Cobalt (III)-EDTA dissociates and chromium (III)-EDTA is slightly more stable under in vitro reducing conditions comparable to those in the rumen

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ABSTRACT

Ideal digesta markers used for feeding studies are inert, unabsorbable, and move with the digesta they are intended to mark. Both chromium (III) and cobalt (III) salts of EDTA (CrEDTA and CoEDTA, respectively) are used as markers of liquid digesta in dairy cattle research. A small portion is absorbed and excreted in urine, but the markers are assumed to remain unreactive and as inert salts in the digesta and animal. The degree to which these colored salts remain bound in solution can be estimated through spectrophotometric measurement at their wavelength (λ) of peak absorbance. The objective of this in vitro study was to evaluate whether CrEDTA and CoEDTA dissociate under reducing conditions that could be experienced in the rumen. In a completely randomized design with 2 replicate analytical runs and samples in duplicate within run, approximately 26 mg/L Cr from CrEDTA or Co from CoEDTA was incubated in a 26-mL reaction volume containing 20 mL of Goering and Van Soest medium without tryptone, 3 mL of CoEDTA or CrEDTA solutions, or water (reagent blanks), and 3.0 mL of a combination of distilled water with 0, 0.25, 0.50, 0.75, or 1.00 mL of reducing solution (RedSol). After incubation for 0.5 h at 39°C, absorbance was read at $\lambda = 535, 465,$ and 560 nm, the peak λ for EDTA salts of Co(III), Co(II), and Cr(III), respectively. Mean reagent blank values were subtracted from CoEDTA and CrEDTA data. The absorbance data at peak λ were analyzed by marker in models that included RedSol with analytical run as a random variable. Contrasts were used to detect linear through quartic effects of RedSol. Samples with RedSol had redox potentials of -250 to -328 mV, which are within the range of reported ruminal measures. As RedSol increased, CoEDTA showed a linear decline of 75% in ABS at 535

nm and a quadratic 4-fold increase followed by a 60% decline at 465 nm. These responses indicate a reduction of Co(III) to Co(II) and subsequent dissociation of Co(II)EDTA. The absorbance of CrEDTA at 560 nm showed a tendency for an 8% linear decrease as RedSol increased. Wavescans from $\lambda = 330$ to 700 nm showed CrEDTA retaining its characteristic 2-peak pattern as RedSol increased, whereas CoEDTA curves deformed entirely. We conclude that CoEDTA is not a stable, inert digesta marker under reducing conditions achievable in the rumen and is therefore unsuitable for use in studies with ruminants. Reexamination of the suitability of available liquid digesta markers is advised.

Key words: CoEDTA, CrEDTA, reducing conditions, liquid digesta, marker

INTRODUCTION

Studies with lactating dairy cattle have used markers for liquid digesta to estimate liquid passage, rumen volume, and ruminal substrate disappearance (Weisbjerg et al., 1998; Shingfield et al., 2008). Ideal digesta markers for use in feeding studies are indigestible, unabsorbable, move with the portion of the digesta they are intended to mark (Faichney, 1993), and should be accurately and specifically measurable. However, all markers have limitations. The EDTA salts of chromium (III) (**CrEDTA**) and cobalt (III) (**CoEDTA**) were developed as liquid markers to allow greater specificity of analysis, with CoEDTA introduced as a non-Cr-containing material to facilitate use of multiple markers (Udén et al., 1980). They are among the most commonly used liquid markers. Small portions of both markers are excreted in urine (Shingfield et al., 2008), which violates the criterion of digesta markers being unabsorbable. A greater concern that has not been well explored is the potential for the metal-EDTA markers to go from their inert salt forms to dissociated ions that may be reactive within the gut or when absorbed by the animal. Shingfield et al. (2008) demonstrated that ruminal infusion of CoEDTA but not CrEDTA resulted in changes in the profile of fatty acids in milk,

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possibly via effects on desaturase enzymes in the cow's tissues. Such findings raise serious concerns regarding the question of marker dissociation and its effects in the conduct of dairy cattle research. Approximately 4% of CoEDTA and up to 14% of CrEDTA has been shown to dissociate in autoclaved, clarified rumen fluid that was not maintained in a reduced state, with differences detected between rumen fluid sources from 2 different cows (Hall and Van Soest, 2019b). Transfer of the metal ions to other soluble components in the rumen fluid appears to be the likely basis for the decline in detectable markers.

