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Effect of Reducing-Equivalent Disposal and NADH/NAD on Deamination of Amino Acids by Intact Rumen Microorganisms and Their Cell Extracts

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When mixed rumen microorganisms were incubated in media containing the amino acid source Trypticase, both monensin and carbon monoxide (a hydrogenase inhibitor) decreased methane formation and amino acid fermentation. Both of the methane inhibitors caused a significant increase in the ratio of intracellular NADH to NAD. Studies with cell extracts of rumen bacteria and protozoa indicated that the ratio of NADH to NAD had a marked effect on the deamination of reduced amino acids, in particular branched-chain amino acids. Deamination was inhibited by the addition of NADH and was stimulated by methylene blue, an agent that oxidizes NADH. Neutral and oxidized amino acids were unaffected by NADH. The addition of small amounts of 2-oxoglutarate greatly enhanced the deamination of branched-chain amino acids and indicated that transamination via glutamate dehydrogenase was important. Formation of ammonia from glutamate was likewise inhibited by NADH. These experiments indicated that reducing-equivalent disposal and intracellular NADH/NAD ratio were important effectors of branched-chain amino acid fermentation.

The importance of redox state in amino acid fermentation by anaerobic bacteria was first demonstrated by Stickland in the 1930s (33). He and later workers showed that highly reduced amino acids could only be fermented if oxidized amino acids were present in the incubation medium as well (22). The reduced amino acids (alanine, leucine, isoleucine, and valine) served as electron donors while the oxidized amino acids (glycine, proline, arginine, and tryptophan) served as electron acceptors in closely coupled reactions.

It has been suggested that Stickland-type reactions might be important in the fermentation of amino acids by rumen microorganisms (12, 14, 16), but results with pairs of oxidized and reduced amino acids were inconclusive (18, 37). Arsenate is an inhibitor of the reduction step in the Stickland reactions (22), and the addition of arsenate to incubations of mixed rumen microorganisms inhibited amino acid deamination (5). These latter experiments did not document whether arsenate was having a selective or specific effect on deamination.

Within the rumen, methanogenesis is a primary means of reducing-equivalent disposal. When H_2 production and methanogenesis were inhibited by carbon monoxide, an inhibitor of bacterial hydrogenases, ammonia production decreased (28). The reduction in ammonia was associated with a large decrease in branched-chain volatile fatty acids (VFA) and little change in straight-chain VFA. Since branched-chain fatty acids are derived from branched amino acids (1, 21), it appeared that a decrease in interspecies H_2 transfer selectively inhibited the fermentation of highly reduced, branched-chain amino acids.

The following series of experiments were designed to: (i) examine the effect of H_2 production on the redox state (NADH/NAD) of intact rumen microorganisms; (ii) examine the effects of NADH/NAD on the deamination of individual amino acids by cell extracts of rumen microorganisms; and (iii) compare the capacity of rumen bacteria and protozoa to deaminate amino acids. Our central hypothesis was that the ratio of intracellular NADH and NAD would exert a significant effect on the deamination of amino acids and in particular branched-chain amino acids.

MATERIALS AND METHODS

Organisms and incubation conditions. Rumen contents were obtained from a 650-kg, nonlactating, rumen-fistulated dairy cow which was fed 2.5 kg of timothy hay and 2.5 kg of commercial concentrate mix twice daily (28). Two hours after feeding, rumen contents were squeezed through four layers of cheesecloth and purged with O_2-free CO_2. The rumen fluid was mixed with the same volume of anaerobic 67 mM phosphate buffer (pH 7.0) and incubated at 39°C for 1 h. The feed debris which had risen up to the surface was removed with a vacuum tube, and the remaining fluid (40% final volume, vol/vol) was anaerobically transferred to a medium containing (milligrams per liter): K_2HPO_4, 292; KH_2PO_4, 292; $(NH_4)_2SO_4$, 480; NaCl, 480; $MgSO_4 \cdot 7H_2O$, 100; $CaCl_2 \cdot 2H_2O$, 64; Na_2CO_3, 4,000; cysteine hydrochloride, 600; $Na_2S \cdot 9H_2O$, 50; and Trypticase (pH 6.7), 15,000. Rumen microorganisms and medium (40 ml) were anaerobically transferred to 160-ml serum bottles. The bottles were capped with butyl rubber stoppers and aluminum seals and placed on a Queue orbital shaker (70 rpm; 35 by 35 cm; 39°C) in a dark incubator. Monensin was dissolved in ether, and the ether was evaporated from the serum bottles before the medium addition (final concentraion, 2 ppm). CO was passed through a copper oxygen-removing furnace, and 120 ml of the O_2-free gas was added to the serum bottles with a syringe. Control and monensin-treated serum bottles received 120 ml of O_2-free N_2 so that the pressure in all bottles was approximately 2.0 atm (202 kPa).

