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Effect of Carbon Monoxide on Fermentation of Fiber, Starch, and Amino Acids by Mixed Rumen Microorganisms In Vitro

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When 1 atm (101.3 kPa) of carbon monoxide was added to mixed rumen bacterial incubations containing timothy hay, methane production was inhibited by 88% without an increase in hydrogen. The molar ratio of propionate to acetate increased from 0.83 to 1.53, extracellular ammonia declined from 5.2 to 2.4 mM, and hemicellulose and cellulose digestions were inhibited by 40 and 27%, respectively. Even low levels of carbon monoxide (less than 0.1 atm [10.13 kPa]) significantly changed the products of fermentation. With starch, methane production was once again inhibited, but the magnitude of starch fermentation was unaffected. Decrease in acetate was accompanied by an equal molar increase in lactate. Ammonia production from the amino acid source, Trypticase, declined 20% as carbon monoxide was increased to 1.0 atm, and 93% of this decrease was explained by a selective inhibition of branched-chain amino acid fermentation.

During rumen fermentations as much as 11% of the energy in the feed is converted to methane, which represents a significant loss of feed energy (5, 20). A variety of feed additives, including chlorinated hydrocarbons (10, 31), sulfite (30), oils (11), and ionophores (6, 7, 24, 25), decrease methane production in vivo, and some of these compounds have increased animal performance. The ionophore monensin is widely used in the beef cattle industry, but its mechanism of action is not entirely clear (13). Methane production decreases without a buildup of hydrogen, the molar ratio of propionate to acetate increases, and amino acid fermentation declines (7, 25, 27, 29). Because methanogenic bacteria are relatively insensitive to monensin (9, 29), it appears that monensin acts on nonmethanogenic species and decreases the transfer of hydrogen to methanogens. Chlorinated hydrocarbons and sulfite are very toxic to methanogenic bacteria, and even low concentrations can completely eliminate methane production (30, 31). Hydrogen gas often accumulates when chlorinated hydrocarbons or sulfite are added to mixed microbial incubations, and they do not appear to have a direct effect on the production of hydrogen (7, 30, 31).

In 1933 Kempner and Kubowitz showed that the hydrogen production by *Clostridium butyricum* was inhibited by carbon monoxide, and subsequent work indicated that this effect was caused by a specific and light-reversible action of carbon monoxide on bacterial hydrogenases (19, 28). From this earlier work it seemed possible that carbon monoxide could alter rumen fermentation in a way that would be similar to monensin treatment—namely, methane inhibition without an accumulation of hydrogen. The following experiments describe the effects of carbon monoxide on rumen fermentation in vitro.

MATERIALS AND METHODS

Cell growth. Rumen contents were obtained from a 682-kg, nonlactating, rumen-fistulated dairy cow that was fed 5 kg of timothy hay twice daily (see Table 1 for composition). At 1.5 h after feeding, contents were squeezed through eight layers of cheesecloth and purged with O_2-free carbon dioxide. More large feed particles were removed from the rumen fluid by passing it through an additional four layers of cheesecloth. The resulting rumen fluid was anaerobically transferred (20% final concentration) to a medium containing 292 mg of K_2HPO, 292 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4,000 mg of Na_2CO_3, 500 mg of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 600 mg of cysteine hydrochloride, and 0.5 mg of glucose per liter. Forty milliliters of microorganisms and medium was anaerobically transferred to 160-ml serum bottles that contained either no addition, 0.580 g of Trypticase, 0.50 g of food starch (Cream Pure Corn Starch; Purex Corp., Lakewood, Calif.), or 0.5000 g of timothy hay ground to a 2-mm particle size (see Table 1). The bottles were capped with butyl rubber stoppers and aluminum seals and injected with 120 ml of N_2, CO, or both that had been passed through hot reduced copper to remove O_2 (21). Since the original gas volume (CO_2) was 120 ml, the addition of another 120 ml of CO or N_2 represented an increase of 1.0 atm (ca. 101 kPa). The bottles were then placed on a Queue orbital shaker (70 rpm; Queue Systems, Parkersburg, W.Va.) and incubated at 39°C for 10 h (starch), 22 h (Trypticase), or 34 h (timothy hay).