The effect of reducing conditions in the rumen environment on metal-EDTA stability has not been explored. The CoEDTA and CrEDTA prepared as markers incorporate the trivalent metal ions Co(III) and Cr(III). The use of hydrogen peroxide in the synthesis of CoEDTA (Udén et al., 1980) converts Co(II) to Co(III), which greatly increases the stability of the CoEDTA marker; the stability constants of Co(II) EDTA, Co(III)EDTA, and Cr(III)EDTA are 16.5, 41.5, and 23.4, respectively (Anderegg, 1977). Reduction of EDTA-associated Co(III) to Co(II) is reported to occur at oxidation-reduction potentials (ORP) of -60 to -68 mV (Ogino and Ogino, 1983), which are less negative than ORP achieved in the rumen in vivo (-231 to -480 mV; Waghorn, 1991; Richter et al., 2010 from figure data; Qin et al., 2017). Variation in ruminal ORP reported in the literature may be related to sample handling, with ORP reported to be less negative for rumen fluid samples processed outside the rumen than for those measured continuously in vivo with indwelling wireless probes in the rumen (Huang et al., 2018). It appears that diet can affect ruminal ORP. If Co(III) EDTA were reduced to Co(II)EDTA under ruminal conditions, the marker would be more prone to dissociation.

A challenge to addressing the question of marker dissociation is the issue of measuring dissociation of a salt in ruminal digesta. In such a complex matrix, it is typically not feasible to measure a compound as an ionically bound salt. However, because CrEDTA and CoEDTA in their bound forms are colored compounds with distinct absorbance patterns across wavelengths, and have absorbance maxima at specific wavelengths, they can be detected spectrophotometrically (Hamm, 1953; Paraneeiswaran et al., 2015). Spectrophotometry has been used to monitor progression of CrEDTA formation in kinetic studies (Hedrick, 1965). Accordingly, measurement of the absorbance of light at specific wavelengths and across wavelengths of visible light can be used to evaluate whether Cr- and CoEDTA are present in their ionically bound, colored forms, or whether they have dissociated. Additionally, the wavelength of

maximum absorption of Co(III)EDTA (535 nm) differs from that of Co(II)EDTA (465 nm) (Xue and Traina, 1996), allowing monitoring of the conversion of the species. The wavelengths of maximum absorption can be slightly affected by pH, with maximum absorption for CrEDTA occurring at 541 nm at slightly acidic pH (Cerar, 2015).

The objective of this study was to determine whether CrEDTA and CoEDTA salts dissociate under reducing conditions in vitro that are comparable to reducing conditions experienced in the rumen. The use of reduced medium rather than rumen fluid with microbes removed the effect of microbial action; the effect of other components present in rumen fluid have been evaluated previously (Hall and Van Soest, 2019b). This approach allowed us to focus the experiment on the effects of reducing conditions and provided solutions that were free of particulate matter and microbes that would interfere with absorbance measurements.

MATERIALS AND METHODS

The lithium salt of Cr(III)EDTA was prepared using Cr(III) acetate hydroxide (Hall and Van Soest, 2019a), and that of Co(III)EDTA prepared according to the method of Udén et al. (1980). Solutions of Cr(III) EDTA (1.0286 g/500 mL) and Co(III)EDTA (0.9260 g/500 mL) were prepared with distilled water. Marker concentrations were set so that when mixed with medium, they provided a concentration equivalent to approximately 26 mg of Cr or Co/L.

