Cytotoxicity in MA-104 Cells and Rumen Protozoa of Some Phytotoxins and Their Effect on Fermentation by Faunated and Defaunated Rumen Inocula

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Toxic compounds present in plants are an important factor limiting the nutritional use of tropical legumes. This work deals particularly with the toxic nonprotein amino acid analogues mimosine, DOPA, and canavanine and structurally related compounds such as nicotinic acid, 3-hydroxypyridine, and catechol. The effect of these toxins was measured on the viability of eukaryote cultured cells (MA-104) and rumen protozoa and on the fermentation activity (production of VFAs, ammonia, and gas) by inocula from faunated and defaunated sheep *in vitro*. Toxins did not increase cell death of protozoa, while catechol and nicotinic acid increased cell death of MA-104 cells. Thus, these toxins affected differentially the viability of eukaryote cells. Mimosine, nicotinic acid, DOPA, and canavanine had a stimulatory effect on fermentation but increased accumulation of methane by faunated inoculum. Catechol drastically depressed the fermentation activity of faunated and defaunated inocula and impaired the ammonia uptake by rumen microbes. Therefore, none of these toxins would be suitable to eliminate protozoa, and they do not appear to improve the overall fermentation balance in the rumen.

INTRODUCTION

Toxic compounds present in plants are an important factor limiting the nutritional use of tropical legumes to feed monogastrics and, in some cases, ruminants. Phytotoxins have presumably evolved as a defense mechanism of the plants against herbivory (Bell, 1981), reducing the nutritional performance of predators that would otherwise be expected from the protein or energy content of the plant consumed (Pusztai, 1986).

The antinutritional constituents of legumes include a wide variety of compounds that affect animals directly if they reach the small intestine, or indirectly, as in the case of ruminants, if they affect the rumen ecosystem. Specialized herbivores with digestive fermentation in the foregut have microbial defensive mechanisms against some dietary phytotoxins (Bell, 1981), presumably derived from a coevolutive process between the plant and the herbivore (Van Soest, 1982).

The secondary compounds in tropical plants are diverse in their chemical nature, and free amino acid analogues are of special interest in this work (Bell, 1981; Rosenthal and Bell, 1979; Cheeke and Shull, 1985; D'Mello, 1989). Among tyrosine analogues, mimosine (Figure 1A) and its primary product of degradation, 3,4-dihydroxypyridine (3,4DHP; Figure 1B), are contained in *Leucaena leucocephala*, *Mimosa pudica*, and *Pithecelobium ondulatum* (Van Veen, 1973; Lucas et al., 1988) and have been reported to be toxic to nonruminants (Wayman et al., 1970; Ross et al., 1980) and to some ruminants (Megarrity and Jones, 1983; Jones and Hegarty, 1984). Mimosine can be degraded by some rumen bacteria (Allison et al., 1987; Domínguez-Bello and Stewart, 1990a) that confer tolerance to ruminants otherwise intoxicated when fed *Leucaena* forage. DOPA 3,4-Dihydroxyphenylalanine (DOPA; Figure 1D) is found in *Vicia faba*, *Macuna* spp., and *Stizolobium decringiamin* (Lucas et al., 1988) and is toxic to insects and humans (Harborne, 1989). Nicotinic acid (Figure 1C) has been shown to be degraded by some aerobic bacteria (Berhman and Stanier, 1957). Catechol (or

