

In Vitro Dissolution and in Vivo Absorption of Calcium [1-¹⁴C] Butyrate in Free or Protected Forms

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ABSTRACT: Butyrate is a byproduct of microbial carbohydrate fermentation that occurs primarily in the large intestine. When added to feed, butyrate quickly disappears in the upper digestive tract. Because butyrate is important for epithelial cell development, mucosal integrity, and animal growth, an encapsulation technique has been developed that allows for the slow release of butyrate into the small and large intestines. The purpose of this study was to describe the in vitro release of calcium [1-¹⁴C]butyrate, formulated into a slow-release (protected) bead, into water and simulated intestinal fluids and to compare the in vivo absorption and disposition of unprotected versus protected calcium [1-¹⁴C]butyrate in broiler chicks. Formulation of calcium [1-¹⁴C]butyrate into protected beads allowed release of 5.8 ± 0.2 and $3.4 \pm 0.2\%$ of the formulated radiocarbon into water and gastric fluid, respectively, after 2 h of incubation. Beads incubated in gastric fluid for 2 h and subsequently incubated in simulated intestinal fluid released a total of $17.4 \pm 0.8\%$ of the formulated radioactivity. Release of respiratory [¹⁴C]CO₂ after oral dosing of aqueous calcium [1-¹⁴C]butyrate in broiler chicks peaked at $15.2 \pm 5.2\%$ per hour 1.5 h after dosing; in contrast, maximal rates of release in chicks dosed with protected calcium [1-¹⁴C]butyrate occurred 4 h after dosing at $9.0 \pm 3.1\%$ per hour. The data suggested an improved efficacy of protected butyrate delivery to intestinal tissues over nonprotected butyrate. This study confirmed that encapsulation strategies designed to enhance delivery of ingredients to improve intestinal health are effective at prolonging intestinal exposure to butyrate. Encapsulation of such ingredients might benefit the food and feed industries.

KEYWORDS: *encapsulation, butyrate, absorption, broiler*

■ INTRODUCTION

Efficient nutrient use of food animals is accomplished in many ways, including maximizing nutrient digestibility and absorption. Digestibility is optimized through efficient digestive enzyme secretion, a balanced intestinal microbial population, and maintenance of mucosal integrity throughout the length of the gastrointestinal (GI) tract. Mucosal integrity is ensured by a proper balance between mitotic rates of crypt stem cells and tip villi apoptosis.¹ For example, disproportionate rates of villi proliferation are counteracted by an increase in apoptosis, and vice versa, which results in a net gain of villi length.^{2,3} Butyrate, a short-chain fatty acid (SCFA) produced by microbial fermentation primarily in the large intestine, is an apoptosis inhibitor of mucosal cells and could have direct effects on mucosal cell proliferation.^{1,3} As a consequence, butyrate is hypothesized to have marked effects on intestinal morphology and function.^{1,4}

Butyrate transport into mucosal epithelial cells is mediated by a specific monocarboxylate transporter (MCT-1), which is expressed in both the large and small intestinal tissues.³ However, because hindgut microbial fermentation is the major source of butyrate, its production in, and availability to, the small intestine is virtually nonexistent. The addition of butyrate in feed may have a strong influence on the balance between mucosal mitosis and apoptosis, and dietary butyrate is thought to play an important role in small intestinal morphology and efficiency.^{1,4} In addition, the bacteriostatic or bactericidal

mechanisms of organic acids in live animals have been hypothesized to occur indirectly through the maintenance of intestinal epithelial integrity.^{1,5} However, SCFA are very quickly⁶ and quantitatively absorbed from sections of poultry GI tracts cranial to Meckel's diverticulum,⁷ which limits their practical use as feed additives to maintain gastrointestinal tract health.

Delivery of bioactive SCFAs in a form that will allow their release along the length of the small and large intestines should improve epithelial cell integrity and morphology of the whole tissue. Although the efficacy of protected organic acids has been demonstrated,^{1,8} data describing the release and disposition of protected butyric acid in target organisms are not available. Therefore, the objectives of this study were to (1) describe the in vitro release of a labeled short-chain fatty acid, (calcium [1-¹⁴C]butyrate) formulated into a protected bead (MicroPEARL), into water and simulated gastric and intestinal fluids and (2) compare the in vivo absorption and disposition of unprotected and protected calcium [1-¹⁴C]butyrate in broiler chicks.

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MATERIALS AND METHODS

Chemicals. Calcium [^{14}C]butyrate (specific activity of 36.0 mCi/mmol; 99.1% radiochemical purity) and Carbosorb E and PermafluorE liquid scintillation cocktails were purchased from PerkinElmer Life Sciences (Boston, MA). Ecolite(+) liquid scintillation cocktail was purchased from MP Biomedicals (Solon, OH). Hog pancreatin was obtained from Sigma Chemical Co. (St. Louis, MO). Purified water (18 M Ω -cm) was generated in-house using a Millipore water-purifying system. Sodium hydroxide, potassium hydroxide, hydrochloric acid, and sodium chloride were obtained from major laboratory chemical suppliers.