After 20 h of incubation, CH_4, H_2, NH_3, VFA, and cellular protein were measured by gas chromatography, colorimetry, high-presssure liquid chromatography, and the Lowry

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method, respectively, as described previously (28). Bacteria and protozoa for cell extracts were obtained from rumen fluid that was free of feed debris (see above). Protozoa were harvested by slow-speed centrifugation (150 × *g*, 3 min, 0°C), and the pellet was washed once in anaerobic phosphate buffer (67 mM, pH 7.0) to remove contaminating bacteria. The supernatant was centrifuged at 10,000 × *g* for 15 min (0°C) to collect bacteria. The cells were stored at −15°C.

Measurement of intracellular NADH and NAD. Culture fluid was transferred to centrifuge tubes containing O_2-free ice and mixed vigorously in an anaerobic glove box. Cells were collected by centrifugation (10,000 × *g*, 0°C, 15 min) and were immediately extracted. NAD was extracted with 0.1 N HCl (final concentration), and NADH was extracted with 0.1 N NaOH (final concentration). The extraction procedure was carried out at 50°C for 10 min (20). NADH and NAD were assayed by the recycling assay of Yamamoto (42). The assay mixture contained 1.7 ml of 0.1 M Tris buffer (pH 7.6), 1.0 ml of extract, 0.03 ml of 0.1 M KCN, 0.1 ml of 1.2 mM dichlorophenol-indophenol, 0.05 ml of alcohol dehydrogenase (100 U/ml; Sigma Chemical Co., St. Louis, Mo.), 0.05 ml of diaphorase (20 U/ml; Sigma), and 0.1 ml of 50% ethanol. The A_{610} was monitored after the addition of ethanol, and purified NAD (Sigma) was used as an internal standard.

Preparation of cell extracts of bacteria and protozoa. Bacterial and protozoal cells were disrupted by sonication (Branson model 200 sonicator, maximum output, 50% duty cycle) for 20 and 8 min, respectively. Cell debris was removed by centrifugation (15,000 × *g*, 20 min, 0°C). The supernatant was dialyzed against 80 mM phosphate buffer (pH 7.0) for 5 to 6 h, and the remaining macromolecules (greater than 10,000 molecular weight) were used as a cell extract. All the treatments during these processes were performed at 0 to 3°C under strictly anaerobic conditions in a glove box. Catalase (50 U/ml of cell suspension; Sigma) was added before sonication to protect against peroxidation (22).

Procedure to determine deamination activity. Cell extracts were incubated with various amino acids and cofactors (indicated in the figures or tables) for 2 h. Routinely, the incubation mixture contained 0.1 to 0.3 ml of cell extract (0.5 to 3 mg of protein) and 3 to 4 ml of phosphate or Tris buffer (100 mM) or both. At pH 6 to 7, only phosphate buffer was used; at pH 7 to 9 the ratio of phosphate to Tris buffer was 3:1; and at pH 9 to 10 the ratio was 1:1. Unless otherwise stated, incubations were performed at pH 8.0. Amino acids were grouped into three categories according to oxidation-reduction state, based on the corresponding oxo-acids as a standard (22): reduced, neutral, and oxidized amino acids. Initially, all incubations were performed aerobically at 39°C, but some NADH was oxidized under these conditions. For this reason, incubations containing NADH were performed in the tubes bubbled with O_2-free N_2 and NADH oxidation was minimized.

After incubation, reactions were stopped by cooling in an ice bath, and ammonia from 1 ml of the incubation mixture was immediately collected by the microdiffusion method of Conway and Byrne (9, 10). Ammonia absorbed into 0.5 ml of 0.01 N H_2SO_4 was measured by a colorimetric reaction as described by Chaney and Marbach (6). Ammonia present in zero time samples was subtracted to obtain the net NH_3 production. This correction accounted for NH_3 in cell extracts and any nonenzymatic NH_3 release from amino acids when the samples were treated with saturated K_2CO_3 in the microdiffusion procedure. Values obtained from incubations without any added amino acid were also subtracted. This subtraction corrected for "nonspecific" NH_3 formation from the amino acids which had not been removed by dialysis or which had been released by proteases and peptidases in the cell extracts. Thus, the reported NH_3 values reflected the deamination of "specific" amino acids that were added to the incubation.