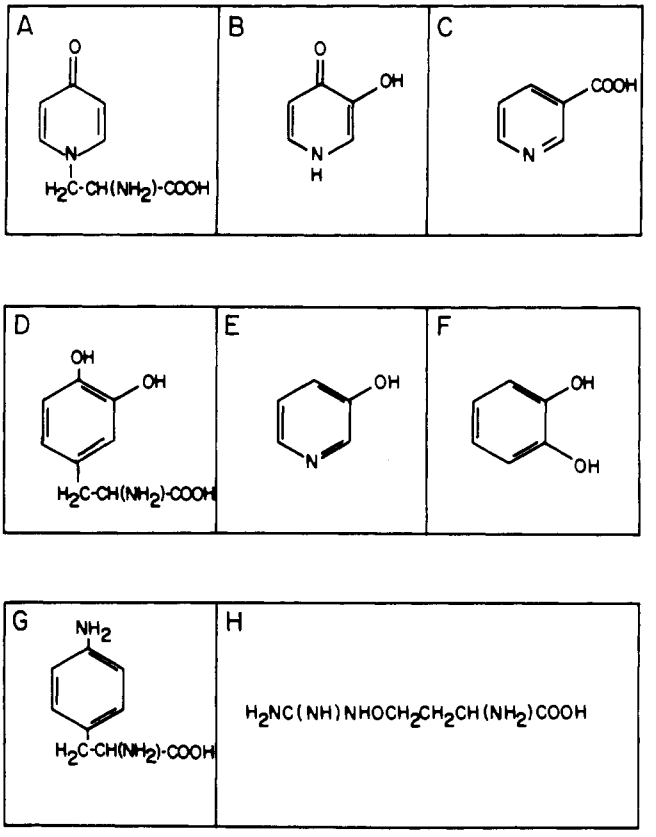

Figure 1. Structure of some free amino acids and analogue compounds in plants. (A) Mimosine; (B) 3-hydroxy-4(1*H*)-pyridone (3,4DHP); (C) nicotinic acid; (D) 3,4-dihydroxyphenylalanine (DOPA); (E) 3-hydroxypyridine; (F) catechol; (G) *p*-aminophenylanlanine; (H) and L-canavanine.

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pyrocatechol; Figure 1F), an intermediate of phenol metabolism (Scheline, 1991), and *p*-aminophenylalanine (Figure 1G) are toxic to humans and insects (Harborne, 1989), respectively. The arginine analogue canavanine (Figure 1H) has been shown to exert toxic effects on a wide variety of organisms, from viruses to prokaryote and higher organisms (Rosenthal, 1970; Schachtele and Rogers, 1968; Liu and Chaloupka, 1984).

A bioactive compound ingested by a ruminant could potentially affect several targets: prokaryote cells (ruminal bacteria), and eukaryote cells (ruminal protozoa and yeasts and the host tissues). Most information on cytotoxicity of plant compounds refers to eukaryotic cells, some to rumen bacteria, and there are no studies on rumen anaerobic protozoa, probably because of technical difficulties in culturing these organisms.

A beneficial effect of defaunation in ruminants fed diets poor in nitrogen content has been indicated (Bird and Leng, 1978). Toxins selectively affecting rumen protozoa might then be of interest in some tropical conditions. This paper presents information on the effect of natural tyrosine analogues and some intermediate metabolites (mimosine, 3,4DHP, DOPA, nicotinic acid, 3-hydroxypyridine, catechol) and an arginine analogue (canavanine) on the viability of eukaryote cells (cultured MA-104 and rumen protozoa) and on the pattern of rumen fermentation *in vitro*.

MATERIALS AND METHODS

Effect of Toxins on the Viability of MA-104 Cells. African green monkey kidney cells (MA-104) were grown on Eagle's minimal essential medium, supplemented with 10% fetal calf serum, in 24-well Linbro plates. After confluency in the cell monolayer, toxins (dissolved in Eagle's minimal essential medium) were added to the medium in concentrations of 4 and 8 mg/L of mimosine, nicotinic acid, DOPA, 3-hydroxypyridine, and catechol and of 0.6 and 1.2 mg/mL of canavanine. The concentrations of tyrosine analogues and intermediate metabolites were at least double those theoretically estimated in a 7-L rumen of a sheep consuming in one pulse 1 kg of *L. leucocephala* forage (2.5 mg/mL of mimosine). Similar calculations give a theoretical canavanine concentration of 0.07 mg/mL, in a 7-L rumen containing 200 g of *Canavalia ensiformis* seeds.

Cell death in MA-104 culture was determined [following the method described by Ruiz et al. (1991)] at 1.8, 4.0, and 5.5 h after the inclusion of the toxins to the medium, adding 50 μM ethidium bromide in phosphate buffer saline for 5 min. The medium was then removed, and fluorescence of the monolayer (a function of the number of dead cells permeable to ethidium bromide) was measured in an inverted microscope (Nikon, Diaphot TMD) equipped with a microphotometer. Total permeabilization was achieved by adding digitonin (40 μg/mL) to the monolayer. The experiments were carried out with four replicates for each toxin, and results were referred to a control cell culture without toxins.