Protected Calcium [^{14}C]Butyrate (Bead) Preparation. Approximately 8 mCi (dissolved in 1.7 mL of water) of calcium [^{14}C]butyrate (0.05 g) was added to 199.95 g of unlabeled calcium butyrate suspended in approximately 250 mL of diethyl ether (J. T. Baker, Phillipsburg, NJ) in a preweighed glass beaker. The suspension was stirred using a magnetic stir bar with slight heating (<34 °C) until the ether was evaporated, and a dry calcium butyrate powder remained having an approximate specific activity of 40 $\mu\text{Ci/g}$. A proprietary excipient (200 g; Kemin Industries) was slowly added to the dried calcium butyrate to form a suspension. Beads containing calcium butyrate and excipient were prepared using a proprietary process for MicroPEARL manufacture. Prepared beads were sized by passing through standardized sieves with only those beads >500 μm , and <2000 μm , retained for experimental purposes. Sized beads were collected and stored in a glass jar at room temperature (20–22 °C).

Radioactivity contained within the beads, on a per unit weight basis, was determined by weighing quintuplicate aliquots (0.025 g) into individual combustion boats (PerkinElmer Life Sciences, Waltham, MA), capping with cellulose wicks and combusting using a Packard model 307 sample oxidizer. Radioactive carbon dioxide was trapped into 9 mL of Carbosorb, and 11 mL of PermafluorE liquid scintillation cocktail were added prior to counting on a Packard model 1900 liquid scintillation counter (LSC). Samples were counted for 10 min each. Background radioactivity was determined by oxidizing quintuplicate samples of unlabeled beads (prepared with unlabeled calcium butyrate).

In Vitro Dissolution in Water. Six 200 ± 20 mg aliquots of calcium [^{14}C]butyrate beads were added to each of six 125 mL Erlenmeyer flasks. Aliquots (50 mL) of warm (37 °C) purified water (18 M Ω -cm) were added to each flask, and flasks were capped with aluminum foil. Flasks were placed in a circulating (60 rpm) water bath (Precision Shallow Form, Thermo Scientific, Waltham, MA) set to 37 °C. Aliquots (100 μL) of water were removed from each flask at 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0 h and placed in 7 mL LSC vials to which 5.5 mL of Ecolite was added. The net volume of water in each Erlenmeyer flask was held constant by adding back 100 μL of water at each sampling time. Radioactivity in each sample was quantified over a 10 min period using LSC.

In Vitro Dissolution in Simulated Gastric and Intestinal Fluids. Six 200 ± 20 mg aliquots of calcium [^{14}C]butyrate beads were added to six labeled 125 mL Erlenmeyer flasks. To each flask was added 50 mL of warmed (37 °C) simulated gastric fluid (SGF), and flasks were placed in a shaking (60 rpm) water bath at 37 °C. SGF (pH 1.2) was prepared according to the method of Maltais et al.⁹ Flasks were capped with aluminum foil, and 100 μL aliquots of each incubation supernatant were collected into LSC vials at 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0 h; LSC contents were suspended with 5.5 mL of Ecolite LSC fluid. SGF volume in each Erlenmeyer flask was kept constant by replacing each aliquot volume with warm SGF fluid. At the end of the 2 h collection period, the beads were separated from the acidic supernatant by filtration (Whatman 541 paper) using Büchner flasks and funnels. Beads were then transferred to previously weighed and labeled 125 mL Erlenmeyer flasks to which 50 mL of 37 °C simulated intestinal fluid (SIF; pH 7.5,⁹ but with no added bile salts) was added. Flasks were incubated at 60 rpm and 37 °C with 100 μL aliquots removed for LSC at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 h relative to the start of the incubation. Samples were counted for 10 min periods.

Water and SIF dissolution data were fit to one- or two-phase exponential association curves, and dissolution rate constants and associated half-lives were calculated using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA). In addition, data were subjected to analysis of variance with repeated measures, and differences in selected treatment means were estimated using Bonferroni's multiple-comparison test.

In Vivo Disposition Study. *Control Article.* Nonprotected calcium [^{14}C]butyrate, the control agent, was prepared by suspending 9.98 g of unlabeled calcium butyrate into a 100 mL graduated cylinder with ice-cold water with 402 μCi (0.02 g) of radiolabeled calcium butyrate. A small magnetic stir rod was placed in the cylinder, and the suspension was mixed thoroughly at dosing (described below).

