Nitrogen source and concentration affect utilization of glucose by mixed ruminal microbes in vitro¹

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ABSTRACT

The availability of rumen-degradable protein (RDP) changes the use of carbohydrates by ruminal microbes. However, the effects of RDP on the simultaneous use of carbohydrate and formation of microbial products are not well described, although such information is needed to understand the potential effect on nutrient supplies for ruminants. The objective of this in vitro study was to compare the effects of different levels of RDP (0.15, 0.31, 0.46 g of N/L) from tryptone (Tryp) or urea (Ur) on product formation from glucose in fermentations with mixed ruminal microbes. The study had a randomized complete block design with 2 replicated fermentation runs and destructive sampling at 0, 0.5, 1, 2, 3, 4, and 5 h. All rates given are first-order rate constants. Glucose disappearance rates and organic acid carbon (C) production rates tended to be or were greater for Tryp (0.64 and 0.58 h⁻¹) than for Ur (0.51 and 0.22 h⁻¹), respectively, but did not differ by N level. Maximum detected microbial N production was 67% greater for Tryp (2.35 mg) than for Ur (1.41 mg), which did not differ from the basal medium (1.47 mg). The pattern of glycogen accumulation over time tended to differ between Tryp and Ur: glycogen peaked and declined earlier in the fermentations with Tryp, resulting in less glycogen remaining at 5 h with Tryp (7.2 mg) than with Ur (11.0 mg). At the point of maximum microbial N accumulation, Tryp and Ur did not differ in the amount of glucose C used (29.4 and 28.9 mg), but did differ in the amounts of cell C (10.1 and 6.0 mg), organic acid C (17.4 and 13.8 mg), glycogen C (3.81 and 6.07 mg), and total microbial product C (35.4 and 29.6 mg) present. This resulted in increased efficiency

for Tryp compared with Ur for cell C produced per used glucose C, corrected for glycogen C (0.40 and 0.27 mg/mg), and it resulted in a tendency for increased yield of cell C per organic acid C (0.59 and 0.44 mg/mg). Total product C exceeded used glucose C for Tryp, likely because of incorporation or fermentation of C from the provided AA. Overall, RDP source altered the temporal patterns of glucose use and the patterns and amounts of microbial product formation.

Key words: protein, glucose, rumen, fermentation

INTRODUCTION

Glucose is a monosaccharide commonly found in fresh legume and grass forages (1 to 5% of DM; Smith, 1973), molasses (1 to 11%; Dionex Corp., 2003), dextrose (100%), and at varying concentrations in byproduct feedstuffs (e.g., 4 to 9% of DM in almond hulls; M. B. Hall, unpublished data). It is reported to be among the most rapidly fermented carbohydrates, with ruminal rates of disappearance of up to 734% h⁻¹ in vivo (Weisbjerg et al., 1998). However, disappearance may not equate to fermentation. Both ruminal protozoa (Oxford, 1951) and bacteria (Gong and Forsberg, 1993) can convert glucose to the intracellular storage polysaccharide, glycogen. Production of this α -(1,4), α -(1,6)-linked glucan delays fermentation of the substrate and requires input of 1 ATP per glucose for synthesis (Stouthamer, 1973). Increases in glycogen formation could reduce microbial protein yield because of reductions in ATP available for protein synthesis.

The availability of RDP alters how ruminal microbes utilize readily available carbohydrate and energy from feeds. The α -dextran (glycogen) content of ruminal bacteria declined by 33 to 45% when dietary protein was increased by adding casein or urea to the hay/straw/flaked maize diets of weaned bull calves (McAllan and Smith, 1974). Lactic acid, which is associated with more rapid flux of carbohydrate through glycolysis (Counotte and Prins, 1981), was increased in the rumens of lactating cows that received a greater proportion of dietary CP as RDP (Hall, 2013). The provision of AA increased the growth efficiency of *Streptococcus*

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bovis and decreased the specific rate of heat production (energy spilling; Russell, 1993). However, despite these associations, little quantitative information is available about the effects of different levels and types of RDP on the time courses of carbohydrate utilization and product formation, and the balance between them.

In vitro studies are often used to investigate the utilization of substrates by microbes, but are not equivalent to in vivo studies for describing what actually happens in the animal. The utility of in vitro fermentations rests in their use as model systems to study specific questions related to the in vivo ruminal system. In vitro studies are conducted if the in vivo system is too complex to allow evaluation of the hypotheses and measures investigated; in vivo studies are typically more suitable for evaluating total diets than specific feed fractions. To put this in perspective, in vitro models may be seen as similar to mathematical models: “Essentially, all models are wrong, but some are useful” (Box and Draper, 1987). Regarding the relationship of model systems to the actual system of interest, statistician George Box commented: “Now it would be very remarkable if any system existing in the real world could be *exactly* represented by any simple model,” and “For such a model, there is no need to ask the question ‘Is the model true?’ If ‘truth’ is to be the ‘whole truth’ the answer must be ‘No.’ The only question of interest is ‘Is the model illuminating and useful?’” (Box, 1979). These statements about mathematical models apply to in vitro models used to explore in vivo systems. In vitro findings can provide information about specific aspects of a system to help us refine our understanding and hypotheses, but they must eventually be related to and evaluated in the context of the in vivo system they attempt to describe.

The objective of this study was to evaluate the effect of the type and amount of supplemented RDP on substrate use, product formation, and fermentation kinetics in fermentations of glucose by mixed ruminal microbes. This mixed-culture study was performed in vitro.

