Effect of Forage to Concentrate Ratio on Disappearance of Vitamins A and E During In Vitro Ruminal Fermentation

W. P. WEISS, K. L. SMITH, J. S. HOGAN, and T. E. STEINER
Department of Dairy Science
Ohio Agricultural Research and Development Center
The Ohio State University
Wooster 44691

ABSTRACT

The effects of forage to concentrate ratio and the commercial form of vitamins A and E on in vitro ruminal disappearance of retinol and α-tocopherol were studied. Ruminally fistulated cows were fed diets with either 80 or 50% forage. In vitro substrates that were similar to those fed to the donor cows were incubated with buffered ruminal fluid for 24 h. Different commercial forms of vitamin E (spray-dried, silicic acid adsorbate, and lipid-encased forms) and vitamin A (gelatin beadlet and lipid-encased forms) were added to the flasks. The vitamin E was all-rac-α-tocopheryl acetate, and the vitamin A was all-trans-retinyl acetate. The amount of α-tocopherol in the flasks was not affected by diet or form of vitamin E and did not change over the 24-h incubation. Retinol disappearance was not affected by form of vitamin A but was substantially higher for the 50% forage diet than for the 80% forage diet (72 vs. 20% at 24 h). These data suggest that ruminal metabolism of vitamin E is minimal and not affected by forage to concentrate ratio. Additionally, vitamin A destruction in the rumen was much higher when cows were fed a typical lactation diet than when fed a typical dry cow diet.

(Key words: vitamin A, vitamin E, rumen)

INTRODUCTION

The importance of vitamins A and E on immune function is becoming well established (3, 5), but fewer data are available on the digestion and metabolism of those vitamins by ruminants. Information about ruminal metabolism of vitamin E is inconsistent. Some studies (2, 8, 9) found essentially no disappearance of vitamin E during ruminal fermentation, but others (1, 13) found substantial ruminal disappearance of vitamin E. Nonlactating dairy cows fed conventional dry cow diets (high forage) may require more vitamin E than lactating cows, which are typically fed a diet that is higher in concentrate. Dry cows often have lower concentrations of α-tocopherol in plasma than do lactating cows even when fed similar amounts of vitamin E (15). No data were found on the effect of dietary forage to concentrate ratio on ruminal disappearance of vitamin E.

High concentrate diets may result in increased ruminal destruction of vitamin A. Rode et al. (11) showed that disappearance of vitamin A during in vitro ruminal fermentation was approximately 80% when donor steers were fed a 70% concentrate diet compared with about 20% when donor steers were fed a high forage diet. Warner et al. (14) reported a trend that was similar but of much less magnitude. In their study, ruminal vitamin A disappearance was about 55% when steers were fed 20 or 40% corn (75 or 50% alfalfa hay) and about 65% when steers were fed 60 or 80% corn (30 or 7% alfalfa hay).

The objective of our experiment was to determine whether ruminal disappearance of α-tocopherol and retinol differed when cows were fed diets that were typical for dry cows or typical for lactating cows. A secondary objective was to determine whether ruminal disappearance of retinol and α-tocopherol differed among various commercial forms of vitamins A and E.
MATERIALS AND METHODS

In Vitro Incubations

Three lactating and three dry Holstein cows were used as donors of ruminal fluid. Dry cows were fed a diet that contained 80% forage (Table 1). Lactating cows were fed a diet with 50% forage (Table 1). Both diets were balanced to meet NRC recommendations (10) except that the diets were devoid of supplemental vitamins A and E. Cows were fed their respective diets for at least 14 d prior to sampling. Ruminal fluid was collected via cannula and squeezed through two layers of cheesecloth. Filtered ruminal fluid (200 ml) was mixed with 800 ml of buffer and 125 μl of mineral solution (4). Sodium sulfite and cysteine-HCl were used as reducing agents in the original method (4), but, for this experiment, 320 μl of 2-mercaptoethanol were used. The buffer solution was maintained at 39°C during preparation. Each in vitro flask contained 50 ml of the inoculum and buffer mixture and .5 g of air-dried substrate. Flasks were purged with CO₂, sealed with vented stoppers, and incubated in an environmental shaker (100 rpm) at 39°C.