Animals. A study protocol was approved by the Institutional Animal Care and Use Committee prior to the initiation of the animal work. Jumbo Cornish \times Rock broiler chicks (pullets and cockerels) were purchased from a commercial hatchery and delivered to the USDA facilities as day-old chicks. Birds were housed in a single 1.6 \times 1.2 \times 1.3 m (L \times W \times H) concrete-floored pen with wood shavings to absorb wastes. The pen was equipped with watering devices, feeders, and duplicate 250 W incandescent lights suspended approximately 0.17 m above the floor as a heat source. Chicks were provided ad libitum access to a commercial unmedicated chick starter-grower

Table 1. Guaranteed Analysis of Nonmedicated Starter-Grower Feed^a

item	minimum (%)	maximum (%)
crude protein	18.0	
lysine	0.85	
methionine	0.30	
crude fat	3.00	
crude fiber		5.00
calcium	0.95	1.15
phosphorus	0.70	
salt	0.10	0.45
sodium	0.19	0.21

^aGrain products, plant protein products, processed grain byproducts, vitamin A supplement, vitamin D supplement, riboflavin supplement, niacin supplement, D-calcium pantothenate, pyridoxine hydrochloride, folic acid, menadione sodium bisulfate complex (source of vitamin K activity), thiamin mononitrate, biotin, L-lysine, calcium carbonate, dicalcium phosphate, monocalcium phosphate, salt, manganous oxide, manganous sulfate, ferrous sulfate, choline chloride, copper sulfate, zinc oxide, zinc sulfate, ethylenediamine dihydride, sodium selenite.

ration (Table 1) and water during the grow-out period. Temperature averaged 23.8 ± 0.6 °C (range 22.2–23.9 °C), whereas humidity averaged $9.2 \pm 2.3\%$ (range 8–18%).

Study Design. A completely randomized design with main effects of calcium butyrate treatment (unprotected and protected) and sampling time was used (Table 2). Four chicks were randomly assigned to treatment each dosing day, with the experiment replicated on five consecutive days for a total of 20 chicks per treatment (4 chicks per treatment per day). Within-day, a single chick from each treatment was euthanized 2, 4, 8, and 12 h after dosing so that each treatment-time period combination was replicated five times. Control chicks, not exposed to calcium butyrate, were maintained as a source of control tissues.

Dosing. Chicks (12–16 days of age; 234 ± 57 g) were weighed immediately prior to dosing. Aliquots (0.1251 ± 0.0006 g) of protected calcium [^{14}C]butyrate beads (2.30 ± 0.01 μCi), containing 0.0626 ± 0.0003 g of calcium butyrate, were weighed into a series of size 0 gelatin capsules (EMS, Hatfield, PA). Aliquots (0.626 mL; 0.0625 g; 2.40 μCi) of nonprotected, aqueous, calcium [^{14}C]-

Table 2. Replication of Treatments and Tissue Collection Times across Study Days

collection time (h)	treatment	weekday					total
		Mon	Tues	Wed	Thu	Fri	
2	protected	1	1	1	1	1	5
	unprotected	1	1	1	1	1	5
4	protected	1	1	1	1	1	5
	unprotected	1	1	1	1	1	5
8	protected	1	1	1	1	1	5
	unprotected	1	1	1	1	1	5
12	protected	1	1	1	1	1	5
	unprotected	1	1	1	1	1	5
per day totals		8	8	8	8	8	40

butyrate, suspended in ice-cold water, were pipetted into size 0 gelatin capsules immediately prior to dosing. At dosing, capsules were lightly lubricated with vegetable oil and inserted into the oral cavity. Capsules were gently massaged until each was felt entering the crop. Immediately upon dosing, each chick was placed into an individual 36 × 23 × 23 cm (H × L × W) Plexiglas metabolism cage and sealed with a Plexiglas lid.

[¹⁴C]CO₂ Collection and Measurement. Air was drawn through each crate with a single-head diaphragm pump (50.8 cm maximum vacuum; Thermo Fisher Scientific, Waltham, MA) connected with Tygon tubing. An air flow meter (Dakota Instruments, Orangeburg, NY) at each metabolism cage restricted the air flow to 0.5 L/min. Traps containing 300 mL of 1 M KOH¹⁰ were placed in-line between the cages and pumps so that the sequential route of air flow was from the atmosphere through the crate, KOH traps, flow meter, and pump. Air drawn through the metabolism crates was bubbled through course-fit, micro gas dispersion tubes (Chemglass Life Sciences, Vineland, NJ) placed in the KOH traps; KOH in each trap was replaced at 4 h intervals. Aliquots (1 mL) of KOH were removed from traps at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 10, and 12 h after dose administration. Aliquots were placed in 20 mL LSC vials and diluted with 15 mL of Ultima Gold LSC fluid (PerkinElmer) for quantitation of radiocarbon. Background radioactivity was determined using three to five blank vials containing 1 mL of control 1 M KOH.

Excreta Collection. Excreta were collected from each chick into preweighed, 384 mL Whirl-Pak bags (Nasco, Fort Atkinson, WI). Bags were secured to the bottom of a pyramidal collector plate placed within each metabolism crate. Excreta were collected by rinsing the cage flooring and the collector with water. Samples were frozen (−20 °C) until analysis.