MATERIALS AND METHODS

Fermentations

Five treatments of different nitrogen (N) types and concentrations were applied in duplicate fermentation runs using modified Goering and Van Soest (1970) media in sealed borosilicate glass fermentation tubes as described by Hall and Weimer (2016). Each tube contained 20 mL of medium, 1 mL of reducing solution, 0.5 mL of autoclaved glucose solution or water, and 5 mL of ruminal inoculum. Purified glucose (G7021;

Sigma-Aldrich, St. Louis, MO) in a 159 mg/mL autoclaved solution was used to deliver 79.5 mg of glucose per tube. Media were used to deliver the protein treatments. The basal medium was modified from Goering and Van Soest (1970) to contain no tryptone (basal; pancreatic digest of casein, T9410; Sigma-Aldrich). The basal medium plus reducing solution supplied 3.54 mg of N from ammonium bicarbonate and 0.56 mg of N from cysteine-HCl in each tube. The other 4 treatments were prepared from the basal medium: basal + 1.17 g of tryptone/L (**TrypL**), basal + 2.34 g of tryptone/L (**TrypH**), basal + 0.329 g of urea/L (**UrL**), or basal + 0.658 g of urea/L (**UrH**), where L and H indicate low and high levels of N. The basal medium and treatment media provided 0.15, 0.31, 0.46, 0.31, and 0.46 g of N/L in the 26.5-mL liquid volume in each tube, for basal, TrypL, TrypH, UrL, and UrH, respectively. Vessels were incubated in tube racks in an incubating orbital shaker at 39°C and 160 rpm (Innova 40 bench top incubator shaker, 19 mm orbit; New Brunswick Scientific, Edison, NJ). Fermentation vessels were destructively sampled at 0, 0.5, 1, 2, 3, 4, and 5 h. Three replicate vessels for each glucose × N treatment were included at each sampling time after 0 h. Individual replicates were analyzed for accumulated microbial N, glycogen, or organic acids/residual carbohydrate. Nine tubes with no substrate (fermentation blanks) in basal medium were collected at 0 h, and the same 3 analyses were applied to 3 tubes each. Two fermentation blanks for each treatment were included at each time point >0 h for organic acid/residual carbohydrate analysis.

Inoculum for each fermentation was obtained from 2 ruminally cannulated, lactating Holstein cows maintained under protocols approved by the University of Wisconsin College of Agriculture and Life Sciences Animal Care and Use Committee. Donor cows were fed a TMR consisting (on a DM basis) of 25.2% corn silage, 24.1% alfalfa silage, 6.4% whole linted cottonseed, and 44.3% mixed concentrate, supplemented with vitamins and minerals to meet NRC (2001) recommendations. Daily DMI averaged 23.8 ± 1.4 kg/cow per day. Dextrose as 1% of diet DM was mixed into the TMR in the 15 d before inoculum collection. Ruminal contents, obtained manually via the ruminal cannula primarily from the ventral portion of the rumen of each cow within 2 h after feeding, were strained through 4 layers of cheesecloth and the ruminal liquor maintained under CO₂. Equal volumes of ruminal liquor from each cow were measured and filtered through an additional 4 layers of cheesecloth, with ruminal fluid from both cows blended together in a common flask maintained at 39°C in a water bath with CO₂ bubbled continuously through the liquor. Inoculum pH values in the fermentation runs were 5.97 and 5.89, approximately

the average ruminal digesta pH of the 2 cows used in each run.

At each sampling time, harvested tubes were placed immediately on ice and chilled for a minimum of 10 min to stop fermentation. At each sampling hour, pH was measured in 1 tube for each treatment and in 2 fermentation blanks for each N treatment. Tubes were inverted to mix and uncapped just before pH was measured; pH did not decline below 6.47 in any vessel. Total contents of each of these tubes were divided between two 20-mL scintillation vials and stored at -20°C until analyzed for organic acids and residual soluble carbohydrate.

Samples were prepared and analyzed for accumulated microbial N and accumulated microbial glycogen as described in Hall and Weimer (2016). For each of these measurements, 1 tube was analyzed for each treatment at each sampling hour >0 h, and 3 fermentation blanks were analyzed at 0 h.

Analyses

Lyophilized fermentation pellets were analyzed for N (Dumas combustion method, VarioMax CN; Elementar Americas Inc., Mt. Laurel, NJ). Nitrogen accreted by microbes was calculated as the hourly sample values minus the average value of the 0-h fermentation blanks to correct for N introduced with the inoculum. The 0-h values were 4.85 mg of N for the first fermentation and 6.10 mg of N for the second. Organic acid concentrations in samples of medium were analyzed by HPLC (Weimer et al., 1991). Values for organic acids were corrected for the average of the fermentation blanks for a given N treatment for the sampling hour. Total organic acid values were the sum of formate, acetate, propionate, butyrate, valerate, and D- + L-lactate (the form of lactate is not differentiated by HPLC). Production of CH_4 and CO_2 were estimated from organic acid production according to the stoichiometric equations of Hungate (1966). These equations assume that no organic acid was produced from substrates other than carbohydrate.

Accumulated glycogen α -glucan analysis was performed by alkaline lysis of cell pellets with 0.2 M NaOH, followed by enzymatic hydrolysis and detection of glucose (Hall and Hatfield, 2016). The calculation for α -glucan or glycogen was detected glucose $\times 0.9$. Net accumulated glycogen was calculated as the hourly sample values minus the average value of the 0-h fermentation blanks, which were 8.18 and 13.02 mg for the first and second fermentations, respectively. The 0-h fermentation blank allowed correction for α -glucan from microbes or undigested feed introduced by the ruminal inoculum.