Substrates for the incubations were similar to the diets fed to donor cows (Table 1). Feedstuffs were ground through a 1-mm screen Wiley mill (Arthur H. Thomas, Philadelphia, PA) prior to mixing the in vitro diets. Substrates contained no supplemental vitamin E or A (blanks), spray-dried (SPD) vitamin E with conventional vitamin A, adsorbate (AD) vitamin E with conventional vitamin A, and ruminally protected (RP) vitamins A and E. The SPD vitamin E was all-rac-α-tocopheryl acetate (500 IU/g) that was manufactured as a spray-dried powder (Rovimix E-50 SD; Roche Animal Nutrition, Nutley, NJ). The AD vitamin E was all-rac-α-tocopheryl acetate (500 IU/g) that was adsorbed onto silicic acid (Rovimix E-50 Adsorbate; Roche Animal Nutrition). The conventional vitamin A was all-trans-retinyl acetate (650,000 IU/g) encased in gelatin beadlets (Rovimix A650; Roche Animal Nutrition). The acetate esters of vitamins A and E are the standard forms used in dietary supplements for cattle. The RP vitamin product contained all-rac-α-tocopheryl acetate (100 IU/g) and all-trans-retinyl acetate (100,000 IU/g) that was protected by a matrix composed of lipid and other polymers (Biopass ADE; Industria Italiana Integratori, TREI, Via Pietro Bembo, Modena, Italy). The final concentrations of supplemental vitamins A and E in all in vitro diets were 600,000 and 600 IU/kg of air-dried feed, respectively, to provide initial concentrations of vitamin A and E of 6000 and 6 IU/L of media. These concentrations were chosen to provide adequate vitamins for reliable quantification and should be similar to concentrations in ruminal fluid in vivo found with practical diets (200,000 IU of vitamin A/d and 300 IU of vitamin E/d, assuming 50 L of ruminal contents).

Ruminal fluid from the three lactating cows was incubated with the 50% forage in vitro diets (all vitamin treatments), and ruminal fluid from the three dry cows was incubated with the 80% forage in vitro diets (all vitamin treatments). Substrates were incubated for 0, 3, 6, 12, and 24 h. Blanks for each cow and each time point were run concurrently to correct for retinol and α-tocopherol provided by the inoculum and basal ingredients. Each incubation was conducted in duplicate (separate runs) for a total of 240 flasks [six cows × four vitamin treatments (including the blank) × two repli-

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**TABLE 1. Ingredient and chemical composition of diets fed to donor cows and substrate used in fermentation vessels.**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Lactation (%)</th>
<th>Dry cow (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa silage</td>
<td>25.0</td>
<td>...</td>
</tr>
<tr>
<td>Corn silage</td>
<td>25.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Grass hay</td>
<td>...</td>
<td>60.0</td>
</tr>
<tr>
<td>Corn grain</td>
<td>29.0</td>
<td>11.0</td>
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<tr>
<td>Ground oats</td>
<td>...</td>
<td>4.0</td>
</tr>
<tr>
<td>Soybean meal, 44% CP</td>
<td>8.5</td>
<td>2.2</td>
</tr>
<tr>
<td>Distillers grains</td>
<td>5.5</td>
<td>...</td>
</tr>
<tr>
<td>Minerals and vitamins</td>
<td>7.0</td>
<td>2.8</td>
</tr>
<tr>
<td>NDF, %</td>
<td>32.3</td>
<td>50.4</td>
</tr>
<tr>
<td>CP, %</td>
<td>17.3</td>
<td>15.2</td>
</tr>
</tbody>
</table>

In vitro substrates

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Lactation (%)</th>
<th>Dry cow (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa silage</td>
<td>25.0</td>
<td>...</td>
</tr>
<tr>
<td>Corn silage</td>
<td>25.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Grass hay</td>
<td>...</td>
<td>60.0</td>
</tr>
<tr>
<td>Corn grain</td>
<td>40.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Soybean meal, 44% CP</td>
<td>10.0</td>
<td>5.0</td>
</tr>
<tr>
<td>NDF, %</td>
<td>29.6</td>
<td>52.1</td>
</tr>
<tr>
<td>CP, %</td>
<td>15.7</td>
<td>14.4</td>
</tr>
</tbody>
</table>

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The mineral solution (4) contained: NaCl, 8.5% (w/v); KH₂PO₄, 4.5% (w/v); MgSO₄·7H₂O, 1% (w/v); CaCl₂·2H₂O, 0.5% (w/v); K₂HPO₄, 0.2% (w/v); NaH₂PO₄·2H₂O, 0.1% (w/v); FeSO₄·7H₂O, 0.02% (w/v); ZnSO₄·7H₂O, 0.05% (w/v); MnSO₄·H₂O, 0.02% (w/v); CuSO₄·5H₂O, 0.005% (w/v); CoCl₂·6H₂O, 0.005% (w/v); and vitamin D₃, 0.5 μg/L.
lates x five time points). Flask was considered as the experimental unit.

