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Transport of Glutamine by *Streptococcus bovis* and Conversion of Glutamine to Pyroglutamic Acid and Ammonia

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***Streptococcus bovis* JB1 cells energized with glucose transported glutamine at a rate of 7 nmol/mg of protein per min at a pH of 5.0 to 7.5; sodium had little effect on the transport rate. Because valinomycin-treated cells loaded with K and diluted into Na (pH 6.5) to create an artificial $\Delta\psi$ took up little glutamine, it appeared that transport was driven by phosphate-bond energy rather than proton motive force. The kinetics of glutamine transport by glucose-energized cells were biphasic, and it appeared that facilitated diffusion was also involved, particularly at high glutamine concentrations. Glucose-depleted cultures took up glutamine and produced ammonia, but the rate of transport per unit of glutamine (*V/S*) by nonenergized cells was at least 1,000-fold less than the *V/S* by glucose-energized cells. Glutamine was converted to pyroglutamate and ammonia by a pathway that did not involve a glutaminase reaction or glutamate production. No ammonia production from pyroglutamate was detected. *S. bovis* was unable to take up glutamate, but intracellular glutamate concentrations were as high as 7 mM. Glutamate was produced from ammonia via a glutamate dehydrogenase reaction. Cells contained high concentrations of 2-oxoglutarate and NADPH that inhibited glutamate deamination and favored glutamate formation. Since the carbon skeleton of glutamine was lost as pyroglutamate, glutamate formation occurred at the expense of glucose. Arginine deamination is often used as a taxonomic tool in classifying streptococci, and it had generally been assumed that other amino acids could not be fermented. To our knowledge, this is the first report of glutamine conversion to pyroglutamate and ammonia in streptococci.**

Streptococci are facultative anaerobes which are found in soil, water, dairy products, and the alimentary tracts of animals (13). Recent nucleic acid hybridization studies, 16S rRNA oligonucleotide cataloging, and chemotaxonomic analyses indicated that the genus *Streptococcus* is a genetically diverse group of organisms, and many of these species have been reassigned to the genera *Lactococcus* and *Enterococcus* (25). *Streptococcus bovis*, a bacterium that flourishes in the rumen when large amounts of starch are fed (16), was retained in the genus *Streptococcus*, but the taxonomic status of this species is in many ways "still unsatisfactory" (25).

Streptococci are generally described as having complex nutritional requirements, but *S. bovis* is able to grow in the absence of amino acids with ammonia as its sole source of nitrogen (29). However, *S. bovis* grows more than twice as fast when amino acids are provided, and recent work has indicated that the bacterium transports amino acids rapidly (10), sometimes by sodium-dependent mechanisms (24). Although some amino acids are transported at a rapid rate, earlier work indicated that ruminal streptococci were unable to produce ammonia (6). Burchall et al. (7) indicated that *S. bovis* had high levels of glutamate dehydrogenase (GDH), an enzyme which should be able to deaminate glutamate. The presence of GDH was difficult to reconcile with the apparent absence of ammonia production.

Amino acid deamination within the rumen is a nutritionally wasteful process (2), but little was known about the transport and metabolism of amino acids by ruminal bacteria. Glutamine and glutamate are the predominant amino acids in most feedstuff proteins. Our results showed that *S. bovis* (i) did not transport or utilize glutamate as a nitrogen source, (ii) took up glutamine by facilitated diffusion and active transport mechanisms, (iii) produced pyroglutamate and ammonia from glutamine, (iv) did not have glutaminase activity, (v) had high concentrations of intracellular glutamate, and (vi) contained concentrations of 2-oxoglutarate and NADPH that inhibited glutamate deamination and strongly favored glutamate formation. To our knowledge, this is the first report of glutamine conversion to pyroglutamate and ammonia in streptococci. Previous workers (6) had failed to detect ammonia production by *S. bovis* because much of the glutamine in commercial amino acid sources (protein hydrolysates) had already been converted to pyroglutamate. Pyroglutamate is often used as a terminal amino acid in short peptides that serve as mammalian hypothalamic releasing hormones (17).

