.[41] In the present study, both the germ-free and conventional animals were deprived of the dams milk, fed the same milk formula and sterilized diet, and housed under similar conditions, eliminating these potentially confounding growth influences. The physiological basis for differences in growth rate is unclear, but may reside with complex host-bacterial interactions.

The enlarged cecum of the germ-free guinea pig is a characteristic finding.[25] In our conventional animals, the cecum and its contents accounted for only 8% of total body weight compared to 27% in the germ-free group, along with the remarkable ability to degrade and decarboxylate ascorbic acid (*Table 1*) in the conventional animals. The smaller heart and liver parenchyma in our germ-free guinea pigs agree with observation by Newton and Dewitt,[41] and others.[20,34] The reduced heart size has been linked to a 30% decrease in cardiac output.[34] Theoretically, at least, the smaller liver size may have accounted for the reduced ability of hepatic decarboxylation mechanisms to degrade [1-^{14}C]ascorbic acid in the germ-free animals. Hepatic biliary production, however, did not appear to be impaired in the present study. Biliary accumulation of labeled ascorbic acid was a striking finding in our germ-free group. The marked elevation of bile radioactivity could conceivably have resulted from the depressed decarboxylation by the gut microflora, or enhanced hepatic concentration of ascorbate. It would be intriguing to postulate a quantitative difference in the enterohepatic circulation of the vitamin in the two animal groups. One plausible scenario may involve the continuous attrition of the available ascorbate supply by ascorbate degrading microflora as it courses through the enterohepatic cycle.

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