Tissue Collection. Chicks ($n = 5$ per time period) were euthanized by decapitation at 2, 4, 8, and 12 h after dosing. Blood was collected into previously weighed, heparinized tubes (Becton-Dickinson, Franklin Lakes, NJ) by gravity flow immediately after decapitation. Gastrointestinal tracts were removed and dissected into three sections consisting of the upper (crop, proventriculus, and gizzard), middle (duodenum, jejunum, and ileum), and lower (ceca, colon, and cloaca) tracts. The pancreas and mesenteric tissue was excised from the duodenal loop at dissection and placed with the “carcass” fraction. Luminal contents in the upper, middle, and lower tracts were rinsed with saline into respective Whirl-Pak bags, and the contents were mixed using a Stomacher 80 (Seward, Sussex, U.K.) at high speed (1 min) prior to being weighed and frozen (−20 °C). Upper, middle, and lower tract tissues were weighed, placed in respectively labeled, individual bags, and frozen until analysis. Livers were removed, weighed, placed into individual Whirl-Pak bags, and frozen (−20 °C). All other portions of the carcass were combined into a “carcass” fraction, which was weighed, placed into a plastic jar, and frozen (−20

°C) until analysis. The duration for each dissection was approximately 30 min.

Tissue Processing. Upper, middle, and lower GI contents were thawed prior to weighing in preparation for radiochemical analysis. Blood samples were thawed and vortexed prior to weighing. Tissues were homogenized with dry ice¹¹ using a Waring blender. Dry ice was allowed to sublime from the powdered tissues prior to weighing and oxidation analyses. The remainder of the carcass fraction was partially thawed and diced into pieces of approximately 8 cm³ prior to grinding with an LEM (Harrison, OH) electric meat grinder. Excreta samples were mixed prior to weighing for radiochemical analysis.

Radiochemical Analyses. Triplicate aliquots (0.1 g) of blood; excreta; upper, middle, and lower GI contents and tissues; liver; and the remainder of the carcass were weighed into combustion thimbles, capped with cellulose wicks, and combusted. Radioactive carbon dioxide was trapped in 6 mL of Carbosorb E trapping solution and diluted with 12 mL of Permafluor E+ prior to quantification by LSC. Radiocarbon was quantified using calibrated Packard model 1900 LSC counters, and quench was corrected using internally stored quench calibration curves. Samples were counted for a minimum of 10 min each.

Limit of Quantitation (LOQ). Background radioactivity was determined for each tissue type by combusting triplicate control tissues with each sample run. Typically six runs were needed to complete a tissue analysis. The average dpm value for all blank replicates ($n = 18$) within a tissue type was defined as background for the tissue; the LOQ was defined as the sum of the mean background and three standard deviations of the mean background (LOQ = BKGRD + 3(SD)).¹² Samples that contained dpm values between the background and the LOQ were said to have detectable, but not quantifiable, residues.

Statistical Analyses. Within fraction (excreta, respiratory gas, individual tissues, etc.) data were analyzed by two-way analysis of variance (ANOVA) with treatment and sampling time as the main effects. Because the equal variance assumption was not met (using the Spearman rank correlation), log or square-root transformations were completed for all data sets and ANOVA were run on transformed data. Treatment differences within time period were determined using the Holm–Sidak test, performed within the SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA) analysis platform. For the lower intestinal tissue data set, transformations were not effective at equalizing variance. In this instance, untransformed means of protected and unprotected activity within the 2, 4, and 12 h time periods were compared using Student's *t* test. The 8 h lower intestinal tissue data required a square-root transformation prior to the equal variance assumption being met; the transformed 8 h data were compared using a *t* test. Within treatment, it sometimes was useful to determine whether means increased or decreased across sampling hours. In such instances, least-squares linear regression of time points within treatment was calculated; *F* tests were used to determine whether best fit slopes were different from zero.

RESULTS

In Vitro Dissolution Tests. Figure 1 (open circles) shows the dissolution of protected calcium [¹⁴C]butyrate beads in water at 37 °C during a 2 h incubation period. Data fit well to a two-phase association curve ($r^2 = 0.9450$) with rapid and slow release half-lives estimated to be 3.5 and 36.7 min, respectively, on the basis of a release plateau of $5.8 \pm 0.3\%$ of total radioactivity within the beads. These release half-lives likely represent dissolution of calcium butyrate from surface and embedded compartments within the beads, respectively. Total release of calcium [¹⁴C]butyrate at 0 min was significantly ($P < 0.001$) less than the mean release of calcium [¹⁴C]butyrate at 60 min; mean release at 60 min was also significantly ($P < 0.001$) less than at 120 min. However, the mean release of calcium butyrate from the beads at 90 and 120 min did not differ ($P > 0.05$).

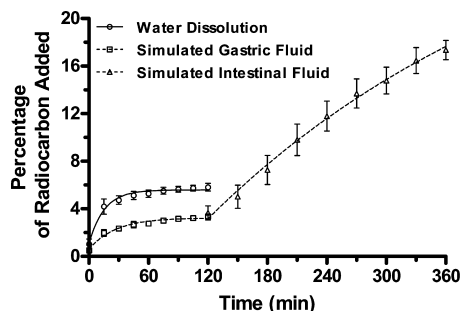


Figure 1. In vitro dissolution of protected calcium $[1-^{14}\text{C}]$ butyrate in water (circles), simulated gastric fluid (squares), and simulated intestinal fluid (triangles). The same beads were used sequentially in the simulated gastric and intestinal fluids. Symbols represent the mean \pm standard deviation of six observations per time point.