Analyses. At the end of the incubation period 0.5 ml of gas was removed from each of the bottles, and methane and hydrogen were detected on a Gow Mac series 550 gas chromatograph (Carbosieve S 8100 mesh column; Supelco, Inc., Bellefonte, Pa.). After gas analyses were performed, the bottles were emptied into tubes and centrifuged (10,000 × *g*, 15 min, 0°C). Cell-free supernatants were stored at

TABLE 1. Composition of timothy hay[a]

Component	%[b]
Neutral detergent fiber	65.0
Acid detergent fiber	38.3
Cellulose[c]	33.1
Hemicellulose[d]	26.6
Crude protein	9.8
Lignin	4.3
Ash	6.4

[a] Determined by the methods of Goering and Van Soest (17).
[b] Calculated on a dry matter basis.
[c] Determined by the sulfuric acid method.
[d] Calculated as the difference between acid and neutral detergent fiber.

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−15°C. Undigested timothy hay in the pellet was immediately analyzed for fiber by the methods of Goering and Van Soest (17). Volatile fatty acids, ethanol, methanol, succinate, and lactate in the cell-free sample were measured by high-pressure liquid chromatography with a Beckman model 334 liquid chromatograph, a model 156 refractive index detector, a model 421 CRT data controller, a CRIA integrator, and a Bio-Rad HPX-87H organic acid column (see reference 14). Extracellular ammonia was measured by the colorimetric method of Chaney and Marbach (8). Color formation from ammonia was decreased markedly by cysteine in the medium. When six times as much reagent was used, color formation was restored and standard curves were linear. The final pH of all incubations ranged from 6.4 to 6.8.

Experimental design. All experiments were performed on duplicate days with three experiments per day (n = 6). Standard errors or linear regressions are reported. The use of duplicate days with hay and Trypticase incubations did not cause a large increase in the error term. When the microorganisms were incubated with starch, however, standard errors were significantly increased by pooling days. Subsequent experiments that measured gas production in a well-lubricated syringe indicated these differences could be attributed to lags in the initiation of starch digestion. For starch, data from a single day (n = 3) are reported, but on each day, overall kinetics of carbon monoxide action were similar.

RESULTS

When mixed rumen microorganisms were incubated in the absence of exogenous carbohydrates or amino acids, there was little increase in methane, hydrogen, or volatile fatty acids (Table 2). Carbon monoxide additions inhibited methane production, but the quantity of volatile fatty acids was not significantly affected. When bottles received hydrogen or formate and no carbon monoxide, methane production increased markedly. However, methane production was nearly eliminated by 0.25 atm (ca. 25.25 kPa) of carbon monoxide. Inhibition of methane production from hydrogen was associated with an increase in formate.

With incubations containing timothy hay, there was a marked decrease in methane production (up to 85%) and little increase in hydrogen as carbon monoxide was increased from 0 to 1.0 atm (ca. 101 kPa) (Fig. 1). Both acetate and propionate declined as the amount of carbon monoxide increased, but the effect on acetate was more dramatic. Extracellular ammonia was also decreased by carbon monoxide addition, but butyrate production was unaffected. At carbon monoxide levels greater than 0.5 atm (ca. 50.5 kPa), there was a small increase in the amount of formate.

Decreases in methane and volatile fatty acids were likewise associated with a reduction in organic matter digestion. After hydrogen, [H] ($H^{\cdot} + e^{-}$), production was calculated from methane and hydrogen gas production by the equations:

$$2[H] \rightarrow H_2$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

There was a positive correlation between [H] production and organic matter digestion. By squaring the correlation coefficient (r^2), 70% of the variation in organic matter digestion, at digestibilities greater than 49%, could be explained by [H] production (Fig. 2). For every 1 mM reduction in [H] (0.125 mM methane), organic matter digestion decreased by 14.66 mg/liter. Hemicellulose and cellulose determinations exhibited greater variability, but in each case greater than 50% of