Sample Preparation

Two incubation runs were performed under anaerobic conditions, with Co(III)EDTA and Cr(III)EDTA included in each run. Goering and Van Soest (1970) media were prepared with no tryptone added and with or without resazurin. The media were prepared the day before an incubation run, sparged for 1 h with CO₂, the flasks stoppered, and stored at 4°C until used the following morning. Incubations were performed in sealed borosilicate glass fermentation tubes (121 mm long, 28 mm outer diameter, 2.8 mm wall thickness, with the end of the tubes formed to be sealed with a crown cap; custom made by Wilmad-LabGlass, Vineland, NJ). Each marker × reducing solution (**RedSol**) treatment was prepared in duplicate in each run. Replication was based on repeatability of EDTA marker absorbance measures found in a previous investigation (Hall and Van Soest, 2019b). Each tube contained 20 mL of medium, 3 mL of marker solution, and 3 mL of a combination of distilled water and RedSol, with the latter added at 0, 0.25, 0.50, 0.75, and 1.00 mL. In each run,

marker solutions and Goering and Van Soest (1970) medium with all solutions except RedSol were brought to 39°C in a water bath. After RedSol was added to all tubes, the headspace was gassed with CO₂ and the tubes were capped with crown caps, inverted to mix, and incubated for 0.5 h at 39°C in a water bath.

After incubation, the color of each tube with resazurin was recorded as an indicator of reduction. Each tube without resazurin was inverted to mix, opened, the pH was measured, and 200 µL of solution was pipetted in duplicate into wells of a 96-well plate using a positive displacement repeat pipetter (Eppendorf Repeater M4, Eppendorf North America, Hauppauge, NY), giving an approximately 5-mm path length. A plate reader was used to allow all samples to be read in less time than would be possible with a spectrophotometer with cuvettes. Once all solutions were pipetted, the absorbances were read in a plate reader spectrophotometer (EONC, Biotek Instruments Inc., Winooski, VT) at wavelengths of 330 to 700 nm in 10-nm increments, and at 465, 535, and 541 nm. Values for replicate wells per sample in the plate reader were averaged for statistical analysis. For each marker and RedSol treatment, the average of the appropriate RedSol reagent blanks containing no marker was subtracted from the values for the samples with marker added. The wavelength of numeric maximum absorbance for Co(III)EDTA and Cr(III)EDTA incubated with no RedSol was selected. Remaining solutions were transferred to scintillation vials and frozen at -20°C for future analysis. The sample solutions from each run were analyzed for minerals by inductively coupled plasma spectroscopy (Official Method 990.08, AOAC International, 2005; model 5100, Agilent Technologies, Santa Clara, CA).

Evaluation of the effect of RedSol dose on ORP was conducted in a separate, single analytical run using duplicate samples prepared with and without resazurin as in the experiment but with no markers added. Samples were measured with an ORP electrode (Mettler Toledo InLab Redox combo electrode, P/N-51343200, VWR, Batavia, IL) on a pH meter (Seven Easy pH meter, Mettler-Toledo, Scherzenbach, Switzerland). Samples were analyzed within 40 to 66 min of addition of RedSol. Upon decapping of tubes containing sample solution, the redox electrode was inserted to within 1 cm of the bottom of the tube and used to stir the samples. This left only a narrow band of liquid at the surface between the probe and tube to interact with air. The ORP was recorded when it reached an apparent minimum value. Redox buffer solution (220 mV, pH 7; #51350060, Mettler-Toledo, Greifensee, Switzerland) used as a standard read as 228 mV with the ORP probe. This offset value of 8 mV was subtracted from the values determined on the samples.

Statistical Analysis

The study was performed as a completely randomized design with the individual tube on which a measurement was taken as the experimental unit. Analytical run was a random variable, and all other variables were fixed and treated as non-numeric classification variables. For the equally spaced RedSol doses (0, 0.25, 0.50, 0.75, and 1.00 mL), contrasts were used to evaluate linear through quartic patterns of response. With all observations made at a single incubation time, there were no repeated measures.

For the response variables of sample solution pH and mineral concentrations, the statistical models included analytical run, marker type [Co(III)EDTA, Cr(III)EDTA], RedSol dose, and the interaction of marker type and RedSol dose ($n = 40$, error $df = 29$). Evaluation of ORP data was performed using data from a single analytical run in a model that included RedSol dose, presence or absence of resazurin, and the interaction term ($n = 20$, error $df = 10$ for all RedSol doses; with 0 mL RedSol omitted, $n = 16$, error $df = 8$). Absorbance responses to RedSol dose of Co(III)EDTA solutions at 535 and 465 nm and Cr(III)EDTA solutions at 560 nm were evaluated separately in models that included analytical run and RedSol dose; in each statistical analysis, $n = 20$, with 14 df for error. No statistics were applied to the wavescan data. Analyses were performed using the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Least squares means values are presented with standard errors of the difference (**SED**) unless otherwise stated. Significance was declared at $P < 0.05$. Data were evaluated for normality using the Shapiro-Wilk evaluation in the NORMAL option in PROC UNIVARIATE of SAS, with the analysis performed on the residuals generated from the statistical models used. If needed, data were transformed to achieve normality with natural log, exponential, inverse, sine, cosine, and tangent transformations evaluated.

