# COMPARISON OF D-ALANINE AND DIAMINOPIMELIC ACID AS BACTERIAL MARKERS IN YOUNG CALVES[1,2,3]

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ABSTRACT

D-alanine (DAL) and diaminopimelic acid (DAP) were compared as markers to estimate proportion of bacterial N in total N reaching the abomasum of young calves. Sixteen Holstein bull calves fed complete pelleted starter or unpelleted starter plus hay and weaned at 4 or 8 wk of age were fitted with ruminal and abomasal cannulas and sampled twice weekly from 2 to 11 wk of age. Isolated ruminal bacterial cells contained more DAL than DAP at all weeks and averaged 7.0 and 5.4 mg N/g N, respectively. Weekly mean marker concentrations were highly correlated (.89) in ruminal bacteria, except at 3 wk of age. Concentration of DAL in abomasal digesta was greater than that of DAP at all weeks and averaged 5.2 and 2.4 mg N/g N, respectively. Weekly mean DAL correlated with DAP .61 in abomasal digesta and correlated .57 and .89 with starter intake, respectively. The proportion of bacterial N in total abomasal N was greater at all weeks when estimated by DAL than by DAP and averaged 77% and 46%, respectively. Estimates by DAL exceeded 100% in several cases and reflected large variation in analytical estimates. Estimates by DAL and DAP correlated .33 and .92 with starter intake. D-alanine was not an acceptable bacterial marker in this study.

(Key Words: Calves, Bacteria, Markers, Alanine, Diaminopimelic acid, Protein.)

## Introduction

Measurement of proportion of microbial and dietary N reaching the lower gut of ruminant animals requires a technique to measure total N and at least one fraction of total N accurately and precisely. A number of marker techniques are available; however, validity of any particular one is difficult to establish because no absolute method exists for quantitating amounts of microbial protein in vivo (Theurer, 1982). A widely used method for estimating contribution of bacterial N to total N reaching the lower gut is diaminopimelic acid (DAP), an amino acid found in the peptidoglycan moiety of some bacterial cell walls. Comparison of DAP-to-N ratios in isolated bacterial cells and abomasal or duodenal digesta allows estimation of proportion of bacterial N in total N at the site of sampling. Discussions of DAP as a bacterial marker are in papers by Ling and Buttery (1978), Stern and Hoover (1979), Siddons et al. (1982) and Theurer (1982).

D-alanine (DAL) has been proposed recently as a marker of bacterial N in digesta of ruminants (Garrett et al., 1982). D-alanine is found only in peptidoglycan and teichoic acid moieties of bacterial cell walls; normally it is not present in eukaryotic cells (Schliefer and Kandler, 1972). Unlike DAP, DAL is found in all bacteria containing peptidoglycan. However, content of DAL in cells varies according to cell type and amount of peptidoglycan in cells, a disadvantage to its use as a bacterial marker (Buttery and Cole, 1977; Ling and Buttery, 1978).

Rapid ruminal development of young calves fed dry feed from 1 wk of age, as indicated by ruminal pH, concentrations of ruminal volatile fatty acids (VFA) and feed intake (Quigley et al., 1985) suggested that the proportion of bacterial N in abomasal N would change with increasing dry feed intake. Changing proportions of bacterial N should permit adequate comparison of bacterial markers. Our objective was to compare DAL and DAP for estimating bacterial

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N in total abomasal N in calves fed diets of complete pelleted starter or unpelleted starter plus hay from 2 to 11 wk of age.