Residual carbohydrate soluble in the medium was analyzed using the phenol-sulfuric acid assay (Dubois et al., 1956) with 5% phenol solution and half the reagent amounts per tube (0.5 mL of 5% phenol, 0.5 mL of sample solution, 2.5 mL of concentrated sulfuric acid); vortexing of samples was performed after each reagent addition except the first. Samples, including fermentation blanks, were thawed at room temperature and inverted to mix, and approximately 1.5 mL was transferred to a 2-mL microcentrifuge tube. Samples were incubated at 60°C for 20 min in a recirculating water bath to completely solubilize all residual soluble carbohydrate. Tubes were inverted to mix, centrifuged at $12,000 \times g$ for 10 min at ambient temperature, and then allowed to sit on the bench for 5 to 10 min for temperature equilibration. Samples of the clarified supernatant were diluted with distilled water as needed and analyzed in triplicate for carbohydrate (Dubois et al., 1956). Glucose was used as the standard for both treatment and fermentation blank vessels. For each sampling hour, a fermentation blank value for that hour was subtracted from values for treatment samples.

Calculations

Branched-chain VFA were the sum of isovalerate, isobutyrate, and 2-methylbutyrate. Milligrams of carbon (C) in organic acids were calculated as the millimolar concentration of an acid $\times 0.0265 \text{ L} \times 12 \text{ mg/mmol}$ of $\text{C} \times 1, 2, 3, 3, 4$, or 5 for the number of moles of C per mole of acid in formate, acetate, propionate, lactate, butyrate, or valerate, respectively. Total milligrams of C in organic acids per sample vessel was calculated as the sum of the values for the 6 organic acids. Milligrams of non-glycogen microbial cell C was estimated as [accumulated microbial N, $\text{mg}/(14 \text{ mg/mmol of N})] \times (5 \text{ C/1 N}) \times 12 \text{ mg/mmol of C}$. This calculation was based on the report of Pavlostathis et al. (1988), who described an average chemical composition of non-glycogen, ash-free, microbial cell mass as $\text{C}_5\text{H}_7\text{O}_2\text{N}$. Carbon in glycogen and in α -glucan introduced with the inoculum were calculated as α -glucan or glycogen $\text{mg} \times 0.44 \text{ mg of C/mg of carbohydrate}$; 0.44 represented the proportion of C in α -glucan.

Lag times and first-order exponential rate constants for the production of organic acid C, microbial N, and glycogen and the disappearance of substrate were calculated using single-pool exponential equations (Weimer et al., 2000). For calculations with accumulated microbial N or glycogen, data after the detected maximum were omitted. All rate calculations were performed using TableCurve 2D, version 5.01 (Systat Software Inc., San Jose, CA).

Table 1. *P*-values for effects of treatment¹ and fermentation hour

Item	Treatment	Hour	Treatment × hour	Basal vs. others	SED ²
Residual glucose, mg	0.27	<0.01	1.00	0.22	6.37
Microbial N, mg	<0.01	<0.01	<0.01	<0.01	0.22
Glycogen, mg	0.14	<0.01	0.96	0.50	2.15
Organic acid C, ³ mg	<0.01	<0.01	0.57	0.06	2.37
Branched-chain VFA, mM	<0.01	0.11	0.21	0.21	0.14
pH of medium	<0.01	<0.01	0.47	0.96	0.04

¹Experimental treatments: basal; tryptone at 0.31 g of N/L; tryptone at 0.46 g of N/L; urea at 0.31 g of N/L; and urea at 0.46 g of N/L.

²SED = pooled standard error of the difference for treatment × hour.

³Carbon in organic acids as the sum of formate, acetate, propionate, butyrate, valerate, and lactate.

Statistical Analysis

References to “minimum” or “maximum” values refer to the smallest or largest values detected for a particular analyte in a fermentation run. The Dixon Q test for outliers (Rorabacher, 1991) was applied to the glycogen data for hour 2 in the first fermentation, resulting in a single data point (UrH) being removed.

The data were analyzed as a randomized complete block design. Data were evaluated in 2 ways to determine the overall effects of treatment, as well as the effects of added N source, N level, and their interaction. The overall effects of treatment were evaluated with models that included treatment (basal, TrypL, TrypH, UrL, and UrH) as independent variables and fermentation run as a random variable. The data set of the entire time course also included terms for sampling hour and treatment × time interaction, and is reported in Table 1. An orthogonal contrast comparing basal with all other treatments was applied to the data analyzed by treatment. The Tukey-Kramer mean separation method was applied when the contrast of basal vs. other treatments was significant, to allow for further interpretation of the result.

Basal data were omitted from analyses that evaluated the effect of type and level of added N from tryptone and urea treatments.

The resulting data sets were analyzed using a model that contained type (tryptone or urea), level (low or high), and the type × level interaction as independent variables; the data set of the entire time course also included terms for sampling hour and the 2- and 3-way interaction terms for type, level, and sampling hour; results are reported in Table 2. Interaction terms were removed from the model if $P > 0.25$ for a term; removal was performed sequentially from highest- to lowest-order interaction terms. The response variables evaluated included the amount of microbial products formed; exponential rates and lag times; selected maxima, minima, and 5 h fermentation values; and microbial product C amounts, efficiency, and proportion of cell C of total product C at the time of maximum microbial N accumulation. These analyses were performed using the MIXED procedure of SAS, version 9.4 (SAS Institute Inc., Cary, NC). Additionally, *t*-tests were performed by fermentation run and treatment on the differences between C from used glucose and C in microbial products at each time point. The tests evaluated whether the difference between product C and used glucose C differed from zero. Values are reported as least squares means with standard errors of the difference (SED). Significance was declared at $P < 0.05$, and tendencies at $0.05 \leq P < 0.15$.