**Chemical Analyses**

At the end of each incubation time, flasks were swirled vigorously, and then three 5-ml aliquots of contents were removed using a wide-tip pipette. Samples were mixed immediately with 10.5 ml of a solution of 5% pyrogallic acid in ethanol and placed into a freezer (-20°C) until analysis. The extraction procedure was similar to that used for milk (6) with minor modifications. After thawing, 1 ml of 10% pyrogallic acid (in ethanol) was added to the mixture followed by 1.5 ml of 50% KOH. The solution was then heated at 70°C for 15 min. After cooling, 20 ml of water and 20 ml of hexane were added. The mixture was shaken vigorously for 20 min, and 5 or 7 ml (depending on anticipated concentrations) of the hexane layer was removed. The hexane was evaporated to dryness and reconstituted in an appropriate quantity of ethanol. The ethanol fraction was injected into an HPLC equipped with a reverse-phase column (Supelcosil LC-18, 25 cm x 4.6 mm; Supelco, Inc., Bellefonte, PA). The solvent (flow rate, 1.8 ml/min) was 83:17 methanol:water that changed linearly to 100:0 (vol/vol) methanol:water over 5 min. Tocopherol was monitored using fluorescence detection (excitation at 295 nm and emission at 330 nm), and retinol was monitored using UV detection (325 nm). All-rac-α-tocopherol (Eastman Fine Chemicals, Rochester, NY) and all-trans-retinol (Eastman Fine Chemicals) were used as external standards. Feedstuffs (diets and substrates) were analyzed for NDF (α-amylase method) and CP (16).

**Statistical Analyses**

Prior to statistical analysis, the vitamin E values for each time point were corrected for background α-tocopherol by subtracting the amount of α-tocopherol in the appropriate blank (no retinol was found in the blanks). All data were expressed relative to the initial sample. Sources of variation included in the ANOVA for vitamin E disappearance were diet (dry or lactation); donor cow nested within diet, type of vitamin E (SPD, AD, or RP); and diet by type of vitamin interaction. The form of vitamin E effect was partitioned into two orthogonal contrasts (SPD vs. AD and SPD plus AD vs. RP). The model for vitamin A disappearance was the same except that form of vitamin A (conventional or RP) replaced type of vitamin E. Data were analyzed within each time point (0, 3, 6, 12, and 24 h) and across times with time as a repeated measure. Analyses were conducted using the general linear models procedure (repeated measures option) of SAS (12).

**RESULTS AND DISCUSSION**

A procedure based on ethanol and hexane was not adequate for extraction of α-tocopherol from ruminal fluid (extraction efficiency was <70%). We have used that procedure routinely for extraction of α-tocopherol from plasma and red blood cells and obtained extraction efficiencies ranging from 85 to 100% (16). The saponification procedure used in this experiment was efficient for both α-tocopherol (92%) and retinol (85%); however, the saponification step precluded measurement of the ester forms of the vitamins.