Animals and Fermentors. An *in vitro* fermentation system (Jouany and Thivend, 1986) was inoculated with pooled rumen contents (50 mL, filtered) from each group of three sheep. Fermentors contained artificial saliva (50 mL) and the components indicated by Jouany and Thivend (1986), with twice the concentration of $NaHCO_3$ and $NaHPO_4$ (18.5 and 14.2 g/L, respectively). The fermentors were fed 3.67 g of starch and 1.25 mL $(NH_4)_2SO_4$ and contained (per duplicate) mimosine, nicotinic acid, 3-hydroxypyridine, DOPA, and catechol, in final concentrations of 1.6 mg/mL, and canavanine, in a final concentration of 0.12 mg/mL. All toxins were purchased from Sigma Chemical Co. (St. Louis, MO). The concentrations of toxins used in this experiments were all much higher than physiological concentrations. The concentration of 3,4DHP in the rumen of sheep fed 50% (w/w) *L. leucocephala* forage is about 0.06 mg/mL (Domínguez-Bello and Stewart, 1990a), and that of canavanine in the rumen of sheep fed 40% (w/w) *C. ensiformis* seeds was about 0.012 mg/mL (measurements performed 4 h after feeding; Domínguez-Bello and Stewart, 1990b).

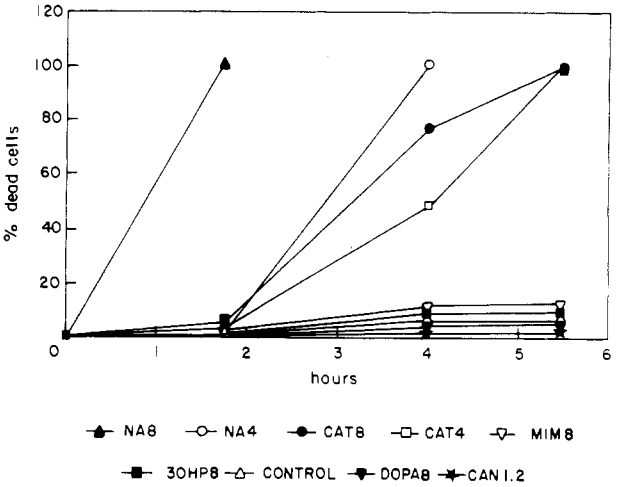

Figure 2. Effect of catechol (CAT), nicotinic acid (NA), mimosine (MIN), 3-hydroxypyridine (3OHP), dihydroxyphenylalanine (DOPA), and canavanine (CAN) on death of cells MA-104. Concentrations were 4 (CAT4, NA4) and 8 (CAT8, NA8, MIM8, 3OHP8, DOPA8) mg/mL of medium. Canavanine concentration was 1.2 mg/mL. Standard deviations were very small (≤1.8%) and lie inside the respective symbols in the figure.

Protozoal counts were recorded on samples taken from the fermentors at 0 and 6 h postinoculation using a Dollfuss cell under a 20× objective lens. Samples were filtered through a double gauze, and 0.1 mL was evenly spread on the chamber. Protozoa were stained by adding one drop of Lugol solution, counts were performed on four columns, and the numbers per milliliter of sample were estimated.

Gas volume was measured by trapping the gas produced into inverted graduated cylinders submerged in water saturated in $CaCl_2$ to avoid solubilization of gas (Jouany and Thivend, 1986).

Samples were taken at different time intervals for determinations of volatile fatty acids (VFA), pH, ammonia nitrogen, and production of gas and at 6 h for the determination of gas composition.

Analytical Methods. VFA were analyzed by gas–liquid chromatography as described by Jouany (1982). Ammonia concentrations were assayed by the method of Van Eenaeme et al. (1969), using a Technicon autoanalyzer. The composition of gases produced was determined by gas chromatography following the method described by Jouany and Senaud (1979).