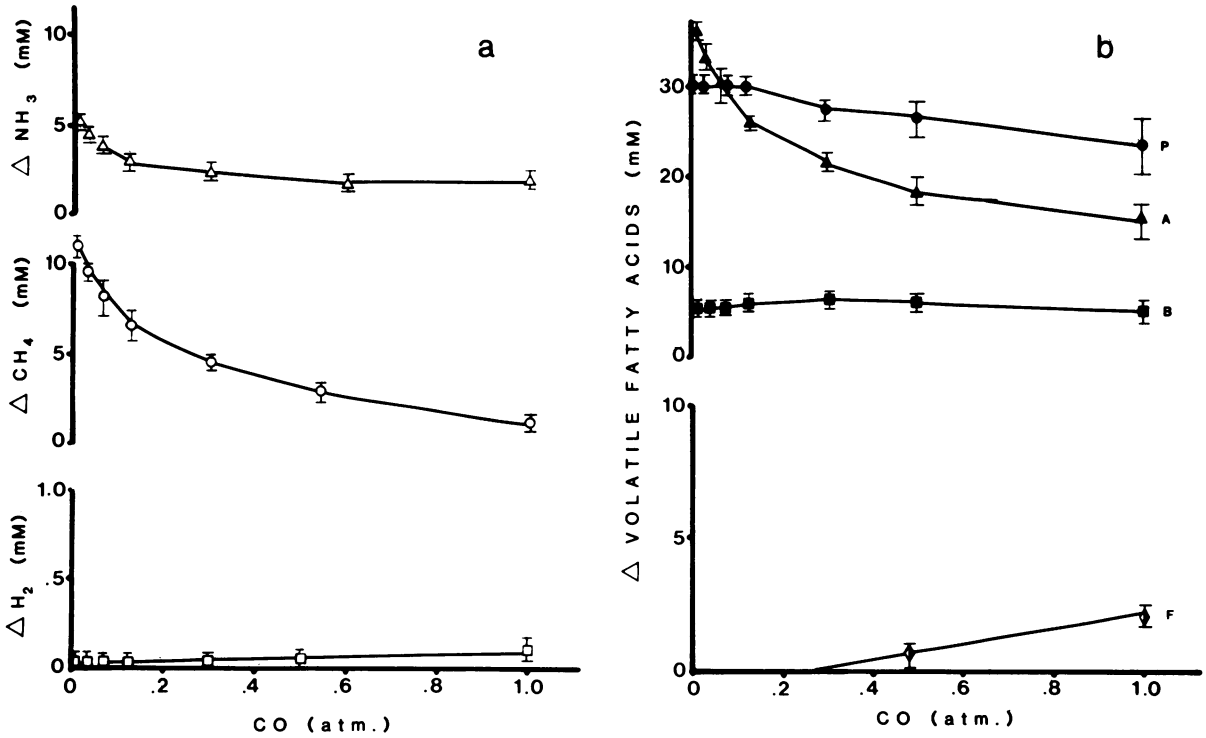

FIG. 1. Effect of carbon monoxide on the fermentation of timothy hay (12,500 mg/liter) by mixed rumen bacteria. One atmosphere of oxygen-free gas (CO, N_2, or both) was injected into each fermentation bottle. Changes in (a) extracellular ammonia (△), methane (○), hydrogen (□), or (b) acetate (A, ▲), propionate (P, ●), butyrate (B, ■), and formate (F, ◆) are reported. Each culture was incubated for 34 h.

TABLE 2. Fermentation products of mixed rumen microorganisms grown in vitro for 34 h with additions of nitrogen, carbon monoxide, hydrogen, and formate[a]

Addition[a]	Concn ± SE (mM) of:					
	H_2	CH_4	Formate	Acetate	Propionate	Butyrate
0 CO + 1 N_2	0.2 ± 0.2	0.8 ± 0.8	ND[b]	5.0 ± 1.8	0.4 ± 0.3	0.7 ± 0.3
1/32 CO + 31/32 N_2	0.2 ± 0.2	1.0 ± 0.6	ND	5.0 ± 0.5	0.9 ± 0.1	1.2 ± 0.1
1/16 CO + 15/16 N_2	0.3 ± 0.2	0.3 ± 0.2	ND	4.6 ± 0.6	0.6 ± 0.3	1.1 ± 0.3
1/8 CO + 7/8 N_2	0.2 ± 0.1	0.2 ± 0.1	ND	5.2 ± 1.2	0.8 ± 0.1	1.1 ± 0.3
1/4 CO + 3/4 N_2	0.1 ± 0	0.1 ± 0.0	ND	5.2 ± 0.6	0.7 ± 0.2	1.0 ± 0.2
1/2 CO + 1/2 N_2	0.2 ± 0.1	0.1 ± 0.1	ND	4.1 ± 1.2	0.7 ± 0.3	0.9 ± 0.6
1 CO + 0 N_2	0.1 ± 0	0.1 ± 0.1	ND	5.2 ± 0.5	1.0 ± 0.2	1.2 ± 0.2
0 CO + 1/2 N_2 + 1/2 H_2	ND	4.0 ± 0.5	ND	5.0 ± 0.4	0.7 ± 0.11	0.7 ± 0.1
1/4 CO + 1/4 N_2 + 1/2 H_2	ND	0.2 ± 0.3	2.7 ± 1.0	4.3 ± 0.8	0.8 ± 0.1	1.8 ± 1.0
0 CO + 1 N_2 + HCOOH	0 ± 0	4.8 ± 0.2	ND	6.3 ± 0.3	0.9 ± 0.1	0.7 ± 0.1
1/4 CO + 3/4 N_2 + HCOOH	0.7 ± 0.2	0.4 ± 0.1	ND	5.8 ± 0.7	1.1 ± 0.3	0.9 ± 0.1