### Experimental Procedure

Sixteen Holstein bull calves were assigned to a factorial arrangement of starter (complete pelleted vs unpelleted starter plus hay) and weaning age (4 vs 8 wk) as reported by Quigley et al. (1985). Calves were fitted with ruminal and abomasal cannulas at an average of 1 wk of age. Ruminal fluid and abomasal digesta were collected twice weekly from each calf and pooled to form weekly samples from 2 to 11 wk of age as in Quigley et al. (1985). Ruminal fluid was strained through two layers of cheesecloth, and approximately 200 ml were centrifuged at 1,500 × g to remove feed particles. The supernatant fluid was transferred to clean bottles and centrifuged at 26,500 × g; the resulting precipitate was resuspended in 200 ml methanol and recentrifuged at 26,500 × g. Precipitated bacterial cells were dried in a convection oven at 60° C to a constant weight, ground through a .64-mm screen and stored in glass vials until analyzed (Quigley et al., 1985). Feed intake, growth rates, ruminal VFA and amino acids in ruminal bacteria and abomasal digesta were reported previously (Quigley et al., 1985).

Nitrogen in bacterial cells and abomasal digesta were determined in duplicate by Kjeldahl process. At each week, samples of isolated bacterial cells and abomasal digesta obtained from calves receiving similar treatments were pooled, resulting in composite samples of bacterial cells and abomasal digesta for each treatment and week. These composite samples were analyzed for DAP and DAL. Diaminopimelic acid was analyzed as reported by Quigley et al. (1985). Samples for DAL analysis were hydrolyzed by addition of 6N HCl to approximately 50 and 100 mg of bacterial cells and abomasal digesta, respectively, in the ratio of 1 ml HCl/20-mg sample. Test tubes were evacuated, sealed, incubated at 108° C for 24 h and dried over NaOH pellets and $H_2SO_4$. Residues were dissolved in 2 ml (bacteria) or 1 ml (digesta) of $H_2O$, filtered through a .22-μm filter and stored at 4° C until analyzed.

D-alanine in hydrolyzed samples was determined in triplicate by a modification of Garrett et al. (1982). D-alanine released by hydrolysis was oxidatively deaminated in test tubes by adding 100 μl of hydrolysate to 1.58 ml oxygenated sodium pyrophosphate buffer (26.21 g/liter $H_2O$), 5 μl flavin adenine nucleotide solution[6] (1 mg/ml $H_2O$), 10 μl catalase[7] (.5 mg/ml $H_2O$) and 10 μl D-amino acid oxidase[6] (5 mg protein/ml). Contents were mixed and tubes were placed in a 39° C water bath for 2 h. Every 20 min, tubes were bubbled with oxygen for 5 s. Pyruvate formed was reduced by adding 1 ml of deamination mixture to 1.96 ml of triethanolamine buffer[6] (.2 M/liter, pH = 8.0, product 665–5) with ethylenediaminetetraacetic acid and 60 μl nicotinamide adenine dinucleotide[6] (10 mg/ml) in a spectrophotometer cuvette. Contents of the cuvette were mixed and allowed to equilibrate; absorbance was determined at 340 nm. Fourteen μl of lactate dehydrogenase[7] then were injected and mixed, and absorbance was determined again after 90 s. Change in absorbance was corrected for endogenous pyruvate by repeating analysis in triplicate with D-amino acid oxidase replaced by water. Standards were prepared by diluting DAL to concentrations of .5, .4, .3, .2, .1, and 0 mg/ml. Standard curves were computed before and after each series of 20 unknowns was analyzed.

Means for bacterial and abomasal N were analyzed in a split-plot experimental design with treatment as main plot and age as subplot (Steel and Torrie, 1960). Nitrogen data are least-squares means. Bacterial and abomasal DAL and DAP each were compared by paired t-test at each week and pooled for all weeks (SAS, 1982). Effect of treatment on DAL and DAP concentration in abomasal digesta and bacterial cells was not analyzed statistically due to pooling of samples before analysis.

