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Effect of the Novel Ionophore Tetronasin (ICI 139603) on Ruminal Microorganisms

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The antimicrobial activity of the novel ionophore tetronasin (formerly ICI 139603) was compared with that of monensin for the growth of ruminal bacteria, protozoa, and an anaerobic fungus. The potency of tetronasin toward most bacteria and the fungus was an order of magnitude or more greater than that of monensin. *Lactobacillus casei* was 55 times more sensitive to tetronasin than to monensin, indicating a potential role for tetronasin in reversing lactic acidosis. Bacteria with a gram-positive ultrastructure were generally sensitive to the ionophores and unable to adapt to grow in their presence. The exception was the cellulolytic *Ruminococcus flavefaciens*, which adapted during successive cultivation on media with increasing ionophore concentrations to grow at 100-fold higher concentrations of tetronasin than were initially lethal to the organism. Gram-negative bacteria were more resistant and generally able to adapt to grow in the presence of both ionophores. An in vivo trial with cattle and in vitro growth experiments indicated that the effect of tetronasin on ciliate protozoa was minor. In vitro experiments measuring hydrogen production by *Neocallimastix frontalis* suggested that this fungus would be unable to survive in ruminants receiving tetronasin.

Tetronasin (ICI 139603) is a novel ionophoric antibiotic produced by *Streptomyces longisporoflavus* which has been shown to increase live weight gain and food conversion efficiency in cattle (5). It is structurally dissimilar to other ionophores studied experimentally in cattle in that it contains a divalent-cation-binding acyl tetronic acid group in place of the more common monovalent-cation-binding carboxylic group of monensin and lasalocid (9). Tetronasin strongly resembles the mode of action of other ionophores in the rumen with a shift toward propionate and a decrease in dietary protein degradation (4, 13).

Changes in ruminal metabolism when carboxylic ionophores are included in the diet have widely been attributed to alterations in the microbial ecology of the rumen caused by the inclusion of the ionophore (2, 10). Thus, the purpose of the present study was to compare the sensitivity of ruminal microorganisms to tetronasin and monensin.

MATERIALS AND METHODS

Bacteria. All bacteria used to initiate growth studies were overnight cultures of stock cultures held in the culture collection of the Rowett Research Institute, Bucksburn, and with the exception of *Methanobrevibacter smithii* were grown in Hungate tubes (Bellco Glass, Inc., Vineland, N.J.) under anaerobic conditions at 39°C in Hobson medium 2 (11). *Methanobrevibacter smithii* was grown on a medium containing (per 100 ml): 15 ml of mineral solutions a and b of Hobson, 20 ml of clarified ruminal digester fluid, 20 mg of cysteine hydrochloride, 0.1 ml of DL-2-methylbutyric acid, and 0.0001 g of resazurin. The medium was prepared and maintained under an atmosphere of 80% H_2–20% CO_2.

Fungus. *Neocallimastix frontalis* RE1 was isolated from the rumen of a sheep and maintained on medium 2 of Hobson with the addition of 300 ml of clarified ruminal fluid, 10 g of glucose, and 10 g of agar per liter.

Effect of ionophores on bacterial growth. Bacterial growth was measured directly in Hungate tubes (0.5 ml of an inoculum in 7 ml of media) by following the change in optical density at 650 nm over 48 h. Antibiotics were added in ethanol solution (1 ml/liter) to the medium before autoclaving. Bacteria were either inoculated directly into the ionophore-containing medium or adapted to the medium in a series of stepwise additions; bacteria grown for 48 h in the presence of the lowest concentration of antibiotic were then inoculated onto the second lowest concentration and so on every 48 h until such time as growth was totally inhibited.

Effect of ionophores on activity of *Methanobrevibacter smithii* and *N. frontalis*. Gas production, measured in duplicate incubations as previously described (19), over 10 days was used as an index of activity for *N. frontalis* (hydrogen production) and *Methanobrevibacter smithii* (methane production) in both the presence and absence of ionophores.