RESULTS

Effect of Toxins on MA-104 Cells. Addition of 3-hydroxypyridine, 3,4DOPA, mimosine (4 and 8 mg/L), or canavanine (0.6 and 1.2 mg/L) had no effect whatsoever on the viability of the confluent MA-104 cell monolayer. However, nicotinic acid and catechol (4 and 8 mg/L) were potent cytotoxins, causing cell death within a few hours (Figure 2).

Effect of Toxins on Rumen Protozoa. Fermentors inoculated with rumen liquor from faunated animals consuming lucerne diet had a predominance of *Entodinium* (75–85%) and *Epidinium* (15–25%; Figure 3) protozoa. Initial protozoal density in the inoculum was 7×10^4 protozoa/mL. There was a 2-fold increase in protozoa numbers in the control flasks after 6 h of incubation, and in the treated flasks, protozoa viability was not reduced by the toxins. Furthermore, mimosine and canavanine caused an increase of about 25% in the numbers of protozoa from the inoculum of animals consuming lucerne diet (Figure 3).

Effect of Toxins on Fermentation Parameters. *VFA.* The production of VFA by the defaunated inoculum

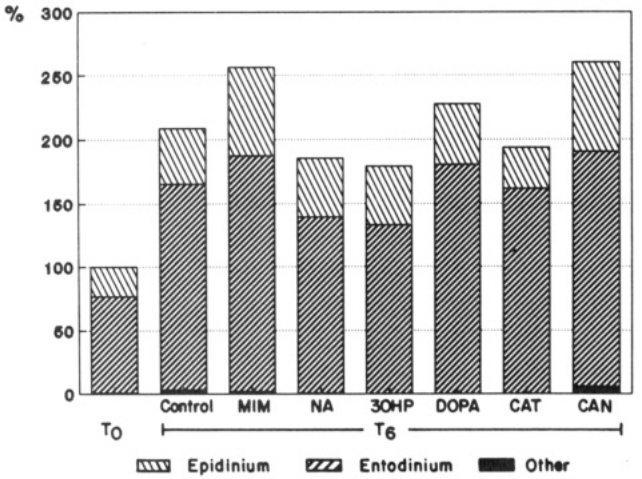

Figure 3. Effect of toxins on the *in vitro* viability of rumen protozoa from animals consuming lucerne diet. Initial density in the inoculum was 6.92×10^4 protozoa/mL of rumen contents.

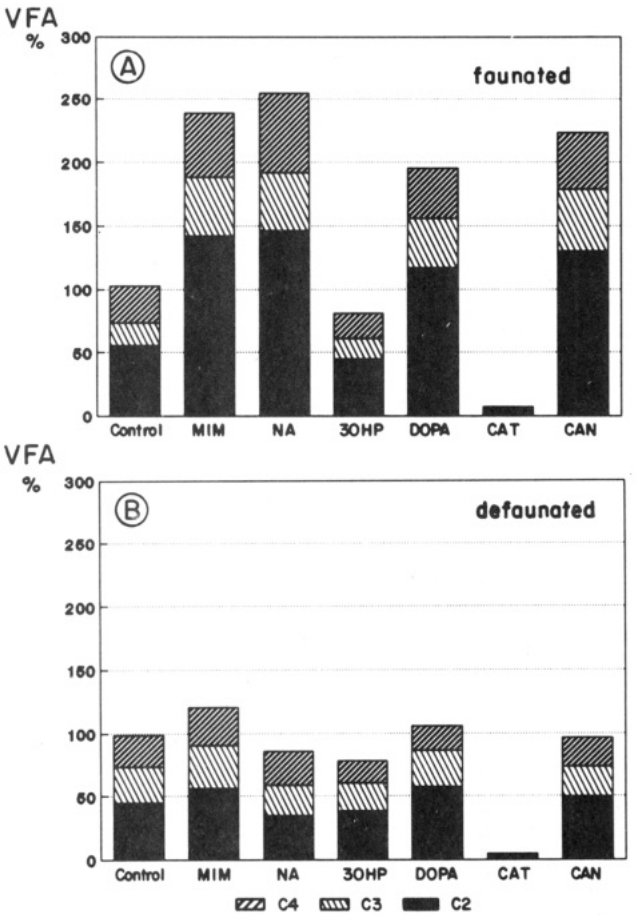

Figure 4. Effect of toxins on the production of VFA by faunated (A) and defaunated (B) inocula from sheep consuming lucerne diet. VFA concentrations in the control flasks after 6 h of incubation were 49.3 (A) and 80.7 mmol/L (B). Toxins are abbreviated MIM (mimosine), NA (nicotinic acid), 3OHP (3-hydroxypyridine), DOPA (3,4-dihydroxyphenylalanine), CAT (catechol), and CAN (canavanine).