Experiments were performed in triplicate, and the data were analyzed statistically and standard errors are reported. Simple comparisons were evaluated by a Student *t* test, and multiple comparisons were analyzed by a Tukey test (32).

RESULTS

Inhibition of H_2 and CH_4 production. When mixed rumen microorganisms were incubated with a pancreatic digest of casein (Trypticase, 15 g/liter), greater than 50 mmol of NH_3 per liter was produced during the 20-h incubation period (Table 1). Acetate was the primary fermentation product, and more than 6 mmol of methane per liter of incubation medium accumulated in the gas phase of the incubation bottles. Treatment with carbon monoxide, a hydrogenase inhibitor (34), or the ionophore monensin inhibited CH_4 production, and there was little increase in H_2. Inhibition of CH_4 production was associated with a decrease in amino acid deamination, but the reductions were not proportional. Carbon monoxide nearly eliminated CH_4 production, and NH_3 production decreased 25%. With monensin, CH_4 production was only inhibited by 31%, but NH_3 was reduced more than 37%.

Acetate, butyrate, and isovalerate plus 2-methylbutyrate were the primary products of amino acid fermentation in control incubations (Table 1). Monensin caused a large decrease in acetate and a significant increase in propionate. Carbon monoxide, in contrast, had a much smaller effect on acetate and did not increase propionate. With carbon monoxide, isovalerate plus 2-methylbutyrate and isobutyrate decreased more than 64%. Monensin treatment was also associated with a decrease in branched-chain VFA, but the effects were less dramatic.

In control incubations, interspecies H_2 transfer and methanogenesis were the primary means of reducing-equivalent disposal, and the ratio of intracellular NADH to

TABLE 1. Effect of carbon monoxide and monensin on the fermentation of amino acids by mixed rumen microorganisms[a]

Parameter	Control[b] (mmol/liter)	Carbon monoxide[b] (mmol/liter)	Monensin[b] (mmol/liter)
CH_4	6.4 ± 0.5	0.7 ± 0.1[d]	4.4 ± 0.2[d]
H_2	0.0 ± 0.0	0.7 ± 0.0[d]	0.1 ± 0.0
NH_3	54.4 ± 1.2	40.6 ± 0.7[d]	34.0 ± 0.7[d]
Acetate	35.7 ± 0.3	32.1 ± 0.6[d]	10.2 ± 0.2[d]
Propionate	1.0 ± 0.1	1.0 ± 0.1	4.7 ± 0.2[d]
Butyrate	14.1 ± 0.2	15.2 ± 0.2[d]	15.9 ± 0.3[d]
Valerate	5.4 ± 0.1	7.7 ± 0.5[d]	2.7 ± 0.1[d]
Isobutyrate	4.3 ± 0.1	1.5 ± 0.0[d]	3.5 ± 0.1[d]
Isovalerate ± 2-methylbutyrate	10.2 ± 0.1	3.6 ± 0.1[d]	9.6 ± 0.4
NADH/NAD[c]	0.39 ± 0.02	0.70 ± 0.02[d]	0.52 ± 0.02[d]

[a] Initial concentration of microbial protein was 1,350 mg/liter.
[b] Mean ± standard error (n = 9).
[c] Ratio of NADH to NAD in cells (n = 4).
[d] Significantly different from control ($P < 0.01$).