Data describing the sequential dissolution of protected calcium $[1-^{14}\text{C}]$ butyrate beads in simulated gastric and intestinal fluids (Figure 1) were fit to one-phase association curves having r^2 values of 0.952 and 0.891, respectively. Estimated time for release of $[1-^{14}\text{C}]$ butyrate in gastric fluid, based on a plateau release of $3.5 \pm 0.2\%$ of the total activity, was 18.3 min. In SIF, release was modeled to plateau at $29.7 \pm 7.4\%$ of the total bead radioactivity with a release half-life being 253 min. Total release of calcium $[1-^{14}\text{C}]$ butyrate into gastric fluid at 60 min was greater than at 0 min ($P < 0.001$). Likewise, release at 120 min was greater than release at 60 min ($P < 0.001$) and 90 min ($P < 0.05$). In SIF, release of radioactivity from beads differed ($P < 0.001$) at successive time points, indicating that within the 4 h intestinal fluid incubation, significant quantities of radioactive butyrate were continually being released.

In Vivo Distribution of Radiocarbon. Table 3 provides the days of age, weights, and dose per kilogram of body weight

Table 3. Bird Weights and Doses (Means \pm SD) of Calcium Butyrate by Study Day^a

study day	age (days)	body wt (g)		dose (mg/kg BW)	
		unprotected	protected	unprotected	protected
1	12	194 \pm 68	192 \pm 59	355 \pm 126	346 \pm 85
2	13	193 \pm 23	201 \pm 23	328 \pm 39	315 \pm 36
3	14	263 \pm 45	243 \pm 32	243 \pm 42	261 \pm 33
4	15	250 \pm 42	248 \pm 82	257 \pm 50	273 \pm 80
5	16	296 \pm 63	264 \pm 38	220 \pm 48	240 \pm 32

^aSignificant ($P < 0.05$) effects of study day on body weight and dose occurred due to the fact that birds were rapidly gaining weight. However, there were no differences ($P > 0.05$) between treatments with regard to bird weights or doses on any given study day.

(BW) across study day for chicks. Because birds were given fixed doses of calcium $[1-^{14}\text{C}]$ butyrate, and because they were rapidly growing during the study period, the dose on a mg/kg BW basis decreased ($P < 0.05$) with study day. However, within a given study day, no treatment differences ($P > 0.05$) in BW or dose per kilogram of BW occurred. Overall, the average dose per mg/kg BW did not differ between treatments ($P = 0.79$).

Figure 2 shows the cumulative and fractional expiration of $[^{14}\text{C}]\text{CO}_2$ from broiler chicks dosed with unprotected and protected calcium $[1-^{14}\text{C}]$ butyrate. Respiratory radioactivity was trapped into KOH solutions,¹⁰ and sample aliquots were

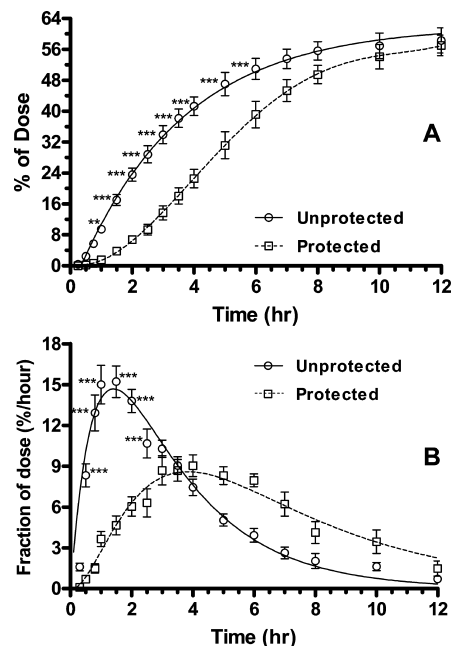


Figure 2. Cumulative (A) and fractional (B) release of $[^{14}\text{C}]\text{CO}_2$ in birds orally dosed with unprotected (circles) or protected (squares) calcium $[1-^{14}\text{C}]$ butyrate. Data are the mean \pm SEM of 20 birds at time points through 2 h; 15 birds at time points from 2 to 4 h; 10 birds at time points from 4 to 8 h, and 5 birds at time points from 8 to 12 h. Points marked with asterisks differ (**, $P < 0.01$; ***, $P < 0.001$) between protected and unprotected at indicated time points.