[a] Fraction of gas that was added to each incubation bottle. Original gas volume (CO_2) was 120 ml and another 120 ml of gas was added to each bottle. Formate was provided at 10 mM.
[b] ND, Not detectable.

the change in digestion could be explained by [H] production. Based on slopes (*b*), hemicellulose digestion was more affected by [H] or methane production than cellulose digestion.

To ascertain the effects of carbon monoxide on starch fermentation, mixed rumen microorganisms were incubated with an excess of commercial food starch. Food starch was selected because it is insoluble and is fermented slowly. Methane production was once again inhibited by carbon monoxide, and there was only a small increase in hydrogen (Fig. 3). Carbon monoxide had little effect on the production of ammonia, propionate, or butyrate, but higher levels of carbon monoxide caused a large decrease in the amount of acetate and a nearly equal increase in the amount of lactate. After the amount of starch fermentation was calculated by the method of Wolin (32), there was no discernible relationship between starch digestion and carbon monoxide treatment. A plot of [H] production versus starch fermentation had an r^2 of only 2% (Fig. 4).

When mixed rumen microorganisms were incubated with Trypticase, an enzymatic hydrolysate of casein, carbon monoxide once again inhibited methane formation (Fig. 5). Ammonia production decreased 20% or 13.3 mM, but the final concentrations of acetate, propionate, butyrate, and valerate were unchanged. Branched-chain volatile fatty acid production was affected, however. Isovalerate plus 2-methylbutyrate decreased by 9.7 mM, whereas isobutyrate decreased by 2.7 mM. Assuming that branched-chain volatile fatty acids are derived from branched-chain amino acids (leucine, valine, and isoleucine), and knowing that these amino acids have a single amino group, 93% of the reduction in ammonia was explained by a reduction in catabolism of branched-chain amino acids.

When ammonia production was plotted against [H] production (resulting from CH_4 and H_2 formation), the r^2 was 0.758 (Fig. 6). This r^2 indicated that at ammonia production greater than 50 mM, 75.8% of the variation in ammonia was explained by [H] production. The slope of the plot indicated that for every 1 mM [H], 0.25 mol of ammonia resulted. This 4:1 stochiometric relationship is in excellent agreement with