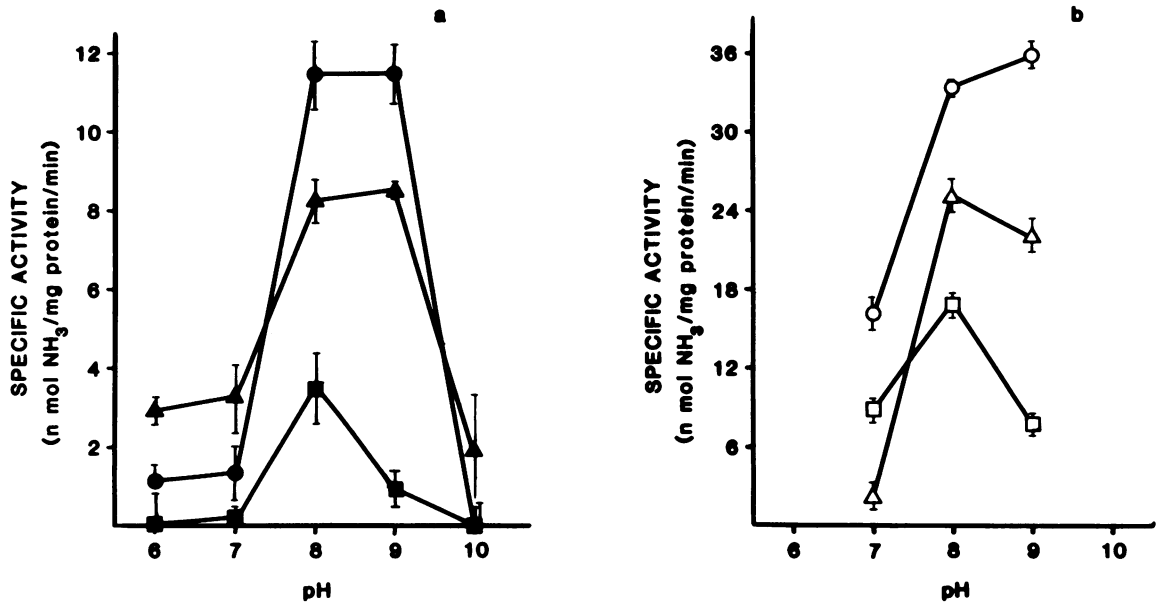

FIG. 1. Effect of pH on the deamination of amino acids by cell extracts of rumen bacteria (a) and protozoa (b). The cell extracts of bacteria and protozoa were incubated anaerobically in the presence of reduced (▲, △), neutral (●, ○), or oxidized (■, □) amino acids (see Table 1 legend), 1.0 mM NAD, and 0.05 mM MB.

NAD was approximately 0.4 (Table 1). When CH_4 production was inhibited by either carbon monoxide or monensin there was a significant increase in intracellular NADH/NAD. This increase prompted us to examine the effects of NADH/NAD on the deamination of specific amino acids with cell extracts of rumen microorganisms.

Effect of pH and NADH on deamination. Preliminary experiments were conducted to elucidate the optimum pH for amino acid deamination by cell extracts. Bacterial extracts showed little activity at pH 6.0, and there was a sharp increase in activity as the pH was increased from 7 to 8 (Fig. 1a). Deaminase activity of oxidized amino acids was lower than that of reduced or neutral amino acids, and the activity decreased as pH was increased to 9 or 10. The protozoal extracts were approximately threefold more active than the bacterial extracts, but the effect of pH was similar (Fig. 1b). In all cases, pH 8.0 allowed optimal or near optimal activity, and all subsequent incubations were performed at pH 8.0.

When cell extracts were incubated aerobically with reduced amino acids in the presence of NAD, NH_3 production was always greater than in the absence of NAD (data not shown). The A_{340} initially increased; however, absorbance eventually declined and it seemed likely that the extracts contained NADH-oxidizing factors. Subsequent treatments with NADH were performed under anaerobic conditions to minimize the oxidation of NADH. Methylene blue (MB), an oxidizing agent capable of oxidizing NADH, was added to comparison incubations to prevent any accumulation of NADH.

When reduced amino acids were incubated with cell extracts in the presence of MB, the deaminase activity was two- to fivefold greater than the rate observed with NADH addition (Table 2). The neutral and oxidized amino acids by contrast showed little or no response to added MB or NADH. Once again the activity of the protozoal extract was at least threefold greater than that observed with the bacterial extract.

Transamination. Glutamate was deaminated at a much faster rate than other amino acids (Table 3), and this high activity suggested that transamination reactions involving glutamate dehydrogenase (GDH) might be involved in net ammonia production. The pH optimum of GDH in cell extracts (Fig. 2) was similar to the pH optima observed in Fig. 1. Ammonia production from glutamate was inhibited by high concentrations of 2-oxoglutarate (greater than 2 mM); however, the addition of catalytic amounts of 2-oxoglutarate (0.3 mM) stimulated the deamination of other amino acids.