MATERIALS AND METHODS

Organism and growth conditions. *S. bovis* JB1 (22) was grown in basal medium which contained the following components (in milligrams per liter): K_2HPO_4, 292; KH_2PO_4, 292; Na_2SO_4, 400; NaCl, 480; $MgSO_4 \cdot 7H_2O$, 100; $CaCl_2 \cdot 2H_2O$, 64; Na_2CO_3, 4,000; $Na_2S \cdot 9H_2O$, 500; and vitamins and microminerals (23). Glucose (final concentration, 2 g/liter), glutamine, glutamate, Trypticase (BBL Microbiology Systems, Cockeysville, Md.) and Casamino Acids (Difco Laboratories, Detroit, Mich.) were sterile-filtered and added separately. All cultures were anaerobically incubated at 39°C at pH 6.7.

Enzyme assays. Cells for enzyme studies were harvested by centrifugation after growth ceased (10,000 × *g*, 0°C, 15 min) and washed once in and resuspended in potassium phosphate buffer (200 mM, pH 7.2). Cell extracts were prepared by sonication for 30 min at 0°C (Branson model 200 sonifier; maximum output, microtip, 50% duty cycle). Toluene-treated cells were incubated with 2% toluene for 5 min.

GDH (EC 1.4.1.4), glutamate cyclotransferase, glutami-

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nase (EC 3.5.1.2), isocitrate dehydrogenase (EC 1.1.1.42), and 2-oxoglutarate dehydrogenase activities were measured spectrophotometrically as the oxidation or reduction of NADs [NAD(H) or NADP(H)] at 340 nm. GDH was NADP linked. When glutamate formation was measured, the GDH assay contained 40 μmol of NH_4Cl, 5 μmol of 2-oxoglutarate, 0.33 μmol of NADPH, and 5 μl of cell extract (approximately 15 μg of cell protein) per ml of potassium phosphate buffer (200 mM, pH 7.2). When glutamate deamination was measured, the GDH assay contained 15 μmol of glutamate, 0.33 μmol of NADP, 0.4 mmol of hydrazine sulfate, and either 15 μl of cell extract or 50 μl of toluene-treated cells (approximately 25 μg of cell protein) per ml of phosphate buffer (200 mM, pH 7.2).

The glutamate cyclotransferase assay contained 15 μmol of pyroglutamate, 0.33 μmol of NADP, 33 μmol of $MgCl_2$, 0.4 mmol of hydrazine sulfate, and either 15 μl of cell extract or 50 μl of toluene-treated cells per ml of phosphate buffer (200 mM, pH 7.2). When glutaminase activity was measured, the assay contained 15 μmol of glutamine, 0.33 μmol of NADP, 33 μmol of $MgCl_2$, 2 U of GDH (Sigma Chemical Co., St. Louis, Mo.), 0.4 mmol of hydrazine sulfate, and either 15 μl of cell extract or 50 μl of toluene-treated cells per ml of phosphate buffer. When ammonia production from glutamine deamination was assayed, the reaction mixture included 15 μmol of glutamine, 5 μmol of 2-oxoglutarate, 0.33 μmol of NADPH, 33 μmol of $MgCl_2$, 2 U of GDH, and either 15 μl of cell extract or 50 μl of toluene-treated cells per ml of phosphate buffer (200 mM, pH 7.2). The isocitrate dehydrogenase was NAD linked, and the assay contained 3.3 μmol of isocitrate, 3.3 μmol of $MgCl_2$, 0.33 μmol of NAD, and 5 μl of cell extract per ml of phosphate buffer (200 mM, pH 7.2). The assay for 2-oxoglutarate dehydrogenase had 15 μmol of 2-oxoglutarate, 10 μmol of NAD or NADP, 1.0 μmol of coenzyme A, and 0.1 mmol of cysteine hydrochloride per ml of phosphate buffer (200 mM, pH 7.2).

Intracellular pools. Cells for intracellular 2-oxoglutarate determinations were chilled quickly by adding crushed ice directly to the culture fluid and mixing vigorously. Once the ice had melted and the temperature had dropped to 5°C, the cells were collected by centrifugation (10,000 × *g*, 15 min, 0°C) and washed once with ice-cold phosphate buffer. Washed cells were then extracted with perchloric acid (final concentration, 10%; 0°C, 5 h). Perchlorate was then precipitated by adding an equal amount of 2.5 M K_2CO_3. Insoluble material was removed by centrifugation (10,000 × *g*, 15 min, 0°C), and the supernatant was frozen until glutamate and 2-oxoglutarate were measured by an enzymatic method (3, 5). Intracellular volumes were estimated from the ratio of cell protein to intracellular space. Previous work indicated that *S. bovis* had a 5-μl volume per mg of protein (21).