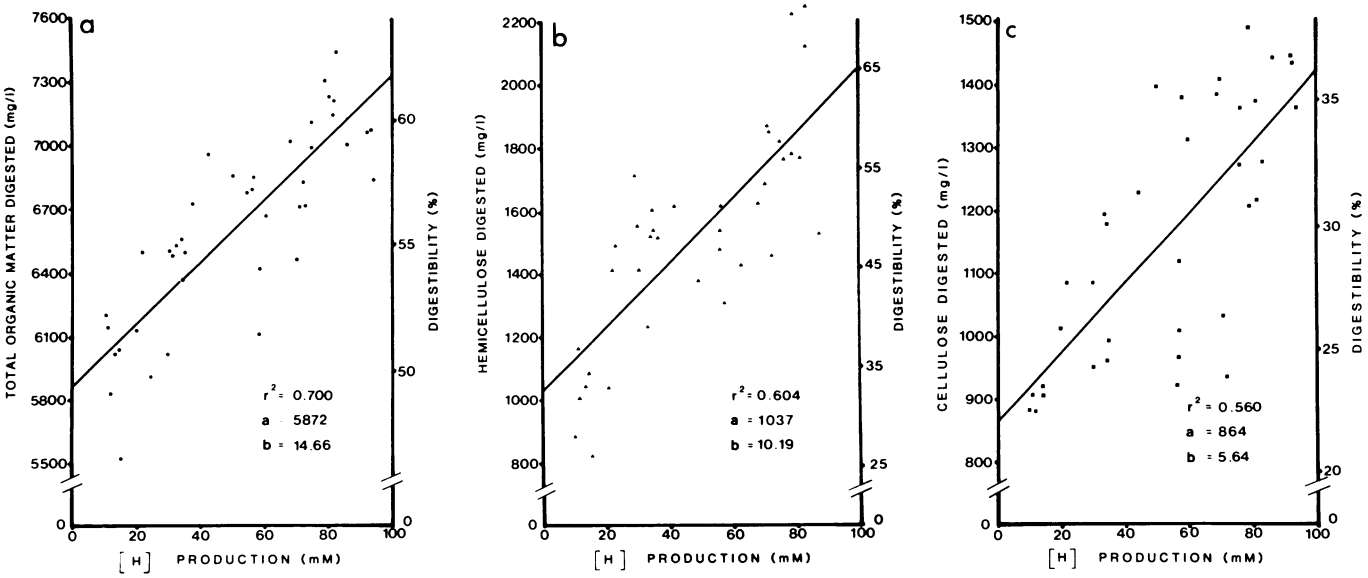

FIG. 2. Correlation between [H] (1/2 H_2) production and organic matter (a), hemicellulose (b), and cellulose (c) digestion by mixed cultures of rumen bacteria. Timothy hay (12.5 g/liter) was the substrate, and the incubation was for 34 h.

probable pathways of branched-chain amino acid fermentation in the rumen (see references 1 through 4):

valine + H_2O → α-ketoisovalerate + NH_3 + 2[H]

α-ketoisovalerate + coenzyme A (CoA) → isobutyl-CoA + CO_2 + 2[H]

isobutyl-CoA + ADP + P_i → isobutyrate + ATP + CoA

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leucine + H_2O → α-ketoisocaproate + NH_3 + 2[H]

α-ketoisocaproate + CoA → isovaleryl-CoA + CO_2 + 2[H]

isovaleryl-CoA + ADP + P_i → isovalerate + ATP + CoA

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isoleucine + H_2O → α-keto-3-CH_3-valerate + NH_3 + 2[H]

α-keto-3-CH_3-valerate + CoA → 2-CH_3-butyrate + CO_2 + 2[H]

2-CH_3-butyryl-CoA + ADP + P_i → 2-CH_3-butyrate + ATP + CoA

DISCUSSION

Previous work has indicated that the rumen bacterium *Eubacterium limosum* can utilize carbon monoxide as an energy source and produce acetate (15). Our gas chromatography method was unable to quantitate low concentrations of carbon monoxide. However, when mixed rumen bacteria were incubated with carbon monoxide as the only potential energy source, acetate concentrations did not increase (Table 2). The absence of CO fermentation was probably related to the diet consumed by the fistulated cow. *E. limosum* has only been found in high numbers in animals fed molasses (16, 26); the diet we used was based on poor-quality forage. An incubation period of 34 h or less was not long enough for *E. limosum* numbers to increase significantly. Daniels et al. (12) showed also that carbon monoxide and hydrogen can be converted to methane by *Methanobacterium thermoautotrophicum*. The lack of methane in incubations containing hydrogen and carbon monoxide (Table 2) and the general inhibition of methane production by carbon monoxide (Fig. 1, 3, and 5) indicated that carbon monoxide was not converted to methane in these experiments.

When carbon monoxide was added to incubations containing mixed rumen microorganisms and timothy hay, effects were similar to those reported for the antibiotic monensin (7, 27, 30). Methane production was inhibited by as much as 88%, the molar ratio of propionate to acetate increased from 0.83 to 1.53, and extracellular ammonia declined from 5.2 to 2.4 mM (Fig. 1). Even low levels of carbon monoxide (less than 0.1 atm [ca. 10.1 kPa]) significantly changed the products of fermentation.

Additives like carbon tetrachloride, chloroform, sodium sulfite, and dichloroacetamide, which exert a toxic effect on methanogens, cause hydrogen to accumulate in mixed rumen bacterial incubations (7, 31). Carbon monoxide treatment was not associated with a significant increase in hydrogen production (Fig. 1), and these results indicated that carbon monoxide was decreasing the hydrogen production of nonmethanogenic rumen microorganisms. At carbon monoxide levels greater than 0.5 atm (ca. 50.5 kPa), an increase in the amount of formate was observed, and this indicated that formate production was not inhibited to as great an extent as hydrogen production.