Protozoa. The effect of tetronasin on protozoal numbers was investigated both in vitro and in vivo. In vitro incubations were done in a system similar to that of Dennis et al. (7). Ruminal fluid from two rumen-fistulated sheep (not receiving antibiotics) fed (1 kg/day) a diet of hay, barley, molasses, and fish meal (5:3:1:1) was collected 1 h after morning feeding and strained through four layers of muslin. Strained ruminal fluid (15 ml) was mixed with an equal volume of Coleman salt solution D (3) in a 50-ml centrifuge tube containing 0.5 g of dried grass. Tetronasin and monensin were dissolved in ethanol and added at 25 μg/ml. The control tube received only ethanol (0.2 ml). The tubes were flushed with carbon dioxide, capped with rubber stoppers equipped with one-way valves, and incubated at 39°C for 8 h. After incubation, the mixture was strained through muslin before protozoa were counted. Each animal was sampled on two different occasions, and duplicates were set up at each incubation.

Samples for the determination of the effect of tetronasin in vivo were obtained from a trial being done by FEEDS Ltd. (Aberdeen, United Kingdom) for Coopers Animal Health Ltd. (Berkhamstead, Hertfordshire, United Kingdom). Thirty castrate Friesian × Hereford crosses were fed a diet of barley, nutritionally improved straw, soya bean meal, molasses, and a mineral and vitamin mix (66.5, 20, 12.5, 2.5, and 2.5% of the dry matter, respectively) ad lib with the

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TABLE 1. Concentration of ionophore required to inhibit growth of various ruminal bacteria by 50% over 48 h with adapted and nonadapted bacterial cultures

Species and strain	IC_{50} of ionophore (μg/ml)[a]			
	Tetronasin		Monensin	
	Direct inoculation	Adaptation	Direct inoculation	Adaptation
Eubacterium ruminantium 2388	0.002	0.002	0.015	0.030
Butyrivibrio fibrisolvens JW11	0.004	0.004	0.128	0.169
Lachnospira multiparus D15d	0.006	0.008	0.128	0.294
Ruminococcus albus SY3	0.004	0.016	0.064	0.064
Streptococcus bovis C277	0.028	0.024	0.32	0.34
Lactobacillus casei LB17	0.012	0.037	1.176	2.050
Bacteroides succinogenes S85	0.024	0.056	0.023	0.194
Bacteroides ruminicola M384	0.024	0.084	2.702	7.132
Ruminococcus flavefaciens 007	0.005	0.588	0.388	0.588
Veillonella alcalescens L59	0.32	2.52	15.3	>16
Bacteroides multiacidus 4615	2.35	7.13	>16	>16
Selenomonas ruminantium Z108	2.35	9.41	>16	>16
Ruminobacter amylophilus WP91	2.35	10.81	1.78	>16
Megasphaera elsdenii J1	8.19	>16	>16	>16

[a] Mean of duplicate experiments.

animals being split into three treatment groups, each of 10 animals, receiving 0, 6, and 10 mg of tetronasin per kg of total diet, respectively. Ruminal samples were taken by stomach tube from all animals 42 and 84 days after the start of the trial.

All protozoal counts were made in a Hawksley cell (16); protozoal genera were identified by using the scheme of Ogimoto and Imai (12).

Chemicals. Tetronasin was from Coopers Animal Health Ltd. Monensin was from Sigma Chemical Co. Ltd. (Poole, Dorset, United Kingdom).

RESULTS

The effect of the ionophores in medium containing serially doubled concentrations of ionophore, starting with 1 ng/ml (i.e., 1, 2, 4, 8, etc., ng/ml) was examined either by direct inoculation or by stepwise adaptation as described above. Results were plotted as a graph of percent growth inhibition against ionophore concentration and are expressed in Table 1 as the concentration of ionophore necessary to reduce growth by 50% (50% inhibitory concentration [IC_{50}]). It was not established whether this reduction was caused by an inhibition of growth rate or a reduction in growth yield. Species are listed in order of their sensitivity to tetronasin after adaptation.