Cells for intracellular NADP and NADP(H) determinations were chilled with oxygen-free ice under a carbon dioxide atmosphere. Once the ice had melted, the cells were washed once in anaerobic phosphate buffer. Cell pellets were immediately extracted in 0.1 N HCl (NADP) or 0.1 N NaOH (NADPH) with a Teflon homogenizer (50°C, 10 min). NADP and NADPH were assayed by the recycling assay of Yamamoto (30) by using diaphorase and alcohol dehydrogenase (Sigma). Intracellular concentrations were based on the cell volume values described above.

Fermentation products. Ammonia production was determined by the colorimetric assay of Chaney and Marbach (8), and bacterial protein was measured by the method of Lowry et al. (18) after the cells were treated with 0.2 N NaOH for 15 min (100°C). Nonvolatile organic acids and pyroglutamate were measured by high-pressure liquid chromatography by using a Beckman model 334 liquid chromatograph equipped with a Bio-Rad HPX-87H organic acid column (0.013 N H_2SO_4 at 0.5 ml/min; 50°C, 20-μl samples). Since propionate and pyroglutamate co-eluted from the high-pressure liquid chromatography column, propionate was assayed by gas-liquid chromatography (*meta*-phosphoric acid-treated samples [final concentration, 6% {wt/vol}]) in a Gow Mac model 580 flame ionization gas chromatograph equipped with a Supelco 1220 column (1% H_3PO_4, 100/120 mesh).

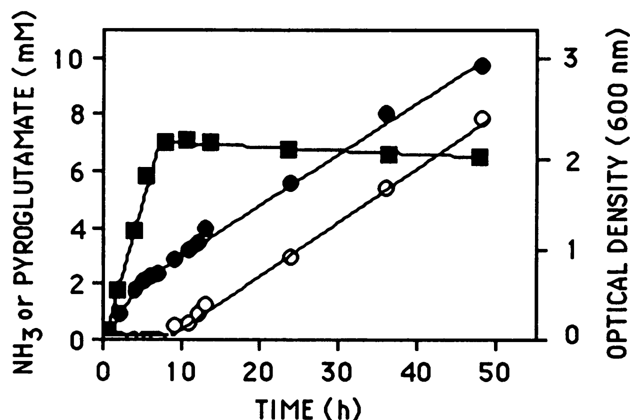

FIG. 1. Growth (■) of *S. bovis* with glutamine as a nitrogen source and production of ammonia (○) and pyroglutamate (●).

Transport assays. Cells (10 ml, optical density of 1.2 at 600 nm) were harvested by centrifugation (8,500 × *g*, 5 min, 0°C), washed twice in potassium phosphate buffer (100 mM, pH 7.0), and resuspended in 150 μl of potassium phosphate buffer. Concentrated cell suspensions (4 μl; 8.8 μg of protein per μl) were added to 200 μl of potassium phosphate or sodium phosphate buffer and energized with glucose for 5 min (10 mM, 28°C). Transport was initiated by addition of 100 nCi of ^{14}C-labeled amino acid (final concentration was usually 1.85 μM) and allowed to continue for 0 to 30 s. Transport was terminated by adding 2 ml of ice-cold 0.1 M LiCl to the reaction mixture and filtering the mixture rapidly through 0.45-μm-pore-size cellulose nitrate membrane filters. In some cases, the cells were not energized with glucose or were deenergized with 2-deoxyglucose (4 mM, 30 min, 39°C after glucose depletion). K diffusion potentials to create an artificial $\Delta\psi$ were conducted with valinomycin-treated cells (5 μM) that were loaded with K (100 mM, 30 min, 0°C) and diluted 50-fold into 100 mM sodium phosphate buffer (pH 6.5).

RESULTS

Growth and ammonia production. When *S. bovis* was inoculated into medium containing glucose (2 g/liter) and glutamate (15 g/liter) as the sole source of nitrogen, little growth was observed and ammonia production was never noted (data not shown). However, when glutamine was the nitrogen source, *S. bovis* grew rapidly (0.54 h^{-1}) and the final optical density was greater than 2.0 (Fig. 1). Glutamine utilization was associated with the production of pyroglutamate, and virtually all of the glutamine utilization could be accounted for by the conversion of glutamine pyroglutamate. There was no increase in ammonia until growth had ceased.