Carbon monoxide decreased the digestion of hemicellu-

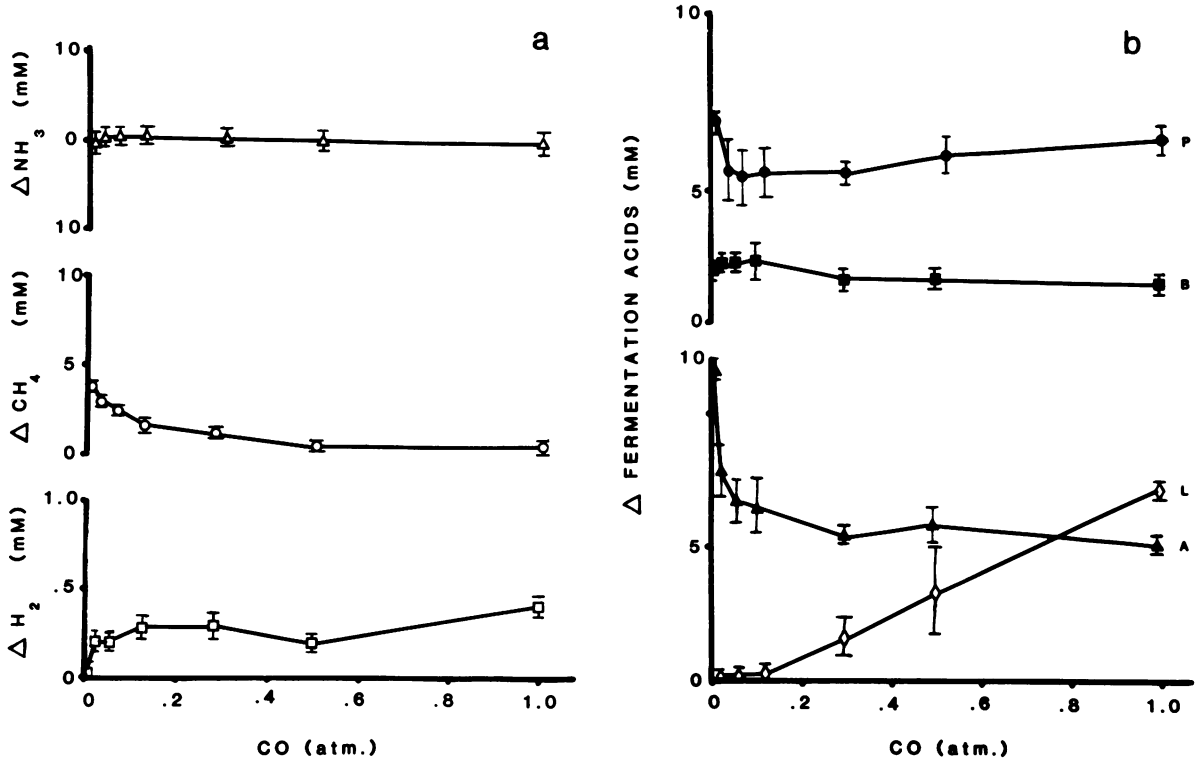

FIG. 3. Effect of carbon monoxide on the fermentations of starch (1.0 g/liter) by mixed rumen bacteria. One atmosphere of oxygen-free gas (CO, N_2, or both) was injected into each fermentation bottle. Changes in (a) extracellular ammonia (△), methane (○), hydrogen (□), or (b) acetate (A, ▲), propionate (P, ●), butyrate (B, ■), and lactate (L, ◇) are reported. Each culture was incubated for 10 h.

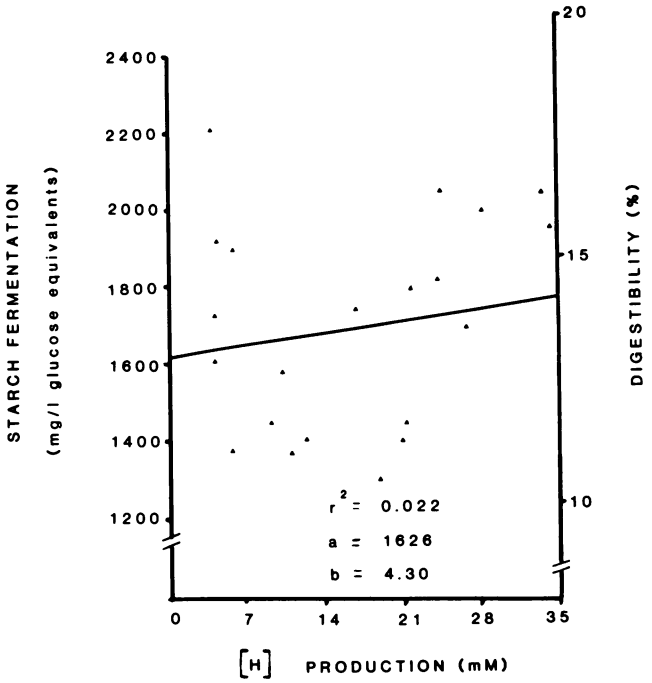

FIG. 4. Correlation between [H] (1/2 H_2) production and starch fermentation. Starch was the substrate, and the incubation was for 10 h.