RESULTS AND DISCUSSION

Normality of Data

The data that were the focus of the experiment—absorbances for each marker at specific wavelengths as affected by reducing solution addition—were normally distributed, as shown by $P > 0.05$ for the Shapiro-Wilk evaluation [$P = 0.40$ for Cr(III)EDTA at 560 nm; $P > 0.93$ for Co(III)EDTA at 535 and 465 nm]. For data included as descriptors of run conditions, pH ($P = 0.93$), ORP ($P = 0.85$), and most minerals ($P = 0.10$ to 0.70) were normally distributed. The 2 exceptions were

Table 1. Mineral concentrations (mg/L) of marker (cobalt-EDTA or chromium-EDTA) by reducing solution treatments¹

Marker	RS ²	Run	Co	Cr	Cu	Fe	K	Mg	Mn	Na	P	S	Zn
None	0	1	0.1	0.1	0.2	0	306	1.9	1.5	1938	456	13.2	0
	0.25	1	0.2	0.1	0.2	0.1	304	1.9	1.5	1970	458	24.8	0
	0.5	1	0.1	0.1	0.2	0	304	1.8	1.5	1987	454	35.3	0
	0.75	1	0	0.2	0.2	0	305	2.0	1.5	2015	459	45.7	0
	1.00	1	0.2	0.1	0.3	0	301	2.1	1.6	2028	458	55.4	0
	0	2	0	0	0.2	0	316	2.4	1.6	2018	472	12.7	0
	0.25	2	0	0	0.4	0	311	2.5	1.6	2020	466	23.0	0
	0.5	2	0	0.3	0.2	0	317	2.6	1.6	2047	479	33.7	0
	0.75	2	0	0.1	0.3	0	314	2.6	1.6	2062	475	44.2	0
	1.00	2	0.2	0.1	0.3	0.2	308	2.6	1.8	2069	470	55.6	0
	0	1	26.5	0.6	0.3	0	302	1.9	1.5	1967	463	12.7	0
	0.25	1	26.6	0.8	0.3	0	313	2.3	1.7	2014	465	24.2	0.3
	0.5	1	26.6	0.8	0.2	0	306	2.2	1.7	2018	463	36.3	0.4
	0.75	1	26.9	0.9	0.2	0	311	2.3	1.7	2053	469	48.8	0.4
	1.00	1	26.6	0.7	0.2	0.1	307	2.2	1.7	2041	466	58.2	0.5
CoEDTA	0	2	26.9	0.9	0.2	0	310	2.5	1.5	2004	470	17.1	0
	0.25	2	26.8	0.4	0.3	0	311	2.5	1.7	2034	473	24.2	0.3
	0.5	2	26.6	0.8	0.3	0	307	2.6	1.8	2041	462	34.0	0.5
	0.75	2	26.8	1.0	0.4	0.2	310	2.5	1.8	2062	470	46.0	0.4
	1.00	2	26.9	0.9	0.3	0.4	307	2.5	1.9	2075	469	58.0	0.3
CrEDTA	0	1	0	26.4	0.2	0	310	2.4	2.2	1988	467	17.4	0.5
	0.25	1	0	26.4	0.2	0	308	2.1	2.0	1997	466	26.6	0.5
	0.5	1	0.1	26.0	0.2	0	302	2.1	1.9	1989	468	35.7	0.4
	0.75	1	0	26.0	0.2	0.2	299	2.2	1.9	2007	462	46.2	0.4
	1.00	1	0	26.1	0.2	0.4	304	2.1	1.9	2041	458	57.1	0.4
	0	2	0.2	26.1	0.4	0	310	2.7	2.1	1994	471	12.8	0.3
	0.25	2	0.1	26.2	0.4	0	308	2.4	2.0	2006	466	22.2	0.2
	0.5	2	0	26.1	0.4	0	308	2.5	2.0	2030	470	35.2	0.2
	0.75	2	0.2	26.1	0.2	0.4	308	2.4	1.9	2057	471	48.4	0.4
	1.00	2	0	26.2	0.2	0.5	309	2.4	2.0	2067	470	56.9	0.3
Replicate SD ³			0.26	0.29	0.05	0.01	10	0.36	0.08	44	9.2	2.2	0.88