### Results and Discussion

Bacterial N in isolated bacterial cells was not affected by age of calf or treatment ($P > .05$); it ranged from 6.9% to 9.1% and averaged 8.2% of dry matter (DM). Bacterial N from mixed ruminal bacteria has been reported to be constant under a variety of conditions, although McAllan and Smith (1977) reported changes in protein content of ruminal bacteria with time after feeding. Smith and McAllan (1974) reported little difference in N content of ruminal bacteria from sheep and cows fed

[6] Sigma Chemical, St. Louis, MO.
[7] Worthington Biochemicals, Millipore Corp., Freehold, NJ.

varying amounts of concentrate and roughage. Average N in ruminal bacteria was 7.2% of DM in sheep and 7.3% in cows (Smith and McAllan, 1974).

D-alanine N in isolated bacterial cells averaged 7.0 mg/g N and ranged from 4.5 to 8.3 mg/g bacterial N (Figure 1). Garrett et al. (1982) reported 10.1 mg DAL N/g N in ruminal bacteria isolated from mature cattle fed alfalfa hay, corn grain, oat straw, soybean meal and urea. Protein in bacterial cells in their study averaged 36.5% of DM. Griefe et al. (1985) reported 3.8 mg DAL N/g bacterial N in cows fed barley, grass silage, and soybean meal; DAL was determined by gas-liquid chromatography.

Concentration of DAP N in isolated bacterial cells averaged 5.4 mg/g N, similar to bacteria isolated from cattle (El-Shazly and Hungate, 1966; Garrett et al., 1982; Theurer, 1982) and calves (Nikolic and Jovanovic, 1973), but lower than that in ruminal bacteria isolated by others (Hutton et al., 1971; Cockburn and Williams, 1984; Rahnema and Theurer, 1986). Range of DAP N (3.7 to 7.3 mg/g N; Figure 1) was slightly less than the range (4.7 to 9.5 mg/g N) cited by Cockburn and Williams (1984). At 2 to 5 wk of age, DAP N concentration was low (4.5 mg/g N), probably due to incomplete establishment of the bacterial flora in calves at this age. That DAL N concentration in isolated bacterial cells was greater ($P < .01$) than DAP N was expected; DAL is normally found in greater concentration in bacterial cells. Many species of ruminal bacteria containing peptidoglycan (and thus DAL) do not contain DAP. Data of Hoogenraad and Hird (1970) indicate more DAL than DAP in isolated ruminal bacteria from sheep fed grass. Content of DAP N in ruminal bacteria has been discussed by Work and Dewey (1953).

Weekly mean concentration of DAL N in isolated bacterial cells increased from 4.5 mg/g N at 2 wk of age to 8.3 mg/g N at 3 wk of age; thereafter concentration was variable until the study ended (Figure 1). Corresponding weekly means for DAP varied with DAL N concentration after increasing from 3.7 mg/g N at 2 wk of age to 4.9 mg/g N at 5 wk of age. From 5 wk of age to the end of the trial, both DAL N and DAP N varied similarly. Variation in DAL and DAP in isolated bacterial cells did not appear to be caused by DAL, DAP or N analyses. Nonrepresentative sampling, or changing populations of ruminal bacteria in young calves, may account for this variation. Ling and Buttery (1978) reported wide variation in concentration of DAP N in isolated ruminal bacteria, attributed to the wide range of ruminal environments existing during the experiment. Similarity of pattern of both DAL N and DAP N in isolated bacterial cells (Figure 1) suggests that, although populations of bacteria in young calves changed with both advancing age and increasing feed intake, ratio of DAL to DAP in peptidoglycan of bacterial cells did not vary markedly except

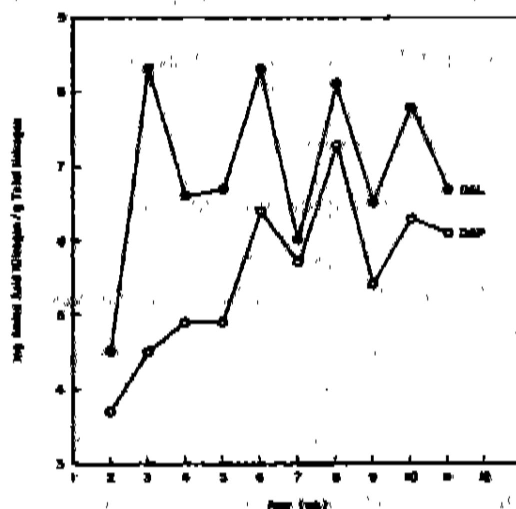


Figure 1. Weekly mean D-alanine (DAL) and diaminopimelic acid (DAP) in ruminal bacteria of calves from 2 to 11 wk of age.