Transport. *S. bovis* cells energized with glucose took up ^{14}C-labeled glutamine at a rate of 7 nmol/mg of protein per

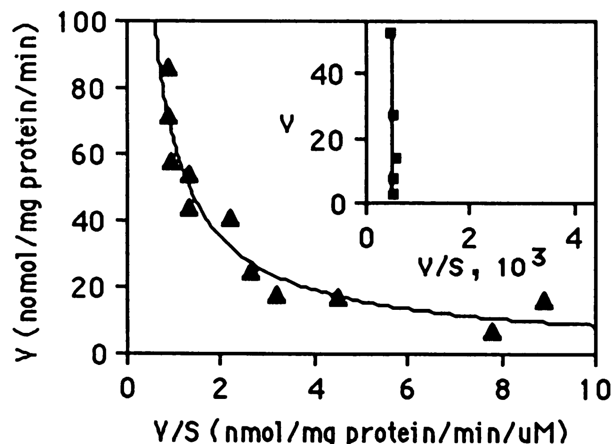

FIG. 2. Eadie-Hofstee plot of glutamine transport by glucose-energized *S. bovis* cells (▲). Ammonia production by whole cells in the absence of glucose is shown in the inset (■).

min (pH 7.5 to 5.0), and there was little change in the transport rate until the pH was 4.5 (4 nmol/mg of protein per min). Cells not glucose energized or loaded with K and diluted into Na to create an artificial $\Delta\psi$ were unable to transport glutamine. The addition of Na to the transport assays had little effect on glutamine transport. Transport of glutamate in the presence or absence of sodium was never observed (data not shown).

When glucose-energized cells were assayed at glutamine concentrations ranging from 1 to 100 μM, the relationship between transport rate (V) and the rate per unit of substrate (V/S) was not linear (Fig. 2). V was abnormally high at high substrate concentrations, and these biphasic kinetics suggested that a diffusion mechanism was also contributing to uptake. Nonenergized cultures produced ammonia from glutamine, but the rate of ammonia production was proportional to the substrate concentration (V/S was constant). The V/S of energized cells was at least 1,000-fold greater than that of nonenergized cells.

Enzyme studies. Toluene-treated cells produced ammonia from glutamine, but no ammonia production was observed with cell extracts (Fig. 3). When toluene-treated cells and cell extracts were incubated with either glutamine or pyroglutamate, glutamate production was never detected. Since glutamate cyclotransferase is a reversible enzyme (20), it appeared that glutamate was not an intermediate in the conversion of glutamine to ammonia and pyroglutamate.

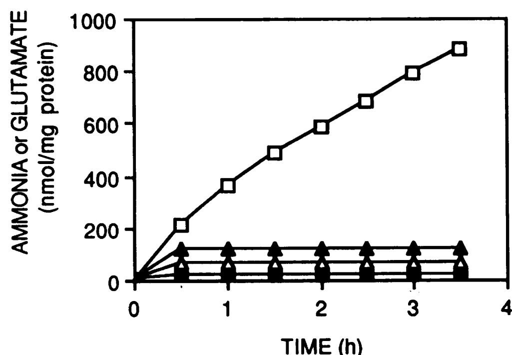

FIG. 3. Ammonia (□ and ■) and glutamate production (△ and ▲) from glutamine by toluene-treated cells (open symbols) and cell extracts (closed symbols) of *S. bovis*.

TABLE 1. Activity of GDH and isocitrate dehydrogenase in toluene-treated cells and cell extracts of *S. bovis*

Enzyme	Activity (nmol/mg per min)	K_m (mM)	V_{max} (nmol/mg per min)
GDH in cell extracts (glutamate formation)	544	0.77[a]	1,013
GDH in cell extracts (2-oxoglutarate formation)	16	10[b]	19
GDH in toluene-treated cells (2-oxoglutarate formation)	10		
ICDH[c] in cell extracts	77		

[a] Affinity constant for 2-oxoglutarate.
[b] Affinity constant for glutamate.
[c] ICDH, Isocitrate dehydrogenase.