When leucine and alanine were incubated with catalytic amounts of 2-oxoglutarate and pyridoxal phosphate, the

TABLE 2. Effect of NADH on deamination of reduced, neutral, and oxidized amino acids by cell extracts of rumen bacteria and protozoa

Amino acid group[a]	Addition[b]	Sp act (nmol of NH_3/mg of protein per min)	
		Bacteria	Protozoa
Reduced	MB	7.0[d,e,f]	23.2[d]
	NADH	1.4[c]	12.1[c]
Neutral	MB	8.5[f]	34.6[e]
	NADH	7.2[e,f]	31.2[e]
Oxidized	MB	4.3[d]	14.7[c]
	NADH	4.9[d]	18.1[c,d]
SE		0.44	1.1

[a] Reduced group was composed of alanine, leucine, isoleucine, and valine. Neutral group was composed of serine, threonine, phenylalanine, and histidine. Oxidized group was composed of glycine, proline, arginine, and tryptophan. Each amino acid was present at an initial concentration of 10 mM.

[b] MB was provided at 0.05 mM and NADH at 2.0 mM. Both contained 1.0 mM NAD.

[c,d,e,f] Within a column, values with different superscripts are statistically significantly different ($P < 0.01$).

TABLE 3. Deamination of glutamate and various other amino acids by cell extracts of rumen bacteria and protozoa[a]

Amino acid	Sp act[b] (nmol of NH_3/mg of protein per min)	
	Bacteria	Protozoa
Alanine	1.41 ± 0.22	9.23 ± 0.54
Leucine	1.61 ± 0.16	5.37 ± 0.44
Valine	1.65 ± 0.13	7.03 ± 0.32
Threonine	13.30 ± 0.21	50.17 ± 1.63
Serine	3.30 ± 0.22	16.01 ± 0.87
Proline	1.68 ± 0.18	9.27 ± 1.41
Arginine	1.74 ± 0.22	7.83 ± 0.94
Glycine	2.38 ± 0.30	11.13 ± 0.59
Glutamate	84.18 ± 1.22	211.33 ± 4.30

[a] Incubation was performed in the presence of 1.0 mM NAD and 0.05 mM MB. Each amino acid was present at an initial concentration of 10 mM.
[b] Mean ± standard error (n = 3).

specific activity of ammonia production increased (Table 4). High rates of neutral amino acid (in particular threonine) deamination in the presence and absence of 2-oxoglutarate suggested that transamination of these amino acids was not important. Oxidized amino acids were deaminated at a slower rate than neutral amino acids, and transamination via GDH did little to increase the rate of ammonia production.

NADH is a product of glutamate deamination, and GDH activity was also sensitive to either the presence or accumulation of NADH (Fig. 3). When GDH activity was measured at 340 nm, initial rates were almost linear but, as time passed, the rates decelerated. If NADH was added to the cuvette, the initial velocity of the reaction was slower and the deceleration was more pronounced. When the ratio of NADH to NAD was 0.6, the initial velocity was apparently zero, and the A_{340} eventually decreased.

Stickland reaction. When the deamination of reduced or oxidized amino acids was compared with the deamination of reduced plus oxidized amino acids, the response was additive but not synergistic (Table 5). With a functional Stickland-type reaction, one would have expected a synergistic response.

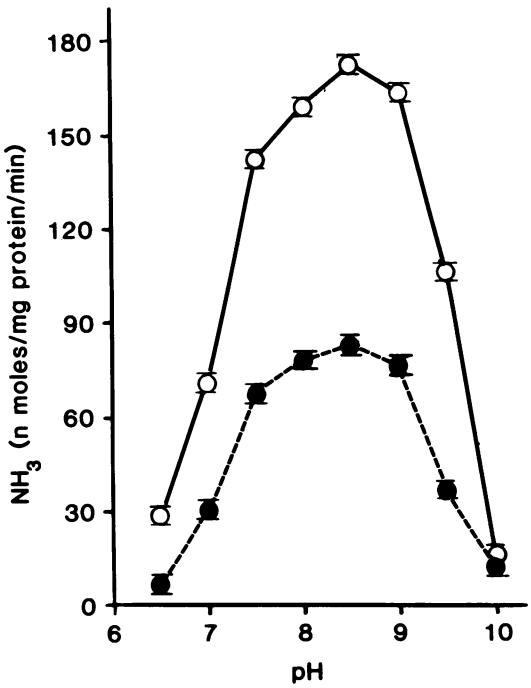

FIG. 2. Effect of pH on the deamination of glutamate by cell extracts of rumen bacteria and protozoa. The cell extracts of bacteria (●) and protozoa (○) were incubated in the presence of 10 mM glutamate, 1.0 mM NAD, and 0.05 mM MB.