during 6 h in the fermentors was higher than that by the faunated one (80.7 vs 49.3 mmol/L). The production of VFA by the inoculum from faunated animals was increased by all toxins except hydroxypyridine, which did not have an effect, and catechol, which drastically reduced VFA production (Figure 4A). Catechol was the only toxin having an effect on the production of VFA by defaunated inoculum, and this effect was markedly inhibitory (Figure

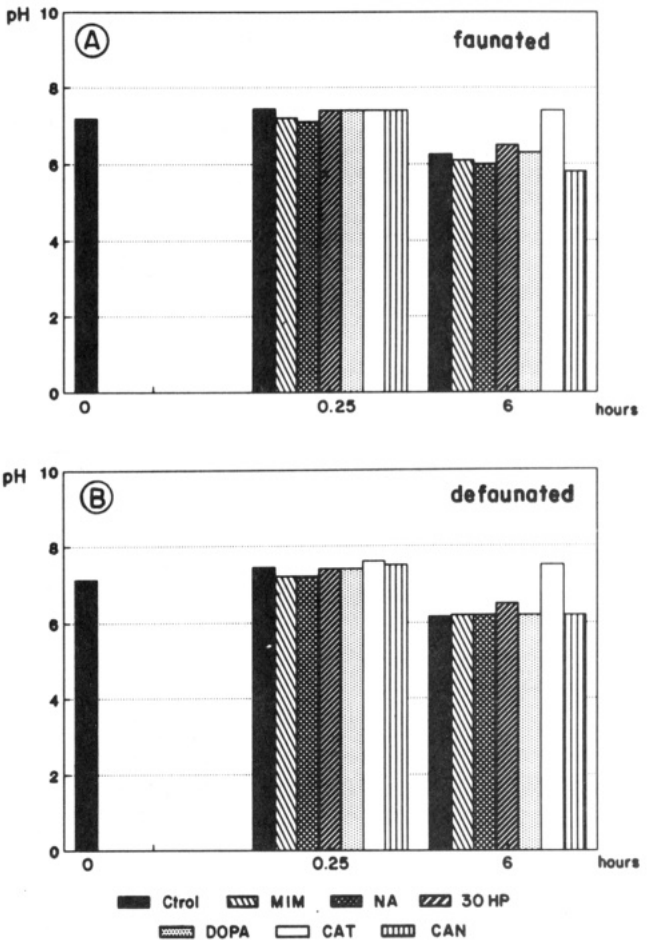

Figure 5. Effect of toxins on pH of media inoculated with faunated (A) and defaunated (B) inocula from sheep consuming lucerne diet. Toxins are abbreviated MIM (mimosine), NA (nicotinic acid), 3OHP (3-hydroxypyridine), DOPA (3,4-dihydroxyphenylalanine), CAT (catechol), and CAN (canavanine).

4B). Fermentors containing catechol developed a dark color after 6 h of incubation.

pH. Mean pH values after 6 h of incubation were close to 6 in control flasks inoculated with either faunated (Figure 5A) or defaunated (Figure 5B) inocula. There was no apparent effect of the toxins, except for an increase in pH by catechol (Figure 5).

NH_3. The production of ammonia by faunated or defaunated inocula after 15 min of incubation was about 500 mg/L (subtracting the values for ammonia at time 0; Figure 6), with no effect due to addition of toxins to the media. Fermentors with defaunated inocula had, after 6 h of incubation, lower concentrations of ammonia (between 150 and 220 mL/L; Figure 6B) than those with faunated inocula (220–380 mL/L; Figure 6A), except for the fermentors with catechol, where ammonia levels remained high (Figure 6).