collected from all birds ($n = 20$ per treatment) at 0.25, 0.5, 0.75, 1, 1.5, and 2 h; from 15 birds at 2.5, 3, 3.5, and 4 h; from 10 birds at 5, 6, 7, and 8 h; and from 5 birds at 10 and 12 h. The sequential decline in sample numbers reflects the fact that five birds from each treatment were euthanized at 2, 4, 8, and 12 h. Cumulative respiratory elimination of radiocarbon was greater ($P < 0.05$) from 1 to 6 h in birds treated with unprotected calcium $[1-^{14}\text{C}]$ butyrate than from birds treated with protected calcium $[1-^{14}\text{C}]$ butyrate beads, whereas fractional rates of $[^{14}\text{C}]\text{CO}_2$ respiration were greater in birds treated with unprotected butyrate ($P < 0.01$) at 0.5, 0.75, 1, 1.5, 2, and 2.5 h after dosing. Although fractional rates of $[^{14}\text{C}]\text{CO}_2$ respiration were numerically greater from 3 h onward in broiler chicks provided protected calcium $[1-^{14}\text{C}]$ butyrate beads, the differences were not statistically significant ($P > 0.05$), most likely due to the decreasing numbers of observations at the later time points. Collectively, the respiratory data indicated that apparent absorption of calcium butyrate was delayed in birds treated with protected beads relative to absorption in birds treated with unprotected butyrate. Although cumulative elimination of $[^{14}\text{C}]\text{CO}_2$ did not differ between the treatments after the seventh hour of the study, the rate of $[^{14}\text{C}]\text{CO}_2$ elimination was clearly greater in birds provided unprotected butyrate.

Table 4 shows the overall disposition of radioactivity in broiler chicks treated with unprotected or protected $[1-^{14}\text{C}]$ calcium butyrate. The upper (crop, proventriculus, gizzard) and middle (duodenum, jejunum, ileum) GI tract contents of birds treated with butyrate beads contained greater ($P < 0.05$) quantities of radioactivity at 2 and 4 h than tracts of birds treated with unprotected butyrate; there were no differences ($P > 0.05$) in radioactivity within contents of upper and middle tracts at 8 and 12 h. Radioactivity in lower GI tract contents

Table 4. Disposition of Radioactivity Dosed as an Unprotected Aqueous Suspension of Calcium [$1\text{-}^{14}\text{C}$]Butyrate (UnPr) or as Protected Calcium [$1\text{-}^{14}\text{C}$]Butyrate (Prot)^a

fraction	time after dosing															
	2 h				4 h				8 h				12 h			
	UnPr (%)	Prot (%)	SEM	P	UnPr (%)	Prot (%)	SEM	P	UnPr (%)	Prot (%)	SEM	P	UnPr (%)	Prot (%)	SEM	P
GI contents ^b																
upper	4.5	38.6	6.5	***	7.2	18.2	5.1	*	0.1	1.6	0.7	NS	0.1	0.1	<0.1	NS
middle	0.6	4.4	1.0	**	0.4	5.2	1.2	**	0.2	0.2	<0.1	NS	0.2	0.3	<0.1	NS
lower	0.3	0.8	0.3	NS	0.2	0.4	0.1	NS	0.1	0.2	<0.1	NS	0.2	0.1	<0.1	NS
total GI content ^c	5.4	43.8	6.9	***	7.9	23.8	5.2	**	0.5	2.0	0.7	NS	0.4	0.5	0.1	NS
excreta	1.4	0.5	0.3	*	2.6	3.6	0.7	NS	2.9	7.6	1.1	NS	3.0	11.2	1.6	*
unabsorbed ^d	6.8	44.2	6.7	***	10.4	27.5	4.8	***	3.4	9.6	1.2	**	3.4	11.7	1.6	***
GI tissues																
upper	2.6	2.5	0.3	NS	2.2	1.9	0.2	NS	2.0	1.4	0.2	NS	2.4	1.1	0.2	***
middle	2.2	1.8	0.2	NS	1.9	2.6	0.2	NS	1.7	2.1	0.1	NS	1.9	2.0	0.1	NS
lower	0.6	0.2	0.1	**	0.4	0.2	<0.1	**	0.4	0.3	<0.1	**	0.4	0.2	<0.1	***
liver	7.4	3.7	1.0	**	4.3	5.0	0.7	NS	2.0	3.0	0.3	NS	2.2	2.3	0.3	NS
blood	0.5	0.1	<0.1	***	0.3	0.2	<0.1	NS	0.3	0.2	<0.1	NS	0.3	0.2	<0.1	**
carcass	16.2	6.9	1.7	***	13.8	9.7	1.3	NS	13.4	10.7	1.1	NS	14.9	11.0	1.1	NS
respiratory	25.3	8.7	3.1	***	39.9	22.0	4.1	**	57.6	51.8	2.1	NS	58.3	57.1	2.0	NS
absorbed ^e	54.7	23.9	5.6	***	62.8	41.7	5.1	**	77.3	69.5	2.2	NS	80.4	73.9	1.8	NS
total recovery ^f	61.5	68.1	2.0	*	73.2	69.2	1.8	NS	80.7	79.1	1.7	NS	83.7	85.6	1.4	NS