TABLE 4. Involvement of transamination to glutamate in the deamination of amino acids by cell extracts of rumen bacteria and protozoa

Type of extract and addition[a]	Sp act (nmol of NH_3/mg of protein per min)					
	Reduced amino acids		Neutral amino acids		Oxidized amino acids	
	Leucine	Alanine	Threonine	Serine	Arginine	Proline
Protozoa						
NAD	3.75^{c}	8.54^{c}	50.30^{b}	13.36^{b}	4.82^{b}	$8.02^{b,c}$
NAD + MB	5.37^{c}	9.23^{c}	50.16^{b}	16.01^{b}	$7.83^{b,c}$	$9.27^{b,c}$
NAD + NADH	-0.30^{b}	-0.44^{b}	54.22^{b}	9.99^{b}	4.89^{b}	13.02^{c}
NAD + 2-OG + B_6	11.73^{d}	14.93^{d}	52.58^{b}	14.55^{b}	$8.20^{b,c}$	$7.29^{b,c}$
NAD + 2-OG + B_6 + MB	16.57^{e}	21.59^{e}	55.66^{b}	17.42^{b}	11.34^{c}	5.99^{b}
NAD + 2-OG + B_6 + NADH	1.04^{b}	7.18^{c}	49.40^{b}	11.91^{b}	$6.89^{b,c}$	$11.11^{b,c}$
SE	0.40	0.65	2.59	1.80	0.85	1.03
Bacteria						
NAD	$1.30^{b,c,d}$	$0.66^{b,c}$	12.30^{b}	$2.82^{b,c}$	1.71^{b}	1.72^{b}
NAD + MB	$1.62^{c,d,e}$	$1.41^{b,c}$	13.30^{b}	$3.18^{b,c}$	1.74^{b}	1.68^{b}
NAD + NADH	$0.72^{b,c}$	0.23^{b}	13.15^{b}	2.32^{b}	2.56^{b}	1.60^{b}
NAD + 2-OG + B_6	$2.48^{d,e}$	$1.17^{b,c}$	$13.60^{b,c}$	$3.40^{b,c}$	1.99^{b}	1.88^{b}
NAD + 2-OG + B_6 + MB	4.67^{f}	1.86^{c}	14.90^{c}	4.25^{c}	2.74^{b}	1.52^{b}
NAD + 2-OG + B_6 + NADH	0.28^{b}	$0.56^{b,c}$	13.20^{b}	$2.81^{b,c}$	2.15^{b}	1.90^{b}
SE	0.20	0.22	0.22	0.26	0.29	0.23

[a] Concentrations in initial incubation mixtures were: NAD, 1.0 mM; MB, 0.05 mM; NADH, 2.0 mM; 2-OG (2-oxoglutarate), 0.3 mM; vitamin B_6 (pyridoxal phosphate), 0.04 mM.
[b,c,d,e,f] Means within a column with different superscripts differ significantly ($P < 0.01$).

DISCUSSION

Amino acids are fermented by rumen microorganisms as an energy source, and significant amounts of ammonia can accumulate in the rumen. Ammonia is absorbed across the rumen wall and eventually is converted to urea by the liver and kidney. Some urea is recycled back to the rumen by salivary secretions, but when ammonia flux from the rumen is great, a larger fraction is excreted in the urine. Therefore, ammonia accumulation in the rumen will decrease nitrogen retention by the animal (31).

The magnitude of ammonia accumulation is often great in vivo (16, 19), but the deaminase activity of rumen microorganisms is low (3, 27). Low specific activity could be due to either a limitation on the uptake of peptides and amino acids or deaminating enzymes. In control incubations with 15 g of Trypticase per liter, 54.4 mmol of NH_3 per liter was produced in 20 h (Table 1). Under these conditions there was no increase in microbial protein, Trypticase was in excess, ammonia increased at a nearly linear rate, and the specific activity was 31 nmol of NH_3 per mg of microbial protein per min. This value for intact cells was within the range of specific activities observed with cell extracts (Tables 2, 3, and 4). Assuming that deamination of individual amino acids is an additive process, total deaminase activity was probably in excess. These comparisons suggested that the uptake of peptides and amino acids could be limiting.

In all cases the specific activity of the protozoal extract was much greater than that of the bacterial extract (Tables 2, 3, and 4). Bird and Leng (2) noted that defaunated sheep fed low-protein diets grew faster than control animals even though there was no significant increase in feed consumption. Other workers have shown that protozoa engulf rumen bacteria and produce ammonia (7, 8, 11, 15) and that rumen ammonia concentrations are about twice as great in faunated as in defaunated sheep (13). These results are consistent with our observation that protozoa have higher deaminase activity than bacteria.