S. bovis was unable to transport glutamate or produce glutamate from glutamine (see above), but cells contained as much as 7 mM glutamate. Cell extracts (and toluene-treated cells) had NADP-linked GDH, but rates of glutamate formation were 34 times higher than those of glutamate deamination (Table 1). GDH had a significantly higher affinity for 2-oxoglutarate than for glutamate, and the V_{max} of glutamate synthesis was at least 50-fold greater than the V_{max} of glutamate deamination. Intact cells contained significant amounts of 2-oxoglutarate, and the ratio of NADPH to NADP was 0.5 (Table 2). When cell extracts were incubated with glutamate and concentrations of 2-oxoglutarate and NADPH that were similar to those found intracellularly (1.94 and 0.03 mM, respectively), deamination was completely inhibited. 2-Oxoglutarate and NADPH alone caused 91 and 49% inhibition, respectively. Ammonia also inhibited glutamate deamination by cell extracts, but more than 10 mM was needed for a 59% inhibition. 2-Oxoglutarate was produced by an NAD-linked isocitrate dehydrogenase. 2-Oxoglutarate dehydrogenase was not detected.

Fermentation of Trypticase and Casamino Acids. Casein contains approximately 7% glutamine, but *S. bovis* was unable to produce ammonia from either an enzymatic (Trypticase) or an acid hydrolysate (Casamino Acids) of casein. High-pressure liquid chromatography showed that more than half of the glutamine in the digests had already been converted to pyroglutamate. *S. bovis* was unable to deaminate pyroglutamate or to use pyroglutamate as a nitrogen source.

DISCUSSION

S. bovis can utilize peptides and amino acids (10), but if amino acid sources are not available, ammonia can serve as the sole nitrogen source for growth (29). Burchall et al. (7)

TABLE 2. Intracellular concentrations of glutamate, 2-oxoglutarate, NADPH, and NADP in *S. bovis* cells and the effect of the compounds on the activity of GDH

Substance(s)	Intracellular concn (mM)	% Inhibition[a]
Glutamate	7.00	
2-Oxoglutarate	1.94	91
NADPH	0.03	49
NADP	0.06	
2-Oxoglutarate + NADPH		100

[a] Inhibition of GDH activity that was caused by the intracellular concentration of the test substance.

showed that *S. bovis* had NADP-linked GDH, and the JB1 strain used here had a similar activity. Since other amino acid dehydrogenases could not be detected, they concluded that GDH was the sole mechanism for ammonia assimilation. Glutamate synthase (26), another enzyme sometimes involved in ammonia assimilation, was not detected (preliminary experiments; data not shown). Even though glutamate must play an important role in amino acid biosynthesis, *S. bovis* could not utilize glutamate as a nitrogen source. Glucose-energized cells were unable to transport ^{14}C-labeled glutamate at a pH between 4.5 and 7.5. Previous studies indicated that *S. bovis* had sodium-dependent transport systems for neutral amino acids (24), but sodium had no effect on glutamate transport.

S. bovis grew with glutamine as the sole nitrogen source, but it was able to transport glutamine rapidly only when glucose was available. Because an artificial $\Delta\psi$, created by K diffusion, was unable to drive glutamine transport, it is likely that glutamine was taken up by a mechanism involving phosphate-bond energy. Since the kinetics of glutamine transport were biphasic (Fig. 2) and the intracellular metabolism of glutamine could have created a concentration gradient across the cell membrane, it appeared that diffusion could also play a role in glutamine uptake. Similar results were noted for leucine transport by *Streptococcus cremoris* (11).

When intact *S. bovis* cells were incubated with high concentrations of glutamine (1.7 to 107 mM) for long periods of time in the absence of glucose, ammonia was produced at a slow rate (Fig. 1). Because the rate of ammonia production was proportional to the glutamine concentration (*V/S* was constant; Fig. 2), it appeared that facilitated diffusion was the sole mechanism driving uptake. Nonenergized cells took up glutamine, but the rate of transport would not have been sufficient for rapid rates of growth. Even with glutamine concentrations as high as 100 mM, the growth rate would have been less than 0.26 h^{-1}. The *V/S* for active transport was approximately 1,000-fold greater than that for facilitated diffusion and could easily account for the growth rate on glucose, 0.54 h^{-1}.

Pyroglutamate and ammonia were the end products of glutamine deamination by *S. bovis*. A reversible glutamate cyclotransferase that catalyzes the conversion of glutamate to pyroglutamate (or vice versa) has been reported in *Pseudomonas cruciviae* (1), but this enzyme was not detected in *S. bovis*. In mammals (17), *Escherichia coli* (14), and *Clostridium welchii* (15), glutamine is deaminated by a glutaminase reaction, but this activity was also absent in *S. bovis*. Since glutamate could not be produced via a glutaminase reaction, it is unlikely that glutamate was an intermediate in pyroglutamate formation. A glutamine cyclotransferase that can produce pyroglutamate directly from glutamine has been found in plants (19) and in *E. coli* (4). Because *S. bovis* cell extracts were unable to produce ammonia, but toluene-treated cells did, it is possible that glutamine cyclotransferase was membrane associated.