Although there was a large difference in the toxicity of the two ionophores, with tetronasin by far the more potent, both showed a similar spectrum of activity. The gram-positive bacteria *Streptococcus bovis*, *Lachnospira multiparus*, and *Eubacterium ruminantium* all showed a high susceptibility to both ionophores, with a 50% reduction in growth at monensin concentrations below 0.32 μg/ml and tetronasin concentrations below 0.024 μg/ml, with little sign of adaptation. *Lactobacillus casei* (gram positive) had a moderate (IC_{50}, 1.176 μg/ml) resistance to monensin but was highly susceptible (IC_{50}, 0.012 μg/ml) to tetronasin. Other *Lactobacillus* species had similar properties (Table 2). Both of the gram-variable ruminococci, *Ruminococcus albus* and *Ruminococcus flavefaciens*, were susceptible to the ionophores but showed some sign of adapting, this adaptation being particularly marked for *Ruminococcus flavefaciens* and tetronasin. Of the gram-negative bacteria tested, only *Bacteroides succinogenes* and *Butyrivibrio fibrisolvens* were highly susceptible to the ionophores (IC_{50}s: for monensin, ≤0.13 μg/ml; for tetronasin, ≤0.024 μg/ml). *Veillonella alcalescens*, *Bacteroides multiacidus*, *Ruminobacter amylophilus*, *Bacteroides ruminicola*, *Selenomonas ruminantium*, and *Megasphaera elsdenii* were all moderately to highly resistant (IC_{50}s: for monensin, 1.78 to >16 μg/ml; for tetronasin, 0.024 to 8.19 μg/ml) to the ionophores and showed marked adaptation.

Growth of *Methanobrevibacter smithii* as indicated by methane production was relatively insensitive to the presence of tetronasin up to fairly high concentrations of the ionophore (Fig. 1). A concentration of 2 μg/ml was necessary to inhibit methane production.

The effect of tetronasin on the rumen fungus *N. frontalis* was determined by its effect on hydrogen production (Fig. 2). Growth of the fungus was suddenly and sharply inhibited by ionophore concentrations above 0.064 μg/ml.

Protozoa. The effect of tetronasin on protozoal numbers in vivo is shown in Table 3. Owing to wide variation in the counts within groups, possibly due to variable contamination of the samples by saliva during sampling, no significant ($P > 0.05$) differences were evident between treatment groups. However, a notable drop in total protozoal numbers occurred with increasing ionophore addition in period 1, largely owing to a drop in the numbers of small entodiniomorphid protozoa. In period 2, there were no clear effects of ionophore addition, suggesting that if the ionophore truly had caused a drop in the count of small entodinia in period 1, then adaptation had occurred in the following 6 weeks.

The effect of tetronasin and monensin on protozoal num-

TABLE 2. Influence of tetronasin and monensin on the growth of *Lactobacillus* species in nonadapted cultures

Species and strain	IC_{50} of ionophore (μg/ml)[a]	
	Tetronasin	Monensin
L. acidophilus D137	0.030	0.600
L. fermentum 1750	0.046	0.240
L. ruminis LB2029	0.045	10.200
L. vitulinus 2030	0.022	0.200

[a] Mean of duplicate experiments.

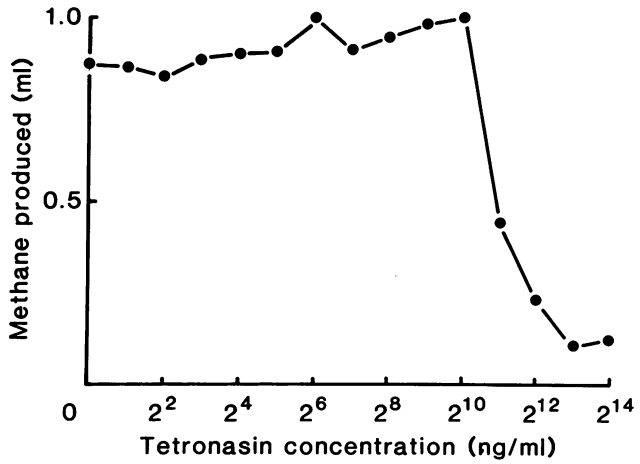

FIG. 1. Effect of tetronasin on methane production by *Methanobrevibacter smithii* in a 10-ml culture.