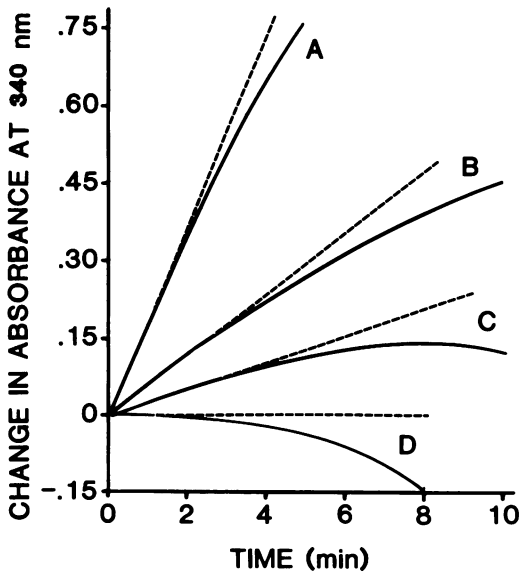

FIG. 3. Effect of NADH/NAD ratio on the reduction of NAD in the presence of glutamate by cell extract of rumen protozoa. The reaction mixture initially contained 10 mM glutamate, 0.2 mM NAD, and 0 (A), 0.03 (B), 0.06 (C), or 0.12 (D) mM NADH (pH 8.0). Initial absorbance was adjusted to 0.

TABLE 5. Coupling of oxidation and reduction in the deamination of amino acids by cell extracts of rumen bacteria and protozoa[a]

Amino acid group[b]	Sp act (nmol of NH_3/mg of protein per min)	
	Bacteria	Protozoa
Reduced	7.17[c,d]	16.65[d]
Oxidized	2.91[c]	7.09[c]
Reduced and oxidized	9.96[d]	22.51[d]
SE	0.68	1.29

[a] Incubated anaerobically with 1 mM NAD.
[b] Reduced group was composed of valine, leucine, and isoleucine. Oxidized group was composed of arginine, glycine, and proline. Each amino acid was present at an initial concentration of 10 mM.
[c,d] Means within a column with different superscripts differ significantly ($P < 0.05$).

In vivo, methanogenesis is a primary means of reducing-equivalent disposal, and the inhibition of methane production by the hydrogenase inhibitor carbon monoxide decreased ammonia production in vitro (Table 1). The decline in ammonia was associated with a large decrease in branched-chain VFA, but there was little change in the straight-chain acids. These results indicated that hydrogenase inhibition was primarily affecting the fermentation of branched-chain amino acids. Similar results were previously reported (28).

When methane and hydrogen productions were inhibited by carbon monoxide, the ratio of intracellular NADH to NAD increased (Table 1). Subsequent experiments with cell extracts indicated that the deamination of reduced amino acids (including branched-chain amino acids) was inhibited by the addition or accumulation of NADH. The neutral and oxidized amino acids were unaffected by NADH. Nisman and Mager (23) likewise noted that the transport of electrons from NADH to the final acceptor is the limiting step in the deamination of reduced amino acids. The sensitivity of reduced amino acid deamination is related to the equilibrium constant of the reaction. Leucine dehydrogenase from *Bacillus cereus* had a $K_{eq} = 11 \times 10^{-14}$ (30), while the alanine dehydrogenase of *Bacillus subtilis* had a $K_{eq} = 9 \times 10^{-14}$ (40). With equilibrium constants this low, ammonia production would only be favored at very small ratios of NADH to NAD. Britz and Wilkinson (4) showed that the oxidizing agent MB enhanced the capacity of *Clostridium bifermentans* extracts to deaminate and decarboxylate leucine.

The addition of catalytic amounts of 2-oxoglutarate to reactions already containing reduced amino acids increased the deamination rate (Table 4), and these results indicated that transamination was probably involved in net deamination. Branched-chain amino acids were more readily transaminated than other amino acids (35). Since GDH was also sensitive to the ratio of NADH to NAD (Fig. 3), much of the effect of reducing-equivalent disposal may have been mediated at this step. Studies by Olson and Anfinsen (24) previously indicated that the equilibrium of GDH was in favor of ammonia assimilation rather than production and that the reaction was inhibited by NADH. Both NAD- and NADP-linked GDHs were found in extracts of mixed rumen microorganisms, but the former was much more important (26).