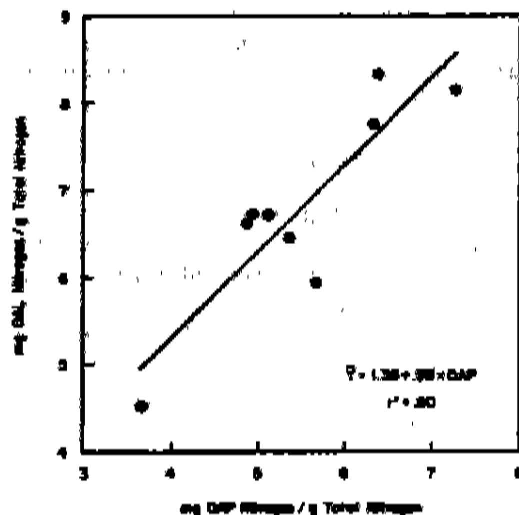

Figure 2. Scatter diagram of D-alanine (DAL) and diaminopimelic acid (DAP) in ruminal bacteria of calves from 2 to 11 wk of age.

at 3 wk of age (Figure 2). Ratio of DAL to DAP at 3 wk of age deviated from regression of DAL on DAP ($P < .07$) and was excluded from the regression in Figure 2. D-alanine and DAP in isolated bacterial cells with and without wk 3 correlated .66 and .89, respectively. Ruminal bacteria of very young calves may vary markedly from bacteria typical of mature ruminants and may change during development of mature ruminal function (Anderson et al., 1986). It is probable that bacteria isolated at wk 3 contained a large portion of these bacteria. Content of other amino acids in ruminal bacteria were constant throughout the study (Quigley et al., 1985) suggesting that the pattern of amino acids contributed by ruminal bacteria was constant throughout ruminal development. Marked fluctuation in content of DAL N and DAP N in isolated bacterial cells illustrates clearly the necessity of measuring marker content in bacteria when estimating bacterial contribution to total N reaching the lower gut.

Nitrogen in abomasal digesta averaged 3.9% of DM and decreased ($P < .05$) from 4.4% to 3.4% of DM from 2 to 6 wk of age, respectively. Nitrogen increased from 6 to 7 wk of age; thereafter it was relatively constant. Decreased N in abomasal digesta reflects lower N in starters and hay than in milk.

Concentration of DAP N in abomasal digesta averaged 2.4 mg/g N and ranged from .6 to 3.5 mg/g N at 2 and 8 wk of age, respectively (Figure 3). As expected, the amount of bacterial marker in abomasal digesta increased with increasing starter intake. It is likely that fermentation in the rumen of young calves fed complete diets was limited by available substrate during the trial. Weekly mean abomasal DAP N concentration correlated .89 with increasing starter intake and was indicative of maturing ruminal function.

Concentration of DAL N in abomasal digesta was greater than that of DAP N ($P < .01$) at all weeks and averaged 5.2 mg/g N. Weekly mean concentration of DAL N in abomasal digesta increased from 3.3 mg/g N at 2 wk of age to 5.4 mg/g N at 6 wk of age (Figure 3) and correlated .61 and .57 with concentration of DAP N in abomasal digesta and starter intake, respectively. At wk 7 and 9, concentration of DAL N in abomasal digesta exceeded that in isolated bacterial cells; abomasal DAL N approached bacterial DAL N at 11 wk, also.