Table 2. *P*-values for effects of nitrogen type, amount, and fermentation hour¹

Item	Type	Level	Type × level	Hour	Type × hour	Level × hour	Type × level × hour	SED ²
Residual glucose, mg	0.03	0.60	0.77	<0.01	0.82	0.98	0.98	6.44
Microbial N, mg	<0.01	0.08	0.75	<0.01	<0.01	0.89	0.82	0.22
Glycogen, mg	<0.01	0.64	0.98	<0.01	0.10	0.98	1.00	2.13
Organic acid C, ³ mg	<0.01	0.74	0.22	<0.01	0.18	0.85	0.81	2.49
Branched-chain VFA, mM	<0.01	0.98	0.66	0.04	0.17	0.30	0.64	0.15
pH of medium	<0.01	0.048	0.36	<0.01	0.01	0.95	0.84	0.04

¹Type = tryptone or urea; level = low (0.31 g of N/L of medium) and high (0.46 g of N/L of medium); hour = fermentation hour.

²SED = pooled standard error of the difference for type × level × hour.

³Carbon in organic acids as the sum of formate, acetate, propionate, butyrate, valerate, and lactate.

RESULTS AND DISCUSSION

Across all response variables, the greatest treatment effect was related to N source, with few effects related to the levels of N (e.g., Table 2). Although N levels were evenly spaced to evaluate the pattern of response, the N concentrations (g/L) used may have been too high to detect significant differences. After the present study and other related studies on fermentations of water-soluble carbohydrates were completed, work was found on pure cultures of rumen microbes that showed responses of cell protein production to increasing concentrations of peptides or AA to be logarithmic or power functions in form at a given concentration of glucose (Cotta and Russell, 1982). In that study, increasing additions of N continued to increase microbial protein production, although the magnitude of response became much less when the ratio of glucose to tryptone decreased below 8:1. The smallest ratio of glucose to tryptone provided in the Cotta and Russell (1982) study was 1.0. The ratios in the present study for the low and high levels N additions from tryptone were 2.6 and 1.3, which, if analogous to the previous work, would be on an almost plateaued portion of the response curve. Designs of future comparisons should take into account the non-linear responses of microbes to added AA and peptides.

In the present study, inoculum donors were not adapted to urea or tryptone via their diet. Urea is supplied to the rumen endogenously, and tryptone is an isolated, water-soluble, laboratory-grade protein source with no matrix that could impede microbial access and use (J. Wallace, Rowett Institute, Aberdeen, UK, and P. J. Weimer, USDA-ARS, Madison, WI; personal communications). Further, ruminal supplementation of casein (230 g/d) in Nellore steers did not cause notable changes in the ruminal bacterial community composition (Bento et al., 2015), suggesting that introduction of an isolated protein source did not cause alteration in the existing microbial community. Theoretically, preadaptation of inoculum donors to natural feed protein sources would apply in adapting microbes to the utilization of the total feed matrix (carbohydrates, fats, and other components) to increase the availability of the feed protein.

Time Course and Kinetics Data

Glucose is utilized very rapidly by ruminal microbes; in vivo rates of disappearance in the rumen increased from 422% to 717 and 734% when cows were preadapted to sucrose or lactose in their diets (Weisbjerg et al., 1998). The increased rates could have been due to the presence of a larger population of glucose utilizers, fostered by the previous sugar supplementation. In

the present study, average glucose disappearance over time was greater with tryptone than with urea ($P = 0.03$); residual glucose in tryptone treatments fell below those of urea after 2 h of fermentation (Figure 1a). We observed no effect of N level ($P = 0.60$; Table 2). These results were reflected in a tendency for greater rates of glucose disappearance with tryptone than with urea ($P = 0.09$, Table 3). Lag times tended to be greater with tryptone than urea, but the differences were small ($P = 0.12$). Glucose disappearance and results for rate and lag time did not differ between the basal and other treatments ($P > 0.17$), and were unaffected by interactions of treatment with time ($P > 0.81$; Figure 1a, and Tables 1, 2, and 3). The rates of glucose disappearance in this in vitro study were substantially less than in vivo rates reported by Weisbjerg et al. (1998), and more similar to the 0.74 h^{-1} rate previously found for glucose in this fermentation system (Hall and Weimer, 2016) with complete Goering and Van Soest (1970) medium, which contains approximately 2.5 g of tryptone/L of medium.