¹Values are arithmetic means of duplicate treatment tubes within run.²RS = milliliters of reducing solution added.³Average of the SD of replicate sample tubes within analytical run for a given mineral. This value gives an estimate of the precision of the analyses but is also affected by variation between sample tubes.

sample solution iron and manganese concentrations ($P < 0.01$ for both). Transformation of the manganese concentration data as its inverse ($1/Y$) normalized the data ($P > 0.05$) but did not change the P -value patterns of significance of treatments. It was not possible to normalize the iron data, likely because of the substantial number of 0 mg/L concentrations in that data, with values describing low concentrations from 0 to 0.6 mg/L. Accordingly, iron data are reported (Table 1) but not evaluated statistically.

Run Conditions

Measurements made on samples conserved at the end of the analytical runs indicated that comparable, targeted amounts of marker were added to each tube and that reagent blanks contained little Co or Cr (Table 1). Both sodium and sulfur concentrations increased linearly with increasing additions of RedSol ($P < 0.01$ for both), reflecting contributions of the NaOH, cysteine HCl, and Na sulfide used in the RedSol formulation (Goering and Van Soest, 1970). There were no differ-

ences in concentrations of copper ($P > 0.50$), potassium ($P > 0.58$), magnesium ($P > 0.64$), or phosphorus ($P > 0.81$) between the Co(III)EDTA and Cr(III)EDTA treatments, RedSol levels, or their interaction. Two of the minerals having the lowest concentrations, manganese and zinc, differed between markers ($P < 0.02$ for both), and manganese differed by RedSol addition and its interaction with marker treatment ($P < 0.01$ for both). Although significant, the differences detected were <1 mg of mineral/L and likely caused by contamination in the original chemicals. Similarly, we attributed the <1 mg of Cr/L detected in the Co(III)EDTA sample solutions to an unknown source of contamination. The pH of the sample solutions differed slightly between the 2 analytical runs (6.95 and 6.88, $P < 0.01$, SED = 0.012), but did not differ by marker ($P = 0.27$), amount of RedSol added ($P = 0.61$), or their interaction ($P = 0.64$) (data not shown).

Solutions without markers became more reduced with increasing RedSol addition ($P < 0.01$) and with versus without resazurin ($P < 0.01$; Table 2). Solutions containing 0.25 to 1.0 mL of RedSol gave ORP values

within the bounds of those measured ruminally in vivo for dairy cattle (−231 to −480 mV; Waghorn, 1991; Richter et al., 2010 from figure data; Qin et al., 2017), suggesting that the experimental conditions were within an appropriate range to evaluate the effect of reducing conditions that could be experienced in the rumen. The interaction of RedSol dose and resazurin usage became nonsignificant when the 0-mL RedSol dose data were omitted. This was likely due to the very large ORP difference (176 mV) between samples with and without resazurin at 0 mL of RedSol compared with the much smaller 10 to 20 mV differences at other RedSol dosages. Resazurin used under ambient lighting has been shown to shorten, by hours, the time required to fully reduce culture medium (Fukushima et al., 2003) and is the likely reason for the more negative ORP values noted with resazurin in the present study. With the 0-mL RedSol data omitted, ORP responses were quadratic for increasing RedSol dosages ($P < 0.01$).

Resazurin-related color change was used qualitatively as a reduction indicator in sample solutions not used for absorbance readings in the main experiment. For reagent blanks with no marker added, those with 0 mL of RedSol stayed blue, the color of unreduced resazurin. Reducing solution dosed at 0.25 mL gave faint pink solutions, and solutions became colorless at greater RedSol volumes. Solutions with Cr(III)EDTA or Co(III)EDTA added showed the same color pattern with some exceptions. The purple color of Cr(III)EDTA was evident in all tubes in which it was included, even with the resazurin color changes. The reddish purple color of Co(III)EDTA treatments was evident in solutions with 0 or 0.25 mL of RedSol, but took on a brown rather than clear color with greater RedSol additions, whether or not resazurin was present. This would indicate that

changes in Co(III)EDTA, not in the resazurin, were the source of the brown color formation.