Gases. Control fermentors with faunated and defaunated inocula produced, respectively, 340 and 370 mL of gas. Total gas production was increased by mimosine, nicotinic acid, and canavanine, while it was markedly reduced by catechol and to a lesser extent by 3-hydroxypyridine (Figure 7). The increase in the production of gas corresponded to an increase in CH_4 (Figure 8A,C) and CO_2 (Figure 8B,D). Catechol drastically inhibited production of CH_4 and CO_2. Defaunation did not alter total gas production, and the effect of toxins was similar to those in faunated inoculum. Defaunation, however, caused an increase in H_2 and a decrease in CH_4 (Figure 8C).

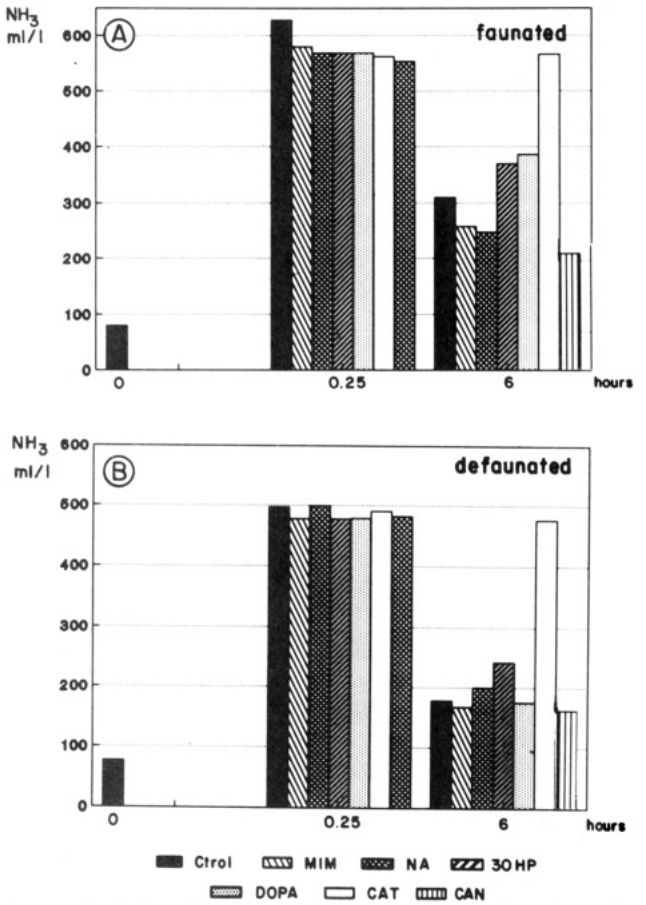

Figure 6. Effect of toxins on ammonia concentrations in media inoculated with faunated (A) and defaunated (B) inocula from sheep consuming lucerne diet. Toxins are abbreviated MIM (mimosine), NA (nicotinic acid), 3OHP (3-hydroxypyridine), DOPA (3,4-dihydroxyphenylalanine), CAT (catechol), and CAN (canavanine).