Microbial cells represent a valuable source of AA for the cow, and microbial N production (microbial growth) showed some of the most marked treatment effects in this study. When microbes were provided with tryptone, N accumulation increased to a greater extent over time than with urea (N type $\times$ time interaction, $P < 0.01$), although we observed no effect on rate or lag time ($P > 0.18$, Table 3). Microbes grew more with tryptone, reaching microbial N maxima that were approximately 67% greater than the urea treatments ($P < 0.01$); the values for UrL, UrH, and basal did not differ ($P > 0.97$; Table 4). The greater microbial growth with tryptone over the other treatments offers a ready explanation for the greater glucose disappearance over time with tryptone: a greater microbial mass was available to consume the substrate. The tendency for an effect of N level ($P = 0.08$) was seen in the greater microbial N production in TrypH over TrypL in hours 2 through 4 of the fermentation; no similar consistent difference was apparent for UrH and UrL (Table 2, Figure 1b). These results were in agreement with studies demonstrating that ruminal microbes preferentially use AA over ammonia-N when given glucose in vivo (Hristov et al., 2005), and that mixed ruminal microbes given mixed soluble carbohydrates in vitro increased microbial cell production as the amount of casein provided to the culture increased (Russell et al., 1983). Across all treatment hours, the basal treatment differed from those with added N ($P < 0.01$) and had a substantially longer lag time ($P < 0.01$; Figure 1b, Tables 1 and 3). The lag phase in bacteria has been associated with trace metal availability (Rolfe et al., 2012), but it is not clear whether that finding relates to

the present study, in which macrominerals, trace minerals, and minerals in the inoculum were available to all cultures. Although we found statistical significance for other kinetic measures, the failure to detect differences in rates of microbial N accumulation may be due to variability in the calculated values (SED = 0.21).

Accumulation of glycogen, an intracellular storage polysaccharide, showed responses that were inverse to those noted for microbial N. The average net glycogen present across all hours of fermentation was 17% greater with urea than with tryptone (10.2 and 8.7 mg, respectively; $P < 0.01$), but the basal treatment

Figure 1. Patterns of substrate disappearance and microbial product appearance over time: (a) residual glucose; (b) accumulation of microbial N; (c) accumulated microbial glycogen; (d) organic acid carbon (formate + acetate + propionate + butyrate + valerate + lactate); (e) branched-chain VFA (BCVFA) concentrations; and (f) pH of medium. Treatments: O = basal medium; □ = TrypL; ■ = TrypH; Δ = UrL; ▲ = UrH; Tryp = tryptone, Ur = urea, L = low level (0.31 mg of N/L), H = high level (0.46 mg of N/L).

was not different from the others ($P = 0.50$; Table 1, Figure 1c). The basal treatment essentially showed the same temporal pattern as the urea treatments (Figure 1c). Glycogen levels in the urea treatments tended to remain elevated longer before declining than in the tryptone treatments (N type $\times$ time interaction, $P = 0.10$; Table 2). Glucose molecules are added to glycogen at a cost of 1 ATP per glucose (Stouthamer, 1973). Because glucose is simultaneously added to and mobilized from the glycogen pool (Prins and Van Hoven, 1977), the longer elevation of glycogen levels in the urea treatments may represent a longer period of glucose addition to glycogen and greater energetic costs of glycogen synthesis in those treatments. Although maximal glycogen amounts did not differ by treatment ($P > 0.15$), glycogen declined to lower values by the end of fermentation with tryptone than with urea ($P < 0.01$; Table 4, Figure 1c). The difference of 4 mg of glycogen remaining at 5 h of fermentation represented approximately 5% more glucose substrate used when tryptone was added. Dietary protein has been shown to affect glycogen storage in ruminal bacteria in vivo, with increases in dietary protein concentration decreasing the proportion of glycogen per unit of isolated dry bacterial cells (McAllan and Smith, 1974).

Organic acid C production showed effects of treatment and time, but not of their interaction. Compared with urea, the addition of tryptone resulted in greater average organic acid production across all hours ($P < 0.01$; Table 2) and a tendency for greater maximal organic acid C production ($P = 0.09$; Table 3). The rate

of organic acid C production tended to be greater with tryptone than urea ($P = 0.05$; Table 3), but tryptone treatments also had a greater lag time ($P = 0.01$). The greater production and rate of production of organic acids with tryptone corresponded with the increased disappearance of glucose and glycogen at earlier hours, as well as the likelihood that VFA were produced from fermentation of some of the added AA. Similarly, the provision of increased dietary concentrations of RDP to dairy cattle has been shown to increase in vivo ruminal organic acid amounts (Hall, 2013) and concentrations (Aldrich et al., 1993; Carruthers and Neil, 1997). Over the course of the fermentation, the basal treatment tended to differ from those with added N ($P = 0.06$; Table 1), but as with both the microbial N and glycogen results, the basal treatment largely appeared to overlay the urea treatments (Figure 1d). There was a tendency for a slightly longer lag time as N level increased ($P = 0.145$). Formate was detected at low concentrations, achieving a maximum of 2.57 mM and averaging 0.63 and 0.15 mM in the first and second fermentations, respectively.

At maximal organic acid C production, valerate C decreased with increasing N levels for both N sources ($P = 0.02$; Table 4), and tended to be greater with tryptone than with urea. Valerate can be produced from the fermentation of AA (El-Shazly, 1952), which could explain the increase with the addition of tryptone versus urea, but does not explain the decline with increasing N. Both acetate and butyrate C production tended to decrease ($P = 0.148$ and 0.12 , respectively) as N level