Marker Response to Reducing Conditions

Colored compounds in solution have characteristic wavelengths at which they achieve maximal absorbance values in spectrophotometric evaluations. Changes in absorbance at these wavelengths are the basis for detecting increasing and decreasing concentrations of analytes in colorimetric assays, as well as for estimating evolution (Hamm, 1953) or, conversely, destruction of a compound of interest. It must be noted that, although there is a wavelength of maximal absorbance for a particular compound or specie, that material also exhibits light absorbance at many other wavelengths, albeit to a lesser degree. Colored compounds have characteristic patterns of absorption across wavelengths that are modified by changes in the valence of associated metal ions or other compositional modifications of the compound (Xue and Traina, 1996).

The Co- and Cr-EDTA markers tested responded very differently to increasingly reduced conditions. The Co(III)EDTA showed a 75% decline in absorbance at its maximum absorbance wavelength of 535 nm (RedSol effect, $P < 0.0001$, SED = 0.008; patterns of response: linear, $P < 0.0001$; quadratic, $P = 0.07$; cubic, $P = 0.03$; quartic, $P = 0.47$; Figure 1a). Absorbances measured at 465 nm, the wavelength of maximal absorption for the reduced Co(II)EDTA, showed a 4-fold increase between 0 and 0.5 mL of RedSol before declining by 61% (RedSol effect, $P < 0.0001$, SED = 0.009; patterns of response: linear, $P = 0.0033$; quadratic, $P < 0.0001$; cubic, $P = 0.16$; quartic, $P = 0.10$). Under the reducing conditions imposed, it appears that a substantial portion, if not

Table 2. Redox potentials (mV) of media without and with resazurin¹

Reducing solution, mL	No resazurin		With resazurin	
	Mean	SD	Mean	SD
0	196	3.5	20.5	3.54
0.25	−250	2.83	−272	4.24
0.50	−285	5.66	−304	7.07
0.75	−314	4.24	−326	0.71
1.00	−328	0.71	−341	0
<i>P</i> -value				
Analysis	SED ²	Reducing solution, mL	Resazurin inclusion	Interaction
All data	3.90	<0.01	<0.01	<0.01
Omitting 0 mL data	3.98	<0.01	<0.01	0.34

¹Values are arithmetic means of duplicate preparations.

²Standard error of the difference.

all, of the Co(III) associated with EDTA was reduced to Co(II); the absolute proportion of Co(III)EDTA reduced could not be determined because Co(III)EDTA normally shows some absorbance at 465 nm (see 0-mL RedSol curve for Co(III)EDTA in Figure 2a), and Co(II)EDTA has some absorbance at 535 nm (Xue and Traina, 1996). With the lesser stability of Co(II)EDTA compared with Co(III)EDTA (stability constants of 16.5 and 41.5, respectively; Anderegg, 1977), we interpreted the increase and then decline in absorbance at 465 nm as feasibly representing generation of and subsequent dissociation of Co(II)EDTA. This scenario was supported by the pattern of absorbance for the Co(III)EDTA treatment across wavelengths from 330 to 700 nm (Figure 2a). Whereas at 0 mL RedSol, the absorbances show the pattern across wavelengths characteristic of Co(III)EDTA (Xue and Traina, 1996), the patterns deform with increasing doses of RedSol, with absorbances at shorter wavelengths increasing and those at 535 nm declining. The gross alteration of the absorbance pattern is indicative of the conversion of Co(III)EDTA to other species. The evolution of visible brown coloration in solutions with ≥ 0.50 mL RedSol may also reflect development of Co(II)EDTA with its wavelength of maximum absorbance of 465 nm. Other brown compounds have been shown to have absorbance maxima at wavelengths between 400 and 500 nm (Tang and Thompson, 2012; Wilson et al., 2016). Finally, increasing RedSol from 0.75 to 1.0 mL resulted in an apparent decline in absorbance across all wavelengths, suggesting dissociation of Co(III)EDTA into species that do not absorb visible light.