bers was investigated by 8-h in vitro incubations (Table 4). The effect of the ionophores was again mainly restricted to the *Entodinium* protozoa, as neither ionophore had a significant effect on the numbers of *Isotricha*, *Dasytricha*, *Diplodplastron*, or *Polyplastron* species. With both the monensin and tetronasin, the numbers of *Entodinium* species (and thus the total count of protozoa) was significantly ($P < 0.05$) lower in the presence of the ionophores.

DISCUSSION

As in previous studies, gram-positive bacteria and bacteria with a gram-positive cell wall ultrastructure (*Butyrivibrio fibrisolvens*, *Ruminococcus albus*, *Ruminococcus flavefaciens*) were found to be highly susceptible to ionophores (2, 10, 14). This is in agreement with the scheme of Chen and Wolin (2) which suggested that an important mode of ionophore action in the rumen is the inhibition of acetate- and butyrate-producing bacteria such as the ruminococci and butyrivibrios and the relative proliferation of ionophore-insensitive propionate-producing bacteria such as *Selenomonas* and *Megasphaera* species.

In general, adaptation of gram-positive bacteria to the two ionophores was slight, with the notable exception of *Ruminococcus flavefaciens* with tetronasin. The ability of this bacterium to adapt to tetronasin suggests that the effect of tetronasin on the ecology of fiber digestion in the rumen differs from that proposed for other ionophores such as avoparcin and monensin (10, 14). With these ionophores, it was proposed that *Bacteroides succinogenes* would fill the niche as the major fiber digester owing to its ability to adapt to higher ionophore concentrations than the ruminococci (10, 14). However, with tetronasin, it is likely that the reverse will occur and *Ruminococcus flavefaciens* will be favored.

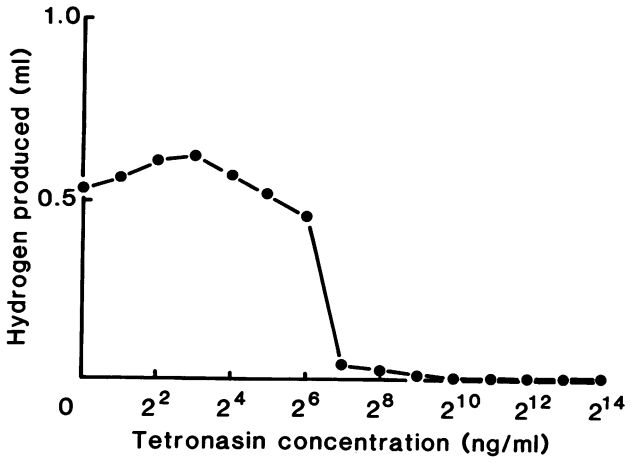

FIG. 2. Effect of tetronasin on hydrogen production by *N. frontalis* in a 10-ml culture.

TABLE 3. Effect of tetronasin on protozoal numbers in vivo

Period[a]	Genus	No. of protozoa (10^3)[b]/ml of strained ruminal fluid from cattle fed tetronasin at (mg/kg):			
		0	6	10	SD
1	*Entodinium*	1,621	1,532	1,271	354
	Isotricha	7.5	13.0	7.9	5.9
	Dasytricha	12.5	3.8	6.2	3.1
	Epidinium	36.2	43.8	82.5	20.5
2	*Entodinium*	1,837	1,967	1,597	264
	Isotricha	5.8	18.3	2.3	8.4
	Dasytricha	5.8	10.0	2.5	6.3
	Epidinium	37.5	44.2	32.5	16.3

[a] Period 1, Tetronasin added to diet for 6 weeks; period 2, tetronasin added to diet for 12 weeks.
[b] Mean and standard deviation of samples taken from 10 cattle on each treatment.