Within each time point, diet (P > .25) and form of vitamin E (P > .25) did not affect disappearance of α-tocopherol (Figure 1). When the data were analyzed across time, diet (P > .25), form of vitamin E (P > .25), and time (P > .25) did not affect disappearance of α-tocopherol. No interactions were observed between form of vitamin E, diet, and time (P > .25). Essentially no disappearance of α-tocopherol occurred during the 24-h incubation for any treatment. The amount of α-tocopherol that remained after 24 h of incubation in vitro was not different (P > .25) from the initial amount for any treatment. The RP vitamin E appeared to have a greater disappearance than the other two vitamin E treatments at 3 and 6 h but not at 12 and 24 h with the high concentrate diet (Figure 1b). The contrast comparing RP to SPD and AD was not significant at any time point (P > .10). Sampling error is a likely reason for the apparent differences. The SPD and AD vitamin E preparations were fine powders, but the RP vitamin E was in the form of small spheres (250 to 750 μm). The standard deviations within each time point, but across diets and cows for the RP treatment, were approximately three times larger than those for the other two treatments.
Results of the present study are similar to those of others (2, 8, 9) but are dissimilar to those of Alderson et al. (1). Leedle et al. (8) examined the disappearance of vitamin E (all-rac-α-tocopherol acetate) in vitro and reported essentially no disappearance during a 24-h incubation with ruminal fluid from steers fed a diet consisting of 82% corn grain. Astrup et al. (2) conducted a similar experiment and found essentially no disappearance of vitamin E (all-rac-α-tocopherol) after 24 h of incubation with ruminal fluid from sheep fed a diet of 50:50 alfalfa chaff and oats. McDiarmid et al. (9) reported that the disappearance of α-tocopherol was essentially nil when incubated in ruminal fluid collected from steers fed a diet of all forage. Alderson et al. (1) measured ruminal disappearance of vitamin E (α-tocopherol; isomeric form not given) in steers by collecting samples via abomasal cannulas. They (1) reported that, when steers were fed a diet of approximately 20% corn grain and 80% alfalfa hay, essentially no ruminal disappearance of vitamin E occurred. When steers were fed diets of 80% corn grain, approximately 42% of the administered vitamin E disappeared prior to the abomasum. Analytical procedures were different among the experiments. Leedle et al. (8) suggested that poor extraction was the reason that some researchers have reported ruminal disappearance of vitamin E. With some techniques, extractability of vitamin E from digesta was less than that from feeds. Furthermore, Alderson et al. (1) used an indirect method to quantify vitamin E; the other experiments, including the present one, quantified vitamin E using chromatography.

Disappearance of retinol was not affected by form of vitamin A, but differed among diets and times (Figure 2). No diet by form of vitamin A interaction was observed (P > .25), but a time by diet interaction existed (P < .01). Disappearance of retinol was two to three times greater for the high concentrate diet at 12 and 24 h than for the 80% forage diet. After 24 h of incubation, 80% (95% confidence interval: 64 to 96) of the added retinol was recovered for the high forage diet (averaged over form of vitamin A). For the 50% forage
diet, 28% (95% confidence interval: 16 to 40) of the added retinol was recovered after 24 h of incubation (averaged over form of vitamin A).

Results from this experiment generally confirmed and expanded the data of Rode et al. (11), who reported that in vitro ruminal disappearance of retinol (retinyl acetate was added initially) was approximately 80% at 24 h when ruminal fluid was collected from steers fed a diet of 70% concentrate. When donor steers were fed all hay diets, retinol disappearance was about 25% after 24 h. Data from the present experiment suggested that ruminal disappearance of retinol can be substantial when diets contained about 50% forage. In contrast, Keating et al. (7) reported that in vitro ruminal disappearance of vitamin A (added as retinyl acetate) at 16 h was much greater when ruminal fluid was collected from steers fed an all forage diet than when steers were fed a 70% concentrate diet (90 vs. 10% disappearance); however, in that study, variation among experiments was large. In one experiment, average disappearance of vitamin A was 35% (at 12 h), and, in another experiment, was 69% (ruminal fluid was from steers fed an all forage diet in both experiments). Retinol was quantified using HPLC in the experiment of Rode et al. (11) and in the present study. Keating et al. (7) used a colorimetric assay to quantify retinol.

Because of the analytical procedure used in the present experiment (saponification), the fate of the vitamin esters could not be determined. The active forms of the vitamins (α-tocopherol and retinol) were monitored. Data from Leedle et al. (8) and McDiarmid et al. (9) showed that the acetate ester of tocopherol was not hydrolyzed during in vitro ruminal fermentation; however, retinyl acetate did not appear to be stable during in vitro ruminal fermentation (11). Between 60 and 100% of retinyl acetate disappeared during a 24-h in vitro fermentation in that study.

Dry cows often have lower concentrations of α-tocopherol in plasma than do lactating cows when fed similar amounts of vitamin E (15). Data from the present experiment suggest that ruminal metabolism of vitamin E is not responsible for that difference. Data from this experiment and others (2, 8, 9) suggest that ruminal metabolism of vitamin E is essentially nil, regardless of diet. Disappearance of vitamin A was substantially greater when the diet contained 50% forage than when the diet contained 80% forage and was not different between conventional vitamin A and a lipid-encased form of vitamin A. Previous data (11, 14) showed that ruminal destruction of vitamin A may be a concern when feedlot cattle are fed high concentrate diets. Data from the present experiment suggest that ruminal destruction of vitamin A may be important when lactating dairy cows are fed diets with 50% forage. The vitamin A requirement for lactating dairy cows may need to be reevaluated.

REFERENCES