Table 3. Exponential rate constants (kd) and lag times

Item	Treatment (Trt) ¹					SED ²	Treatment <i>P</i> -values ³		Tryp vs. Ur <i>P</i> -values ⁴			
	B	TrypL	TrypH	UrL	UrH		Trt	B vs. Tryp + Ur	Type	Level	Type $\times$ level	SED ²
Glucose disappearance												
Lag, h	0.16	0.24	0.25	0.18	0.19	0.05	0.47	0.28	0.12	0.65	1.00	0.05
kd, h ⁻¹	0.50	0.63	0.65	0.51	0.51	0.06	0.18	0.18	0.09	0.87	0.85	0.07
Microbial N, mg												
Lag, h	1.86	0.55	0.76	0.93	0.64	0.28	0.04	<0.01	0.58	0.87	0.35	0.32
kd, h ⁻¹	0.35	0.38	0.60	0.68	0.39	0.21	0.46	0.36	0.78	0.85	0.19	0.22
Glycogen, mg												
Lag, h	0.00	0.00	0.07	0.00	0.08	0.06	0.50	0.46	0.90	0.12	0.92	0.06
kd, h ⁻¹	0.75	0.68	1.04	0.70	0.98	0.24	0.53	0.63	0.92	0.13	0.85	0.27
Organic acid C, ⁵ mg												
Lag, h	0.19	0.39	0.58	0.10	0.18	0.12	0.06	0.26	0.01	0.145	0.55	0.12
kd, h ⁻¹	0.50	0.53	0.64	0.27	0.18	0.17	0.20	0.53	0.05	0.95	0.52	0.20

¹Experimental treatments: B = basal, TrypL = tryptone at 0.31 g of N/L, TrypH = tryptone at 0.46 g of N/L, UrL = urea at 0.31 g of N/L, UrH = urea at 0.46 g of N/L.

²SED = pooled standard error of the difference for comparisons of treatments or comparison of nitrogen types and levels.

³*P*-values for the comparison of the effects of all treatments, including results of the contrast basal vs. tryptone + urea.

⁴*P*-values for the evaluation of the effects of protein type (Tryp or Ur), level [low (0.31 g of N/L of medium) or high (0.46 g of N/L of medium)], and their interaction for tryptone and urea treatments; basal data were omitted from this comparison.

⁵Carbon in organic acids as the sum of formate, acetate, propionate, butyrate, valerate, and lactate.

Table 4. Selected detected minima, maxima, and 5 h end points for microbial products

Item	Treatment ¹				Treatment <i>P</i> -values ³			Tryp vs. Ur <i>P</i> -values ⁴			SED ²	
	B	TrypL	TrypH	UrL	UrH	SED ²	Trt	B vs. Tryp + Ur	Type	Level		Type × level
pH, minima	6.58	6.50	6.52	6.60	6.66	0.03	0.03	0.84	<0.01	0.17	0.42	0.03
Maxima	1.47	2.23	2.47	1.44	1.38	0.17	<0.01	0.04	<0.01	0.48	0.27	0.16
Microbial N, mg												
Glycogen	16.4	15.8	14.8	16.4	15.8	0.65	0.25	0.24	0.15	0.15	0.80	3.71
At 5 h, mg	9.42	7.24	7.18	10.7	11.4	0.95	0.03	0.73	<0.01	0.64	0.62	0.95
Gas C, mg	3.52	4.70	3.63	4.45	3.53	0.65	0.35	0.33	0.68	0.07	0.88	0.63
Lactate C, mg	4.02	1.27	10.9	1.17	0.77	5.89	0.48	0.92	0.34	0.38	0.36	6.57
Organic acid C, ⁵ mg	12.6	18.7	21.4	16.4	13.3	3.24	0.19	0.13	0.09	0.94	0.28	3.08
Organic acids (OA) at total OA maxima												
Acetate C, mg	5.05	6.05	3.86	5.94	5.14	1.12	0.42	0.84	0.52	0.148	0.48	1.22
Propionate C, mg	5.40	7.96	4.62	6.72	5.90	1.83	0.51	0.57	0.99	0.22	0.45	2.05
Butyrate, mg	1.98	3.35	2.27	2.96	1.91	1.01	0.59	0.47	0.53	0.12	0.98	0.89
Valerate C, mg	0.14	1.09	0.56	0.76	0.19	0.21	0.04	0.04	0.08	0.02	0.88	0.24
Lactate C, mg	0.05	0.28	10.1	0.00	0.16	6.30	0.50	0.63	0.36	0.37	0.40	7.02

¹Experimental treatments: B = basal, TrypL = tryptone at 0.31 g of N/L, TrypH = tryptone at 0.46 g of N/L, UrL = urea at 0.31 g of N/L, UrH = urea at 0.46 g of N/L.
²SED = pooled standard error of the difference for comparisons of treatments or the comparison of nitrogen types and levels.
³*P*-values for the comparison of the effects of all treatments, including results of the contrast basal vs. tryptone + urea.
⁴*P*-values for the evaluation of the effects of protein type (Tryp or Ur), level [low (0.31 g of N/L of medium) or high (0.46 g of N/L of medium)], and their interaction for tryptone and urea treatments; basal data were omitted from this comparison.
⁵Carbon in organic acids as the sum of formate, acetate, propionate, butyrate, valerate, and lactate.

increased, but this appeared to be an artifact of elevated lactate production in TrypH at the organic acid maximum in 1 fermentation (Table 4). All treatments, including the basal treatment, contained measurable lactic acid at some point, but the time of maximal production varied across fermentations and treatments (data not shown). Lactic acid is transient, and the organic acids in the treatments reflect the presence or metabolism to other VFA of previously produced lactic acid (Hino and Kuroda, 1993). The tendency for the maximum C in methane and carbon dioxide to change with N level ($P = 0.07$; Table 4) may also represent the effect of substantial lactic acid production in TrypH in 1 fermentation, because the stoichiometric equations used for estimation predict that no gas is produced with lactic acid (Hungate, 1966), a temporary condition until the lactate is metabolized.