Concentration of marker in digesta might be greater than that in bacteria if cells lyse between the rumen and digesta sampling site with a resulting increase in bacterial cell wall content after lysis (Nikolic and Jovanovic, 1973; Buttery and Cole, 1977). However, in such a case, concentration of DAP N also would be expected to increase dramatically because both are constituents of cell walls. Ratio of DAL to DAP in ruminal bacteria was not different except at 3 wk of age (Figure 2). Therefore, it is not likely that variation in DAL N at wk 7, 9 and 11 was due to changes in bacterial cells or to changing populations of bacteria. A scatter diagram of abomasal DAL and DAP (Figure 4) indicates considerable variability around a center line. Greater variability in ratio of DAL to DAP in abomasal digesta compared with isolated bacterial cells suggests that some error must be associated with analysis of one of the markers in abomasal contents.

We encountered a large amount of variation within samples when analyzing DAL in abomasal digesta. Coefficients of variation of DAL (mg/g DM) were 42% and 29% in abomasal digesta and in isolated bacterial cells, respectively. Further, abomasal digesta at wk 7, 9 and 11 were analyzed approximately 3 mo after other abomasal samples were analyzed. Possibly, alanine racemase, an enzyme responsible for isomerization of L-alanine to the D-isomer in the synthesis of bacterial cell walls, might have increased DAL concentration during storage. Hoogenraad and Hird (1970) reported the

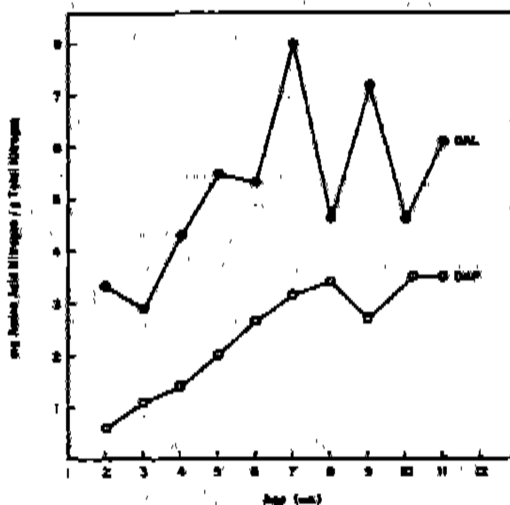


Figure 3. Weekly mean D-alanine (DAL) and diaminopimelic acid (DAP) in abomasal digesta of calves from 2 to 11 wk of age.

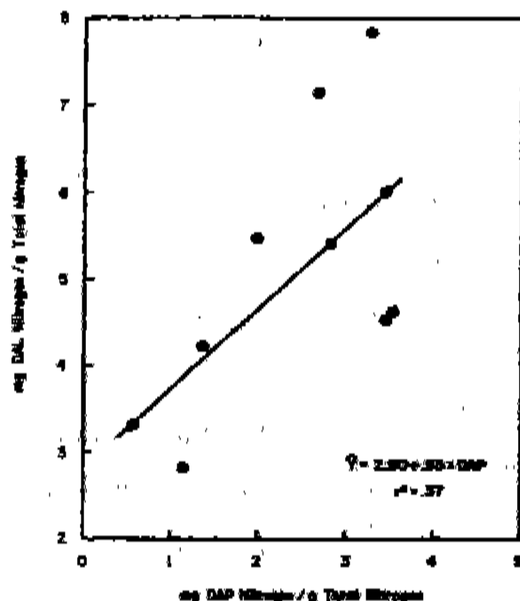

Figure 4. Scatter diagram of D-alanine (DAL) and diaminopimelic acid (DAP) in abomasal digesta of calves from 2 to 11 wk of age.

presence of active alanine racemase in cell walls of freeze-dried bacteria isolated from sheep fed grass.