S. bovis was able to use glutamine as its sole nitrogen source, but since pyroglutamate was a product, only 50% of the nitrogen (ammonia) from glutamine was available to the organism (Fig. 4). The inability of *S. bovis* to produce glutamate via a glutaminase reaction meant that glutamate could be formed from ammonia only via a GDH reaction. Because the carbon skeleton of glutamine was lost as pyroglutamate, glutamate production had to occur at the expense of glucose.

Although *S. bovis* was unable to transport glutamate,

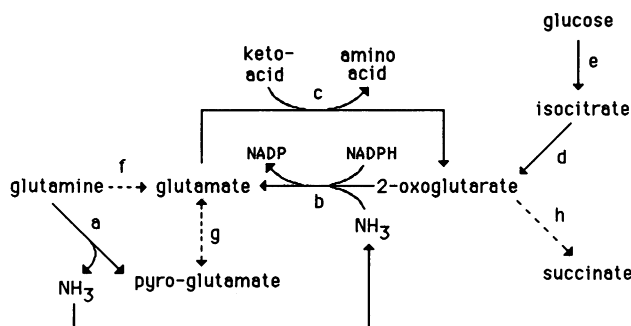

FIG. 4. Schematic of glutamine metabolism by *S. bovis*, showing (a) glutamine cyclotransferase, (b) GDH, (c) amino acid transaminases, (d) isocitrate dehydrogenase, and (e) glucose fermentation. Glutaminase (f), glutamate cyclotransferase (g), and 2-oxoglutarate dehydrogenase activities (h) were not detected.

intact cells contained more than 7 mM glutamate. Cell extracts and toluene-treated cells deaminated glutamate, but the GDH favored glutamate formation over deamination (Fig. 4). Since the equilibrium constant for glutamate deamination is very low, 4.5×10^{-14} (12), product accumulation could have had a significant effect on catalysis. When the GDH of *S. bovis* was assayed with concentrations of 2-oxoglutarate and NADPH that mimicked those found intracellularly, glutamate deamination was completely inhibited.

In aerobic organisms, the tricarboxylic acid (TCA) cycle is often a pathway for energy derivation, but anaerobic organisms lack oxygen-linked electron transport systems and are usually unable to reoxidize reduced cofactors arising from this cycle. *S. bovis* had significant isocitrate dehydrogenase activity, but 2-oxoglutarate dehydrogenase was not detected (Fig. 4). Because 2-oxoglutarate could not be converted to succinate, it is not surprising that 2-oxoglutarate accumulated (Table 2). Some organisms are able to reoxidize reduced cofactors via reactions involving hydrogenases. No such activity could be detected in *S. bovis*, and intracellular NADPH concentrations also inhibited glutamate deamination.

Recent work indicated that a ruminal peptostreptococcus was able to convert glutamine to pyroglutamate (9), but to our knowledge this is the first report of pyroglutamate production in streptococci. Considering the fact that the amide nitrogen of glutamine is easily removed by strong acids (27) and that pyroglutamate can be produced from glutamate by autoclaving (28), it is not surprising that commercial amino acid sources (e.g., Trypticase and Casamino Acids) contained considerable amounts of pyroglutamate or that previous workers were unable to detect ammonia production by ruminal streptococci (6).

Pyroglutamate is sometimes found as the terminal amino acid in proteins (17). It had generally been assumed that pyroglutamate arose as a posttranslational modification of terminal glutamyl residues (20), but in *E. coli*, glutamine cyclotransferase, the enzyme producing pyroglutamate, is also able to convert glutaminyl-tRNA to pyroglutaminyl-tRNA (4). In animals, pyroglutamate can occur as the terminal amino acid in hypothalamic releasing hormones. For example, thyrotropin-releasing hormone is a tripeptide consisting of pyroglutamate, histidine, and proline (17). The impact of ruminal pyroglutamate production on host metabolism has never been considered. Recent studies in our laboratory indicated that pyroglutamate accumulated in vitro when mixed ruminal bacteria were treated with monensin,

an animal growth promoter (J. B. Russell and G. Chen, J. Anim. Sci., in press).

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