The branched-chain VFA (BCVFA) available in the fermentation are of interest, because these breakdown products of protein are used by microbes in the de novo synthesis of AA from NPN (Allison and Bryant, 1963). The BCVFA concentrations were affected by treatment ($P < 0.01$) and time ($P = 0.11$ for all treatments; $P = 0.04$ for comparison of tryptone and urea treatments), but not by N level or interactions with time (Tables 1 and 2; Figure 1e). That the concentrations of BCVFA were greater with tryptone is not surprising, considering that the AA it provided could be broken down to produce BCVFA. At 1 h of fermentation, BCVFA decreased markedly in the urea treatments (Figure 1e) corresponding with the start of increased microbial N accumulation in those treatments (Figure 1b).

Medium pH declined over time ($P < 0.01$) as was expected with increasing accumulations of organic acids. Urea treatments gave higher pH values over time ($P < 0.01$ for N type, $P = 0.01$ for N type $\times$ hour; Table 2, Figure 1f) and for pH minima ($P < 0.01$; Table 4) than tryptone treatments, likely the result of hydrolysis of urea to release ammonia, which is alkaline, and lower production of organic acids on the urea treatments. The greater level of added N gave higher pH values than the lower level ($P = 0.048$; Table 2).

Carbon at Cell N Maxima: Products, Efficiencies, and Balance

Efficiencies for products formed and substrates used were evaluated on a C basis to reduce the noise introduced by varying amounts of oxygen and hydrogen entering the conversions (Table 5). At the detected maxima for microbial N, we observed no difference among added N treatments in the amount of carbohydrate substrate C used, whether expressed as used glucose C ($P > 0.25$) or used glucose C minus the amount of gly-

cogen C present ($P > 0.15$). There was a tendency for less glucose C to be used in the basal treatments than when additional N was added ($P = 0.11$). Consistent with the lower use of substrate, the total C in products as the sum of microbial cells, glycogen, organic acids, and estimated gas production also tended to be less for the basal treatment ($P = 0.05$).

Despite equivalent amounts of used glucose C, more total C was incorporated into products with tryptone than with urea (35.4 and 29.5 mg of C, respectively; $P = 0.02$), with respective averages of 6.0 and 0.6 mg of product C in excess of the glucose C consumed ($P = 0.05$). Both the inoculum and tryptone could provide C in addition to that provided by glucose. The substantially greater cell production supported with the addition of tryptone likely represented direct incorporation of AA from tryptone, because microbes that utilize glucose can incorporate AA (Hristov et al., 2005). What is not clear is the degree to which the provision of AA spares C from other substrates to be fermented or converted into other molecules required to produce cells. There was a tendency for less product C to be formed as the N level increased (decrease from 34.3 to 30.7 mg of C; $P = 0.09$), but at the same time, the proportion of cell C in product C tended to increase (22.8 to 26.2%; $P = 0.11$).

Tryptone improved the efficiency of cell C production from glucose C over urea, but neither treatment affected the yield of organic acid or gas C. For each milligram of glucose C that was used but not converted to glycogen, tryptone addition gave a yield of 0.395 versus 0.269 mg for urea ($P = 0.04$; Table 4); AA that could be directly incorporated likely contributed to this improvement. Organic acid and gas C production, although not affected by N source ($P = 0.23$ and 0.58, respectively), tended to decline with increasing N levels ($P = 0.11$ and 0.14, respectively). The yield of glycogen C at the microbial N maxima tended to be greater with urea ($P = 0.11$), and reflects the greater amount of glycogen remaining in the cells, representing glucose that had not yet been fermented. The yield of cell C per unit of organic acid C tended to be greater with tryptone (0.59 mg/mg) than with urea (0.44 mg/mg; $P = 0.08$). This difference describes a 34% greater efficiency of cell production on a fermented carbohydrate basis. It may reflect greater availability of energy and C for cell growth based on reductions in the energy and C required for synthesis of AA and glycogen, and possibly energy available from fermentation of AA.

The differences noted between consumed glucose C and product C also appear in the overall C balance of the fermentations (Figure 2). The figure shows the dashed unity line representing C in consumed glucose, and the gray line above that represents the C provided

Table 5. Amounts of carbon (C) in substrate used, microbial products, and efficiency of product yield at the time of maximum detected microbial N accumulation

Item	Treatment (Trt) ¹					Treatment <i>P</i> -values ³			Tryp vs. Ur <i>P</i> -values ⁴		
	B	TrypL	TrypH	UrL	UrH	SED ²	Trt	B vs. Tryp + Ur	Type	Level	Type × level
Used glucose, mg	26.9	30.0	28.8	29.1	28.7	1.39	0.41	0.11	0.46	0.26	0.60
Used glucose – glycogen, mg	22.3	26.1	25.0	22.2	23.5	2.64	0.56	0.40	0.16	0.98	0.53
Cells, mg	6.31	9.56	10.59	6.18	5.82	0.68	<0.01	0.03	<0.01	0.51	0.22
Glycogen, mg	4.64	3.82	3.83	6.90	5.24	1.40	0.32	0.80	0.10	0.47	0.49
Organic acids, ⁵ mg	12.6	18.8	16.0	14.8	12.8	2.03	0.15	0.14	0.04	0.12	0.76
Estimated gas, mg	3.52	4.70	3.64	3.88	3.42	0.70	0.47	0.52	0.33	0.18	0.59
CH ₄ -C/CO ₂ -C	0.44	0.37	0.32	0.41	0.41	0.046	0.24	0.16	0.05	0.41	0.37
Total product, mg	27.1	36.8	34.0	31.7	27.4	2.47	0.06	0.05	0.02	0.09	0.69
Product – used glucose, mg	0.20	6.84	5.20	2.61	–1.36	2.70	0.15	0.22	0.05	0.23	0.62
Efficiency											
[Product/(used glucose – glycogen)]											
Cells, %	28.4	36.6	42.4	29.0	24.9	5.04	0.10	0.29	0.04	0.84	0.30
Organic acids, ⁵ %	56.8	71.8	63.8	67.0	54.8	6.82	0.24	0.23	0.23	0.11	0.72
Estimated gas, %	11.0	13.2	11.0	12.4	10.3	1.75	0.53	0.60	0.58	0.14	1.00
Glycogen/used glucose, %	17.4	12.8	13.3	24.1	18.3	5.75	0.41	0.94	0.11	0.55	0.51
Cells/organic acids, %	49.9	51.5	66.7	42.9	45.6	8.03	0.18	0.79	0.08	0.23	0.40