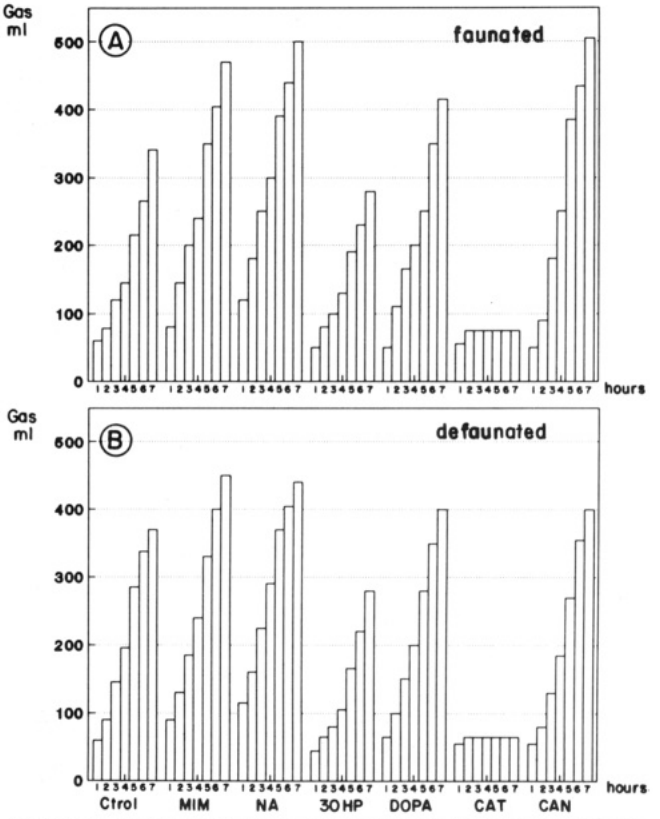

Figure 7. Effect of toxins on gas production by faunated (A) and defaunated (B) inocula from sheep consuming lucerne diet. Toxins are abbreviated MIM (mimosine), NA (nicotinic acid), 3OHP (3-hydroxypyridine), DOPA (3,4-dihydroxyphenylalanine), CAT (catechol), and CAN (canavanine).

DISCUSSION

At the concentrations of toxins included in the cultures of MA-104 cells, only catechol and nicotinic acid increased cell death. The experiments performed did not measure, however, toxic effects such as inhibition of cell division or any effect other than that on cell viability. Some toxins may affect cell metabolism without being lethal. Canavanine, for example, as an arginine analogue, might interfere with the physiological processes regulated by diamines and polyamines, important factors for growth regulation in bacteria and eukaryote cells (Bender, 1985). Mimosine has been shown to chelate metal ions necessary for enzyme activities (Stunzi et al., 1980; Cheeke and Shull, 1985; Jones et al., 1978) and to interfere with the metabolism of tyrosine and methionine (Christie et al., 1979; Cheeke and Shull, 1985; Hegarty et al., 1976). Viability of rumen protozoa appeared to be unaffected by the addition of the toxins to the media in concentrations that did cause alterations in the fermentation parameters. In general, protozoa seem to be less sensitive to pesticides and a number of antibacterial agents than are rumen bacteria (Prins, 1978), although they appear to be more sensitive to detergents and a low pH (Prins, 1991).

Defaunated inocula showed less production of VFA and methane, and higher ammonia concentration than faunated inocula, as has been reported in the rumen (Hobson and Jouany, 1988). Protozoal numbers were stimulated by mimosine and canavanine, while the fermentation activity of faunated inocula was stimulated by mimosine, nicotinic acid, DOPA, and canavanine (e.g., increased VFA and gas—methane and carbon dioxide—production, without a detectable decrease in pH). These changes were more marked in fermentors with faunated inocula, indicating that protozoal activity or that of the protozoa–bacteria system was affected by these toxins.

Bacteria, as suggested by the results with defaunated inoculum, were not affected in their fermentation activity by canavanine. Some rumen bacterial strains are able to degrade canavanine *in vitro*, and rumen bacterial sensitivity to canavanine was dependent on the composition of the culture medium (Domínguez-Bello and Stewart, 1990b).

The stimulatory effect of mimosine, nicotinic acid, DOPA, and canavanine on bacterial fermentation contradicts previous results with pure cultures of rumen bacteria. DOPA, nicotinic acid, and 3-hydroxypyridine have been shown to inhibit the growth of pure cultures of rumen bacteria, although some of these compounds can be degraded by rumen bacteria (Domínguez-Bello, 1989).

Catechol markedly depressed the fermentation activity and produced an impaired capability of the microflora to use ammonia. Catechol has been proved to be an inhibitor of bacterial growth of pure rumen bacterial strains (Domínguez-Bello, 1989), which agrees with our results. The dark color of flasks containing the potent toxic catechol might be due to the formation polymers of conjugated quinones. The metabolism of catechol in mammals involves oxidation to quinone derivatives and formation of benzoquinone and glutathione conjugates of the quinone (Sadler et al., 1988; Bhat et al., 1988). These compounds may suffer further polymerization, yielding products that give the urine a dark appearance (Miller et al., 1973).