Bacterial N in total abomasal N estimated by DAL was greater than that estimated by DAP ($P < .01$), averaging 77% and 44%, respectively. Weekly mean bacterial N in total abomasal N as estimated by DAP increased from 23% at 2 wk of age to peak at 68% at 11 wk of age (Figure 5). Estimates by DAP correlated well with increasing starter intake (.92) and followed the expected pattern of ruminal development. Estimates of bacterial contribution by DAL, on the other hand, were erratic and did not correlate well with increasing starter intake (.33). Weekly estimates at wk 7 and 9, and individual estimates at week 11, exceeded 100% (Figure 5). Griefe et al. (1985) used DAL as a marker of bacterial N in total duodenal N and reported estimates of quantity of total microbial N that were significantly greater than those estimated by $^{35}$S. Some estimates of quantities of microbial N entering the small intestine based on DAL and D-glutamic acid (also tested in the study) exceeded the total quantities of non-$NH_3$-N entering the small intestine (Griefe et al., 1985). Because $^{35}$S serves as a marker for both bacterial and protozoal N, and DAL detects bacterial N only, estimates by DAL appeared less realistic (Griefe et al., 1985). The authors proposed that DAL of feed origin escaped fermentation within the rumen and thus artificially inflated estimates of bacterial N entering the small intestine. In the present study, DAL of feed origin was assumed to be negligible because no fermented feedstuffs were fed; however, we did not analyze our feed. Racemization of D-amino acids during acid hydrolysis of samples of digesta and bacteria have been reported (Hoogenraad and Hird, 1970; Griefe et al., 1985). Extents of racemization reported were 1.87% and 3% to 5%. Though small, these errors will affect estimates by the DAL procedure.

Numerous comparisons of methods for separating digesta into bacterial, protozoal, microbial and dietary N have been reported (Nikolic and Jovanovic, 1973; Ling and Buttery, 1978; Smith et al., 1978; Cockburn and Williams, 1984; Rahnema and Theurer, 1986). Diaminopimelic acid generally underestimates total microbial N flow to the lower gut compared to other methods, because it does not account for the protozoal contribution to microbial flow (Ling and Buttery, 1978; Siddons et al., 1982). On the other hand, Siddons et al. (1982) reported greater proportions of microbial N were estimated by DAP than by $^{35}$S or $^{15}$N in sheep.

Excellent correlation, which we noted, between bacterial N in abomasal N estimated by DAP and starter intake suggests that the DAP

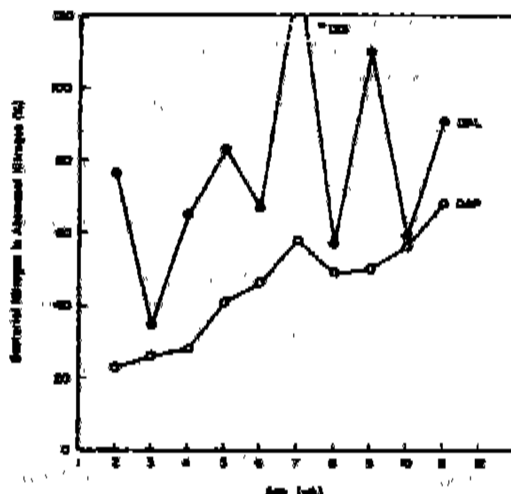


Figure 5. Weekly mean contribution of bacterial N in abomasal N as estimated by D-alanine (DAL) and diaminopimelic acid (DAP) in calves from 2 to 11 wk of age.

technique was adequate to allow inferences to be made regarding ruminal development, as made by Quigley et al. (1985). Correlation between estimates of bacterial N in abomasal N estimated by DAL and starter intake suggests that DAL was not a satisfactory marker.

D-alanine correlated with DAP in isolated bacterial cells from calves fed commercial starters and weaned at 4 or 8 wk of age. However, as a result of variability in abomasal DAL concentrations, due to the presence of alanine racemase in abomasal digesta, racemization during hydrolysis and(or) procedural variability, DAL was not acceptable marker of bacterial N in this study.

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