^aData are expressed as the mean and standard error of the mean of five broiler chicks per treatment within each time period. Significant differences within time period and fraction are indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, $P > 0.05$. ^bUpper tract contents contained pooled digesta from the crop, proventriculus, and gizzard; middle tract contents contained digesta from the duodenum, jejunum, and ileum; lower tract contents contained digesta from the ceca, colon, and cloaca. ^cTotal GI contents, summation of radioactivity in upper, middle, and lower GI tract contents. ^dApparent percentage of dose that was not absorbed; summation of radioactivity in GI tract contents and excreta. The fraction also contains radioactivity present in urine, which is excreted with fecal matter in avian species. ^eApparent absorption, summed percentage of radioactivity in GI tissues, liver, blood, carcass, and respiratory gases. ^fSum of radioactivity present in GI contents, excreta, tissues, and respired gases.

never exceeded 1% of the dose, regardless of treatment, at 2, 4, 8, and 12 h. Radioactivity in excreta was greater in birds treated with unprotected butyrate at 2 h ($P < 0.05$), but at 12 h it was greater ($P < 0.05$) in the excreta of birds treated with butyrate beads. Radioactivity in excreta from birds treated with butyrate beads was numerically greater at 4 and 8 h, but means were not statistically different ($P > 0.05$).

Assuming that urinary excretion of radioactivity contributed relatively minor quantities of radioactivity^{13,14} to the total excretory radiocarbon output, one can estimate unabsorbed activity by summing the activity present in the upper, middle, and lower gastrointestinal contents with excreta. Unabsorbed activity was significantly greater ($P < 0.01$) in birds dosed with protected calcium [$1\text{-}^{14}\text{C}$]butyrate at all sampling times relative to birds receiving the unprotected dose.

In general, upper and middle GI tract tissue radioactivity did not differ ($P > 0.05$) between treatments. Lower GI tract radioactivity was always greater ($P < 0.01$) in birds treated with unprotected butyrate than in birds dosed with butyrate beads. For liver, blood, and carcasses, few treatment differences were observed after 2 h; at the 2 h sampling point liver, blood, and carcasses of birds treated with unprotected butyrate always contained more ($P < 0.01$) radioactivity than those tissues from birds treated with protected butyrate. Such results reflect the rapid absorption of radioactivity in birds treated with the aqueous calcium butyrate suspension.

Birds treated with control butyrate respired more ($P < 0.01$) radioactivity than birds treated with protected butyrate at 2 and 4 h. By 8 h, differences in respiratory activity were not apparent ($P > 0.05$), and this similarity was maintained at 12 h.

Absorbed activity was calculated as the sum of all tissue and respiratory radioactivities. At 2 and 4 h, birds dosed with unprotected butyrate had absorbed greater ($P < 0.01$) quantities of radioactivity than birds dosed with protected butyrate (54.7 vs 23.9% at 2 h and 62.8 vs 41.7% at 4 h); no differences in absorbed activity ($P > 0.05$) were apparent at 8 and 12 h.

Total recovery of radioactivity was similar ($P > 0.05$) between treatments at 4, 8, and 12 h, but was slightly greater ($P < 0.05$) for birds dosed with protected butyrate at 2 h than for birds treated with unprotected butyrate. Regression analysis indicated a linear relationship ($P < 0.001$) between total recovery and sampling time for both treatment groups.

DISCUSSION

This study describes the in vitro release of a labeled SCFA, (calcium [$1\text{-}^{14}\text{C}$]butyrate), formulated into a slow-release bead (MicroPEARL) and compares the in vivo absorption and disposition of unprotected versus protected calcium [$1\text{-}^{14}\text{C}$]butyrate in broiler chicks.

In vitro dissolution studies using water and SGF clearly demonstrated that the sustained release beads provided a barrier for release of radioactivity into aqueous solution. In water, <6% of the total amount of [$1\text{-}^{14}\text{C}$]butyrate contained in suspended beads was in solution after a 2 h incubation. Even less radioactivity (<4%) was released into SGF, no doubt due to the fact that the pK_a (4.8) of butyrate dictates that it would exist primarily in the fully protonated form at pH 1.2. At the higher pH of the SIF (pH 7.5) butyrate solubility increased markedly relative to water; nevertheless, total release of

radioactivity in the sequential 2 and 4 h incubations in SGF and SIF, respectively, was about 18% of the total activity added.

In the 6 h incubation with SGF <20% of the total radioactivity within the beads was released; unsolubilized beads were clearly present in the incubation matrix at the end the incubation. Although stirred, the simulated intestinal incubations lacked the physical contact of the bead with intestinal wall and chyme that would be present in the intestinal tract of a live animal. In addition, the milieu of gastrointestinal bacteria that could accelerate bead dissolution was absent from the SIF. Nevertheless, the data indicated that dissolution into aqueous solution under acidic conditions (pH 1.2) was less than dissolution in water and that dissolution was accelerated in SIF. These data suggest that under physiological conditions the release and utilization of protected butyrate could occur at sites caudal to the proventriculus and be available to a dosed animal.