lose and cellulose by 40 and 27%, respectively (Fig. 2). Because hemicellulose was degraded at a faster rate than cellulose, it appeared that the influence of [H] production was related to the inherent rate of digestion. Inhibition of fiber digestion also indicated that cellulolytic bacteria could not easily alter their fermentation, produce more reduced products, and adapt to a decrease in interspecies hydrogen transfer.

With starch as the energy source, methane production was likewise inhibited (Fig. 3), but the magnitude of starch fermentation was largely unaffected (Fig. 4). One atmosphere (ca. 101 kPa) of carbon monoxide decreased acetate production, but there was an equal molar increase in lactate (Fig. 3). In this case, the bacterial population was able to produce more reduced products, and the extent of fermentation did not decline. Carbon monoxide additions caused a small increase in hydrogen, and this could mean that the hydrogenases of starch-fermenting bacteria are less susceptible to carbon monoxide.

Ammonia production from the amino acid source, Trypticase, decreased 20% as carbon monoxide was increased to 1.0 atm (Fig. 5), and 93% of this decrease could be explained by an inhibition of branched-chain volatile fatty acid production. Because branched-chain volatile fatty acids are derived from branched-chain amino acids, it appeared that only the fermentations of valine, leucine, and isoleucine were significantly affected. A plot of increase in ammonia versus [H] production likewise indicated that the molar ratio was 4:1 (Fig. 6). This ratio was in agreement with theoretical pathways of branched-chain amino acid fermentation (see