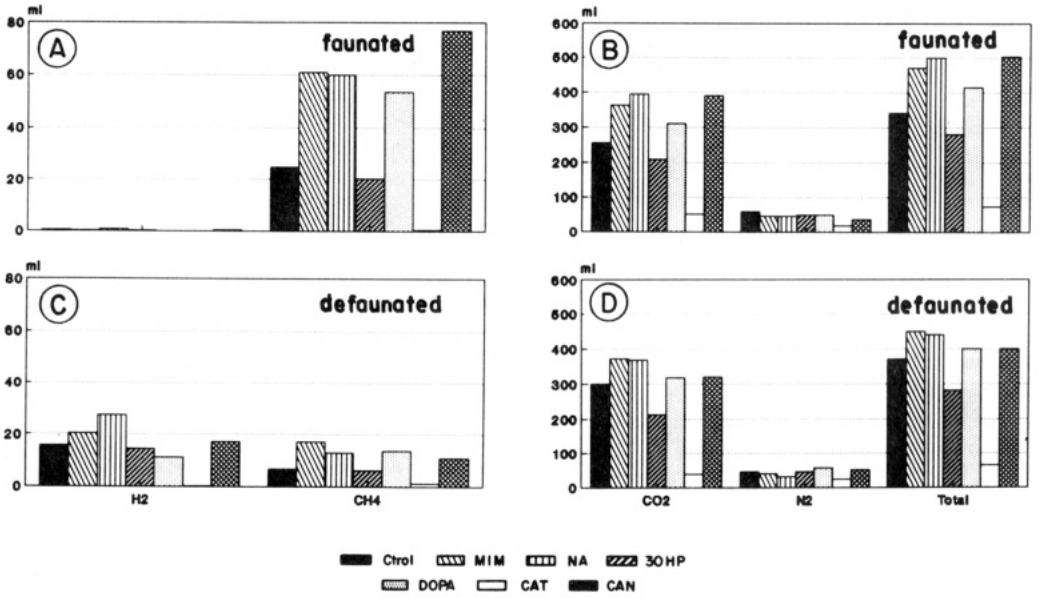

Figure 8. Effect of toxins on the composition of gas produced by faunated (A and B) and defaunated (C and D) inocula from sheep consuming lucerne diet. Toxins are abbreviated MIM (mimosine), NA (nicotinic acid), 3OHP (3-hydroxypyridine), DOPA (3,4-dihydroxyphenylalanine), CAT (catechol), and CAN (canavanine).

Defaunation caused accumulation of hydrogen in detriment of methane production. This result is consistent with previous findings (Jouany et al., 1981; Whitelaw et al., 1984), which attribute the reduction in methane production to the elimination of methanogens associated to protozoa (to their surface; Vogels et al., 1980) which provide methane precursors (Hobson and Jouany, 1988). Results from previous works have indicated a positive effect of defaunation on the intestinal nitrogen supply, mainly due to a lower proteolysis and deamination (Itabashi and Katada, 1976), reduction of N recycling in the rumen, and a higher flow of microbial protein into the duodenum (Jouany and Thivend, 1986; Ushida et al., 1990). The higher fermentation activity leading to VFA and gas in this study was inconsistent with the reported decrease (of 37%) in organic matter digestion after defaunation (Jouany and Thivend, 1986). However, fermentation by bacteria is quicker and more intense than that by protozoa (Jouany and Thivend, 1986), and this could explain the higher productions of VFA by defaunated inocula relative to that by faunated in this study. Thus, the selective direct effect of a toxin against a particular rumen population of bacteria or protozoa can also indirectly affect the density and activities of other populations.

The composition of rumen microflora may be different among animals consuming different diets, and the responses to toxins can therefore vary. We performed an experiment similar to the one reported in this paper, inoculating the fermentors with rumen contents from faunated animals consuming timothy diet (1.5 kg of timothy hay and 0.3 kg of pelleted barley grain) and found no effects of the toxins used in this work on the production of VFA and ammonia uptake (results not shown). The fact that the chemical environment (e.g., diet) may affect the action of a given toxin on the microbes from the rumen makes the toxicological problem even more complex.

Understanding the toxicology of plant phytotoxins on all posible targets exposed during the ruminant digestive process and their digestive microbial degradation is fundamental to the understanding of the rumen function to achieve biological solutions to problems of toxicity and in the practice of defaunation using tropical plants containing defaunating agents. The complexity of the rumen ecosystem, the variation of bacteria and protozoa in their response toward toxins, and the effects due to interactions among different populations are of relevance if the rumen composition is to be manipulated.

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