Because respiratory elimination of [^{14}C]CO₂ could not have occurred without prior GI absorption of butyrate, respiratory elimination of radioactivity serves as an excellent marker for butyrate absorption. Data presented in Figure 2B suggest that peak absorption of unprotected calcium butyrate occurred between 1 and 1.5 h after dosing and confirmed that protection of butyrate in beads delayed maximal absorption until 3–4 h post dosing. Half-lives of digesta passage in 14-day-old broiler chicks consuming barley-based diets were 5.5–8.9 h and were 3.4–4.8 h in 6-week-old cockerels.¹⁵ Consistent with Almirall and Esteve-Garcia,¹⁵ lower tract contents, total GI tract contents, and excreta data (Table 4) suggest that in the chickens used for this study, passage rates were 4–8 h. Passage rate data from this trial are also within expected norms as reported in previous studies.¹⁶ For example, lower tract contents always contained <1% of the dosed radioactivity, regardless of time point. Excreta analysis established that in birds treated with protected butyrate, excretory elimination of activity at 8 h was 211% of the 4 h period value and was 147% of the 8 h value at 12 h; during the same time intervals, excretory radioactivity of birds dosed with unprotected butyrate increased only 11.5 and 3.4%, respectively. To have beneficial effects in the intestinal tract, sustained release of butyrate from beads should occur during transit at all points along the length of the GI tract. Radioactivity contained within the protected beads in this study was clearly demonstrated to have been retained longer in the GI tract as evidenced by its greater appearance in fecal matter.

Total butyrate absorption was greater ($P < 0.01$) in birds treated with unprotected butyrate than in birds treated with protected material at 2 and 4 h, but by 8 and 12 h, total absorption of radioactivity was not statistically different ($P > 0.05$; Table 4) between the two experimental treatments. Whereas these data indicated that protection of butyrate delayed absorption, they also indicated that protection did not affect overall butyrate availability. Data from GI contents and unabsorbed butyrate indicate that upper and middle butyrate contents were significantly ($P < 0.05$) higher in chicks treated with protected material. Thus, the availability of butyrate in the intestinal lumen for utilization by the epithelial cells was greater with protected butyrate. Unlike the *in vitro* dissipation studies in which the total amount of butyrate released into solution was modest, the *in vivo* studies indicated that butyrate on the protected beads was available to the whole animal and that a majority of the butyrate associated with beads was available for metabolism.

Total recoveries of radioactivity were lower than what would typically be desirable, ranging from 61.5% (unprotected treatment at 2 h) to 85.6% (protected treatment at 12 h). Although the source of radioactive loss is not known, the two main sources were probably (1) loss of gaseous radioactivity from the metabolism crates at slaughter and (2) loss of gaseous radioactivity from carcass components during dissection. The metabolism crates had a volume of 19 L; with an airflow rate of 0.5 L/min, 19 L represents approximately 32% of the total air volume at the 2 h sampling time [$19 \text{ L}/(120 \text{ min} \times 0.5 \text{ L/min}) = 0.317$]. Because this air volume was not passed through the CO₂ traps at the end of each collection period, [^{14}C]CO₂ present in the closed system at slaughter would be lost, reducing overall recovery. Two pieces of circumstantial evidence support this supposition. First, the linear ($P < 0.001$) improvement of recovery with time, regardless of treatment, indicates that the intact system was capable of trapping radiocarbon with >80% efficiency. Second, slopes and intercepts obtained from the least-squares regression of total recovery and time did not differ ($P > 0.05$) between treatments, indicating that treatment was not a variable in the recovery of radioactivity. When treatment means were compared (Table 3), the only statistical difference in total recovery occurred at 2 h: recovery was greater ($P < 0.05$) for the protected butyrate treatment than for the unprotected treatment. Such results would be expected at the earliest time point because the protected butyrate had relatively greater quantities of radiocarbon sequestered and unavailable for gaseous loss.

The second major source of radioactivity loss was likely from tissues themselves during dissection. Each bird took approximately 20–30 min to euthanize, bleed, and dissect. The most time-consuming process was dissecting the upper, middle, and lower GI tracts and stripping their contents. Due to the apparent rapid rate of metabolism of butyrate once it was liberated from the protected beads, cumulative losses from tissues could be significant.

Collectively these data strongly suggest that protected butyrate has a greater propensity to interact with larger portions of the chicken's GI tract than unprotected or aqueous suspensions of butyrate. Additionally, the data suggest a mechanism for the observed improved efficacy of protected organic acids relative to nonprotected organic acids; that is, protection affords a greater availability of organic acid to larger segments of the intestinal tract or to microbial populations therein. Several studies have demonstrated that butyrate has a positive direct influence on intestinal mucosa health and microbial populations within specific segments of the GI tract.^{1,8,17–19} The present study confirms that encapsulation technologies may have application to feeding strategies directed to the management and improvement of intestinal health. For example, use of protected SCFA in livestock diets may allow the reduction of antibiotic growth promoters while reducing bacterial resistance or use in postnatal diets to allow optimal development of the intestinal tract mucosa.

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Notes

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