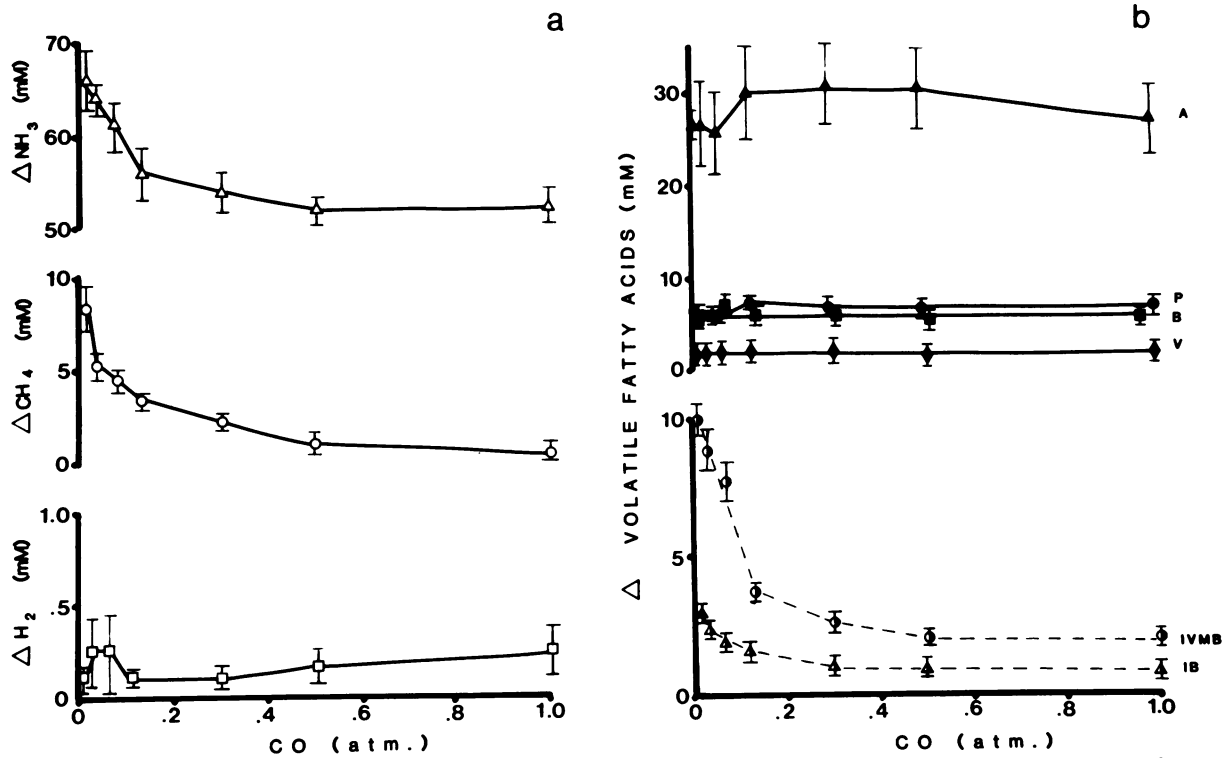

FIG. 5. Effect of carbon monoxide on the fermentations of Trypticase (15 g/liter) by mixed rumen bacteria. One atmosphere of oxygen-free gas (CO, N_2, or both) was injected into each fermentation bottle. Changes in (a) extracellular ammonia (△), methane (○), hydrogen (□), or (b) acetate (A, ▲), propionate (P, ●), butyrate (B, ■), valerate (V, ◆), isovalerate plus 2-methyl butyrate (IVMB, ◨), and isobutyrate (IB, ◭) are reported. Each culture was incubated for 22 h.

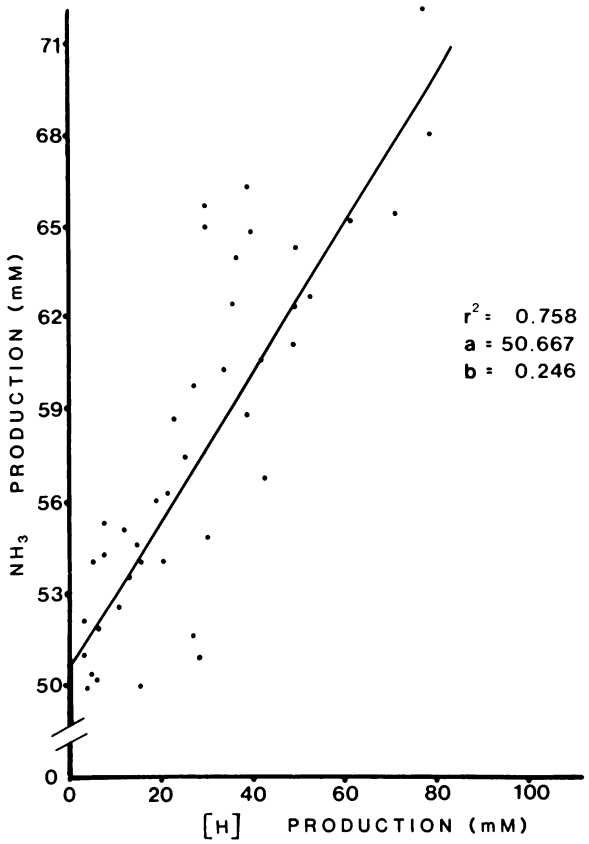

FIG. 6. Correlation between [H] (1/2 H_2) production and ammonia production from Trypticase (15 g/liter) after incubation for 22 h. The Trypticase was 11% nitrogen, so ammonia production of 71 mM represented a digestion of ca. 60%.

above). The plot of [H] versus ammonia did not pass through the origin, and the intercept was proportional to the concentration of branched-chain amino acids in Trypticase. Compositional data from BBL indicated that valine, leucine, and isoleucine account for 17.7% of the amino acids in Trypticase.

The importance of redox state to amino acid fermentation by anaerobic bacteria was first demonstrated by Strickland in the 1930's (18, 23). He and later workers showed that highly reduced amino acids like leucine, valine, and isoleucine could only be fermented if acceptors of hydrogen, oxidized amino acids, were available. In our mixed-culture incubations, methanogenesis was a primary hydrogen acceptor, and a decrease in interspecies hydrogen transfer selectively inhibited the fermentation of highly reduced amino acids.

Carbon monoxide has no value as an in vivo methane inhibitor because of its well-documented interaction with cytochrome a_3 of aerobic organisms (22). However, carbon monoxide may have value as an in vitro tool. Previous work indicated that carbon monoxide caused a light-reversible inhibition of bacterial hydrogenases (28), and strict anaerobes do not contain type *a* cytochromes (22). Because carbon monoxide inhibited hydrogen production as well as methane formation, it may provide a specific means of inhibiting interspecies hydrogen transfer in mixed-culture, ruminal incubations. Experiments are currently being conducted to compare the effects of carbon monoxide and monensin on in vitro ruminal fermentation. Such studies may indicate whether monensin responses are due to its general properties as an antibiotic or to a more specific action on rumen bacterial hydrogenases.

ACKNOWLEDGMENTS

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