¹Experimental treatments: B = basal, TrypL = tryptone at 0.31 g of N/L, TrypH = tryptone at 0.46 g of N/L, UrL = urea at 0.31 g of N/L, UrH = urea at 0.46 g of N/L.

²SED = pooled standard error of the difference for comparisons of treatments or the comparison of nitrogen types and levels.

³*P*-values for the comparison of the effects of all treatments, including results of the contrast basal vs. tryptone + urea.

⁴*P*-values for the evaluation of the effects of protein type (Tryp or Ur), level [low (0.31 g of N/L of medium) or high (0.46 g of N/L of medium)], and their interaction for tryptone and urea treatments; basal data were omitted from this comparison.

⁵Carbon in organic acids as the sum of formate, acetate, propionate, butyrate, valerate, and lactate.

by glucose and tryptone in TrypL calculated as tryptone mg $\times$ 0.51, with 0.51 as the average proportion of C in proteins. All data points fall between or close to the gray and unity lines, with most falling closer to the unity line. Glucose C consumed minus product C detected did not differ from zero in the 2 respective fermentation runs for basal (SEM = 0.90; -0.14 mg of C, $P = 0.84$; -1.22 mg of C, $P = 0.33$), UrH (SEM = 0.58; 0.09 mg of C, $P = 0.85$; -0.51 mg of C, $P = 0.50$), and UrL in the second fermentation (SEM = 1.26; -1.35 of mg C; $P = 0.33$). In contrast, treatments that produced more product C than glucose C consumed in the 2 runs were TrypH (SEM = 1.65; -3.83 mg of C; $P = 0.12$; -5.16 mg of C, $P = 0.01$), TrypL (SEM = 1.55; -4.04 mg of C, $P = 0.10$; -3.33 mg of C, $P = 0.03$), and UrL in the first fermentation (SEM = 0.47; -1.54 mg of C, $P = 0.03$). The accuracy of detection methods, calculations, and assumptions do affect the accuracy of C recovery estimates. The good agreement between the basal and 3 of 4 urea treatment runs supports the concept that the detection methods successfully accounted for substrate disappearance and product appearance when supplemental peptides and AA were not provided. By the end of the fermentations, all basal and urea treatments had product C (mg)/consumed glucose C (mg) that were between 95 and 105%; the tryptone treatments ranged from 111 to 134%. As previously discussed, microbes in the tryptone treatments could derive C from glucose, inoculum, and AA from the tryptone. That even many of the basal or urea treatment data points fell slightly above the unity line may be indicative of incorporating C from sources in the inoculum.

CONCLUSIONS

Nitrogen source had a marked effect on microbial growth, as well as on the use of glucose substrate in fermentations with mixed ruminal microbes. The addition of true protein or peptides could increase microbial cell production over that supported by urea (Russell et al., 1983). The increases in glucose and glycogen disappearance and increase in organic acid C production with tryptone over urea suggest that perhaps the increased cell growth also mediated changes in carbohydrate use. This, in turn, has implications for how types and amounts of protein could alter supplies of organic acid or α -glucan nutrients to the cow. That the temporal pattern of responses to urea treatments frequently overlaid the results from the basal medium, which contained the least amount of nitrogen, suggested limited use of urea as a nitrogen source compared with AA and peptides by microbes that use glucose, a point that is in agreement with in vivo results (Hristov et al., 2005).

Figure 2. Amounts of carbon in microbial products relative to utilized glucose carbon for each treatment in each fermentation. The dashed unity line describes utilized glucose carbon = carbon in products; the gray line describes the amount of carbon in the utilized glucose and that provided by the lowest level of tryptone. Products include microbial cells, organic acids, calculated carbon dioxide and methane, and accumulated glycogen. Treatments: $\diamond$ = basal; $\triangle$ = TrypL; $\blacksquare$ $\square$ = TrypH; $+$, $\times$ = UrL; $\bullet$ $\circ$ = UrH. Closed symbols (and $+$) are from the first fermentation and open symbols (and $\times$) are from the second fermentation. Tryp = tryptone, Ur = urea, L = low level (0.31 mg of N/L), and H = high level (0.46 mg of N/L).

The determination of patterns of responses to changing nitrogen sources and concentrations with other carbohydrates and the basis for the substantial lag in microbial growth at the lowest nitrogen concentration could be of use in understanding how different combinations of nitrogen and carbohydrate sources affect nutrient supply to the ruminant.

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