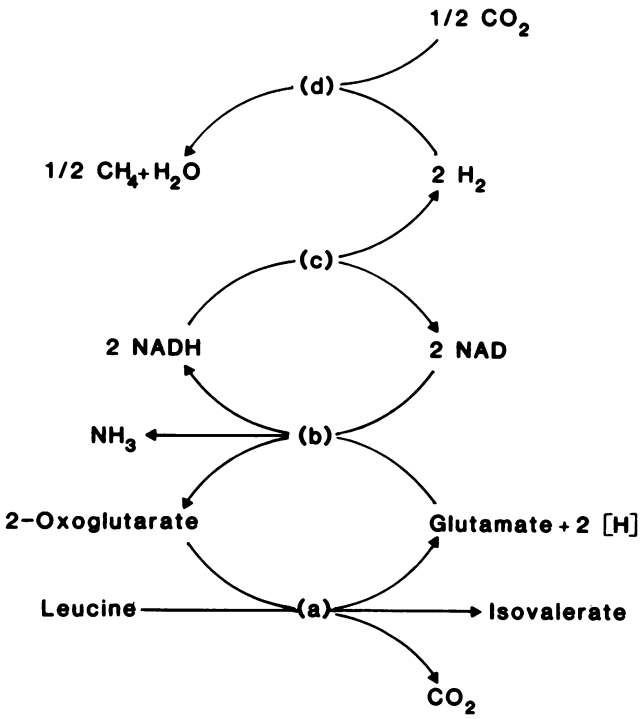

FIG. 4. Schematic representation of the relationship between leucine fermentation and methane production. (a) Transamination and decarboxylation; (b) deamination and hydrogenation; (c) hydrogenase activity; and (d) methanogenesis.

Rates of leucine deamination were increased two- to threefold by the addition of 2-oxoglutarate (Table 3), but there may have been some deamination via reactions not involving transamination. Threonine was deaminated at a rapid rate in the presence and absence of 2-oxoglutarate, and transamination was probably not important (Table 4). Threonine dehydratase has been demonstrated in rumen bacteria (17, 36), and Walker (39) showed that serine was metabolized by the same enzyme as threonine.

Glycine can be deaminated by reductive deamination (37), but Wright and Hungate (41) suggested that another pathway involving oxidative metabolism is more important. Arginine was first metabolized to ornithine and then reductively deaminated to valerate via 5-aminovalerate (38). Proline is also reductively metabolized to form valerate by rumen bacteria (12, 38). Arginine and proline are metabolized similarly by protozoa to 5-aminovalerate (25). Because degradation pathways of oxidized amino acids were known to proceed via reduction reactions, we had anticipated that NADH would stimulate the deamination rate. This tentative hypothesis was not substantiated (Table 4).

Stickland reactions involving the simultaneous oxidation and reduction of reduced and oxidized amino acids, respectively, were suggested for rumen microorganisms (12, 14), but attempts to show the reaction were inconclusive (18, 37). In our experiments a combination of reduced and oxidized amino acids was additive, but the interaction between the two groups was insignificant ($P < 0.05$). In vivo NADH formed by fermentation of reduced amino acids can be oxidized by other mechanisms (hydrogenase activity and methanogenesis or lactate and propionate production). These observations suggest that the Stickland reaction is not necessarily required.

Monensin decreased methane and ammonia production, but the mechanism of action appeared to be different from the effect of carbon monoxide (Table 1). Monensin also increased the intracellular NADH/NAD ratio and decreased isobutyrate, but there was a large and overriding decrease in acetate. These results once again substantiate the hypothesis that the mechanism of monensin action in the rumen cannot be explained by a simple inhibition of hydrogen production (29).

The probable relationship between methanogenesis and the fermentation of leucine, a highly reduced, branched-chain amino acid, is summarized in Fig. 4. Leucine is transaminated and decarboxylated as 2-oxoglutarate, is converted to glutamate and two moles of reducing equivalent (a), deamination by GDH produces ammonia and catalyzes the conversion of NAD to NADH (b), the enzyme hydrogenase then produces H_2 via an oxidation of NADH (c), and methanogens use the H_2 in the production of CH_4 (d). Our data indicated that these steps were closely coupled and regulated processes. When H_2 and CH_4 production was inhibited by carbon monoxide, intracellular NADH/NAD increased and ammonia and branched-chain fatty acids, including isovalerate, all decreased. These experiments indicated that reducing-equivalent disposal and the ratio of intracellular NADH to NAD were important effectors of branched-chain amino acid fermentation.

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