The Cr(III)EDTA showed maximum absorbance at 560 nm at the nearly neutral pH of the experimental media compared with 541 nm under slightly acidic conditions (Cerar, 2015). Accordingly, evaluations of Cr(III)EDTA were performed with absorbances determined at 560 nm. In contrast to Co(III)EDTA, the effect of RedSol additions ($P = 0.32$; SED = 0.003) was only detected as a tendency for a linear decline (linear, $P = 0.14$; other patterns of response: quadratic, $P = 0.29$; cubic, $P = 0.66$) with a maximum of 8% decrease in absorbance (Figure 1b). The absorbances across wavelengths of visible light retained the basic pattern characteristic of Cr(III)EDTA but with absorbance declining with increasing additions of RedSol, and apparently more so at shorter wavelengths (Figure 2b). The redox potential at which Cr(III) is converted to Cr(II) is -420 mV (at 298.15 K and acidic pH; Bratsch, 1989), which is within the range of ORP values reported for the rumen. However, Cr(II) is easily oxidized back to Cr(III) through reactions with O_2 and water depending on the acidity and anions present (Mandich, 1997). It is possible that some reduction of Cr(III) resulted in a

Cr(II)EDTA salt that is less stable than Cr(III)EDTA (respective stability constants of 13.6, Vinokurov and Bondar, 2003; and 23.6, Anderegg, 1977). As with Co(II)EDTA, if Cr(II)EDTA dissociated and did not reassociate as Cr(III)EDTA, the released ions would no longer be incorporated in colored compounds and absorbance would decline. A pattern of absorption across wavelengths for Cr(II)EDTA was not found in the literature.

The potential complete dissociation of Co(III)EDTA and partial dissociation of Cr(III)EDTA under reducing conditions lend themselves to explanation of the *in vivo* work of Shingfield et al. (2008). In that study, CoEDTA but not CrEDTA altered the fatty acid profile of milk

Figure 1. Absorbance values at wavelengths (λ) of peak absorption for sample solutions of Co(III)EDTA and Cr(III)EDTA with different volumes of reducing solution added. Values are least squares means, and error bars represent SEM. (a) Co(III)EDTA absorbance at $\lambda = 535$ nm, ○, SED = 0.008 and $\lambda = 465$ nm, ▲; SED = 0.009. (b) Cr(III)EDTA absorbance at $\lambda = 560$ nm, ○, SED = 0.003.

Figure 2. Wavelength absorbance data from wavelengths of 330 to 700 nm for (a) Co(III)EDTA, and (b) Cr(III)EDTA sample solutions with different volumes of reducing solution added. Data are arithmetic averages of sample replicates from the second analytical run. Lines show wavelength scans for individual additions of reducing solution.

in lactating cows. In our previous work with unreduced, autoclaved, clarified rumen fluid, we had shown that CrEDTA and, to a lesser extent, CoEDTA could dissociate (14 and 4% by 24 h, respectively) and potentially bind to phenolics or other ligands present (Hall and Van Soest, 2019b). If bound to other compounds, the minerals have the potential to remain nonreactive. However, unbound Co from extensively dissociated CoEDTA could generate the observed *in vivo* effects in dairy cattle through increased production of vitamin B₁₂ analogs or as absorbed Co. A study in Wistar rats showed that vitamin B₁₂ deficiency relative to folic acid adequacy or excess alters activity of hepatic desaturases (Wadhwani et al., 2012). An effectual B₁₂ deficiency could theoretically be produced if the infused CoEDTA dissociated and greatly increased the ruminally available Co and

increased ruminal formation of vitamin B₁₂ analogs (Kawashima et al., 1997). The analogs can inhibit enzyme activity by binding in place of coenzymes derived from B₁₂ but without their activity (Lengyel et al., 1960). Alternatively, Co as cobalt acetate administered orally (3.5 g Co/d) or intravenously (175 mg Co/d) has been shown to alter mammary desaturase milk fatty acid desaturation products in dairy cows (Taugbøl et al., 2010); the oral dose of Co was approximately 1,500× recommendations (NRC, 2001), and the venous dose was 5% of the oral dose to be comparable to urinary Co excretion noted with markers. The carryover effect on mammary fatty acid desaturation products appears to vary by Co dose level, and may be greater than 10 d (Karlengen et al., 2013).