It has been suggested that carboxylic ionophores could reduce the risk of acidosis in grain-fed ruminants by controlling the numbers of *Streptococcus bovis* (6), one of the organisms most active in the early stages of the disorder. Results from the present experiments suggest that tetronasin may be expected to have a similar effect in preventing the onset of acidosis. However, it will have the additional advantage over monensin in controlling *Lactobacillus* numbers in animals in which acidosis is firmly established.

As with monensin (2, 17), tetronasin had little effect on methane production by the methanogen *Methanobrevibacter smithii*, and any influence it may have on methane production in the rumen will be indirectly through reducing the supply of metabolic hydrogen. It should be noted, however, that *Methanobrevibacter smithii* was not isolated from the rumen (14).

Tetronasin was highly active against the ruminal fungus *N. frontalis*. The significance of this antifungal action in terms of ruminal fermentation is unclear, since although the ruminal fungi have been shown to be both proteolytic and fiber

TABLE 4. Effect of tetronasin and monensin on numbers of ruminal protozoa in vitro

Genus	No. of protozoa (10^3/ml)[a] after incubation with:		
	Control	Tetronasin (25 μg/ml)	Monensin (25 μg/ml)
Diploplastron	1.2	1.2	0.9
Polyplastron	0.6	0.8	0.7
Isotricha	7.5	5.8	6.0
Dasytricha	1.1	1.9	1.5
Entodinium	518.8	348.8*	375.0*
Total	529.4	358.5*	384.1*

[a] Means within rows followed by asterisks differ from the mean of the control ($P < 0.05$).

digesting (1, 19) their quantitative significance to the total microbial activity in the rumen is unknown. Elliott et al. (8) have suggested that part of the increase in propionate concentrations seen when ionophores are fed is due to the inhibition of fungi and their associated methanogenic bacteria, leading to a change in the hydrogen flow pattern in the rumen and an increase in propionate. The reduction in protein digestion in the rumen seen in in vivo trials with tetronasin (13) would also tally with the inhibition of these highly proteolytic organisms (19). The effect of monensin was not determined in the present study, but comparison with the results of Stewart et al. (15) suggests that for the fungi, as with most of the bacteria, tetronasin is about an order of magnitude more potent than monensin.

The effect of tetronasin on ruminal protozoa was equivocal. In vivo results from the present study failed to show any statistically significant effect, although they do suggest that in agreement with results with monensin (7), any toxic effect of the ionophore will be manifest mainly in the small entodinia and that these organisms adapt to the ionophore. Results from the in vitro incubations confirm that the effect of the ionophores was mainly limited to the small entodinia, although it should be noted that the concentrations of ionophore used in this system were, by necessity, far higher than those likely to occur in vivo, owing to the intrinsic insensitivity of using a short-term incubation (8 h) in the study of factors affecting the growth of organisms with a doubling time of 12 to 24 h.

In summary, it would appear that tetronasin and monensin have a broadly similar spectrum of activity in the rumen. Thus, the observed effects of tetronasin in vivo in altering the stoichiometry of the ruminal fermentation can be explained by changes in the microbial ecology of the rumen similar to those proposed for monensin (2). However, differences in potency and in the ability of individual bacteria to adapt to the two ionophores suggest that they will differ in their specific effects in the rumen; possible differences in the areas of lactate metabolism and fiber digestion are indicated in the present study.

Effects of tetronasin on ruminal protein metabolism in vivo (13) are harder to explain in terms of an ecological shift in the rumen, because of the number of different proteolytic species present (18). Nonetheless, the ionophore is active against several potentially protein-digesting organisms including *N. frontalis*, *Bacteroides ruminicola*, and *Streptococcus bovis*, and it seems reasonable that a reduction in ruminal proteolytic activity could result from the presence of tetronasin.

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