It seems less likely that CrEDTA is substantially dissociated in the gut. Dobson et al. (1976) investigated ruminal absorption of [⁵¹Cr]EDTA from the ventral sac of nonlactating dairy cows. In that study, eluate from urine applied to an anion exchange column “had a faint purple color” with a peak absorbance at 542 nm. They concluded that the CrEDTA “passed from the rumen into the blood without appreciable degradation.” This is suggestive, but not definitive, of a greater stability of CrEDTA as an intact molecule than CoEDTA.

Consequences of Marker Dissociation

To be useful in research, markers used to assess digesta kinetics or diet digestibility must not interfere with responses of the animal or the microbiome. Markers that become reactive under conditions found in the animal violate this standard and raise the question of whether responses seen in studies where they were used were a function of the treatments or interactions of treatments and the marker. This may also affect how treatment inferences can be applied to the larger population of animals that do not receive markers. It is clear from the *in vivo* data with Co(III)EDTA (Shingfield et al., 2008) and the results of the present study that Co(III)EDTA is not suitable as a digesta marker in ruminants because of its bioactivity and dissociation. The status of Cr(III)EDTA is slightly less clear. That it does partially dissociate in rumen fluid (Hall and Van Soest, 2019b) and under reducing conditions makes it less than ideal, and we do not know what the combination of reducing conditions and solutes in rumen fluid would do to the extent of dissociation. However, Shingfield et al. (2008) did not note an effect of CrEDTA on the specific measures they made in lactating dairy cattle, although the study was not suited to evaluation of lactation performance, having used 5 cows in a 5 × 5 Latin square. Reports of effects of CrEDTA on animal or microbial metabolism or diet digestibility

were not found in the literature. Questions that require further investigation are whether any dissociated Cr recombines with indigestible compounds for it to stay inert and a marker in the liquid fraction, and whether CrEDTA affects other ruminal or animal responses of interest. With either EDTA marker, dissociation will release EDTA, which is not likely to degrade under anaerobic conditions (Tiedje, 1977) and could bind other minerals.

An alternative liquid digesta marker is polyethylene glycol (**PEG**; molecular weight 4,000 to 6,000) which was the earliest viable marker of liquid digesta investigated for use in nutrition studies (Shaffer and Critchfield, 1947). Polyethylene glycol of molecular weights 4,000 and 6,000 are reported not to be absorbed from the gut in rats or humans, although absorption of PEG of molecular weights 1,000 and 1,540 can be slight (Shaffer and Critchfield, 1947). Polyethylene glycol has been reported to not (Teeter and Owens, 1983) or minimally (Downes and McDonald, 1964) be recovered in urine in ruminants and has not been found to be appreciably degraded in the gut of ruminants (Teeter and Owens, 1983) or swine (Ishikawa, 1965). However, criticisms of PEG include that it interacts with tannins in feeds and has required analysis of undried samples using a turbidimetric method that is affected by technique. Feeds containing tannins, such as cottonseed hulls and a variety of tropical forages, may complex with PEG (Clark et al., 1972), thus removing the marker from the liquid pool. Use of PEG in diets with tanniniferous feeds is not recommended. Work has been done to stabilize the turbidimetric assay of Hydén (1955) and make it less sensitive to technique (Malawer and Powell, 1967), and PEG (molecular weight 6,000) has been reported to be detected by near-infrared reflectance spectroscopy in oven-dried feces from lactating dairy cows (Ahvenjärvi et al., 2018). Although PEG is not as ideal in its precision of measurement as the Co- and Cr-EDTA salts, it provides an alternative that may be less prone to absorption and bioactivity in the animal.

CONCLUSIONS

We conclude that Co(III)EDTA is not suitable for use as a liquid digesta marker in ruminants because of its dissociation under reducing conditions achievable in the rumen and the potential bioactive effect on animal and microbial responses. Chromium (III)EDTA shows less propensity for dissociation; however, it has not been fully investigated under reducing and chemical conditions present in the rumen to fully warrant its use. We suggest reexamining the suitability of available liquid digesta markers for use in ruminant research.

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