CHANGES IN THE RUMEN BACTERIUM, *BACTEROIDES RUMINICOLA*, GROWN IN THE PRESENCE OF THE IONOPHORE, TETRONASIN

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Introduction

Tetronasin is a feedlot ionophore which, although more potent than monensin and lasalocid, has a similar spectrum of antimicrobial activity in that it suppresses the growth mainly of Gram-positive rumen bacteria (Chen and Wolin, 1979; Newbold et al., 1988). The nutritional effects of ionophores have usually been interpreted in terms of the known activities of these bacteria and the likely metabolic consequences of their deletion from the rumen ecosystem. Much less is known about how ionophores affect the population of intrinsically resistant bacteria that survives while ionophores continue to be added in the diet. These predominantly Gram-negative bacteria show evidence of adaptation to tetronasin during cultivation in the presence of the ionophore *in vitro* (Newbold et al., 1988), and this adaptation results in a lower deaminative activity against amino acids (Newbold et al., 1989). The present experiments demonstrate that *Bacteroides ruminicola* grown in the presence of tetronasin has a decreased affinity for binding the ionophore, and that a reduced rate of hydrolysis of phe_4, but not phe_3 or phe_2 accompanies this decrease. A common mechanism involving a decreased permeability of the cell envelope is proposed.

Materials and Methods

Bacteroides ruminicola M384 was grown in medium 2, and was adapted to grow in the presence of tetronasin by subculturing in medium containing increasing concentrations of the ionophore, as described by Newbold et al. (1988) up to 0.1 μg/ml. The culture was maintained in medium containing this concentration of tetronasin for several months before use.

The binding of tetronasin to *B. ruminicola* was measured using [^{14}C] tetronasin, a gift from Coopers Animal Health Ltd. Overnight cultures were centrifuged (18,000 g, 3 min) and bacteria were washed once before finally resuspending at their original cell density in 0.12 M Tris HCl, pH 7.0. Cell suspensions (0.5 ml) were incubated with unlabelled tetronasin for 10 min, then 0.1 μCi each of [^{14}C] tetronasin (1.35 mCi/mmol) and [^{3}H] inulin (3.9 Ci/mmol) were added. Incubations were stopped by rapid filtration through a glass-fibre filter on top of a 0.45 μm cellulose nitrate filter, followed by washing with 2 ml of distilled water. Extracellular liquid trapped on the filter was estimated from the count of ^{3}H, and bound tetronasin was calculated from the difference between the total ^{14}C and the extracellular [^{14}C] tetronasin calculated from the inulin volume. EDTA treatment to remove the outer membrane was carried out as described by Leive (1968).

Peptidolytic activity was determined using phenylalanine peptides, phe_2, phe_3 and phe_4. Overnight cultures of *B. ruminicola* were harvested and resuspended in anaerobic buffer (1 M Tris HCl, pH 7.8, containing 0.5% glucose). Suspensions of *B. ruminicola* adapted to grow in the presence of ionophore also contained 0.1 μg tetronasin/ml. Peptides were dissolved in 70% acetic acid, and were added in a 30 μl volume to 1.2 ml of cell suspension, giving a final pH of the incubation mixture of 6.8 and a final peptide concentration of 112 μM. Suspensions were incubated at 39°C under CO_2, the reaction was stopped by the addition of H_3PO_4 to a final concentration of 0.175 M, and peptide concentrations were determined by HPLC (Newbold et al., 1989). Protein was determined using the Folin reagent.

Results and Discussion

Tetronasin rapidly became associated with *B. ruminicola* (figure 1), such that after 3 min the [^{14}C] tetronasin bound to cells was 5.4×10^5 dpm/mg protein. Since the cell volume of *B. ruminicola* is about 3 μl/mg protein, this is equivalent to 1.8×10^8 dpm/ml of cell water, or an apparent accumulation ratio of 400 over the extracellular count of 4.4×10^5 dpm/ml. It was not established if this apparent accumulation was caused by an active uptake of tetronasin or to binding of tetronasin to cell material, although

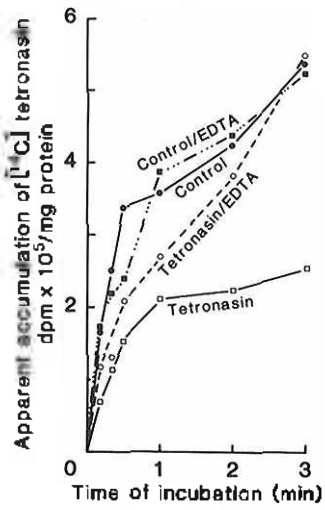

Figure 1. Apparent accumulation of [^{14}C] tetronasin by *B. ruminicola* adapted to tetronasin, and influence of washing with EDTA.

the latter seems more likely. The binding of [^{14}C] tetronasin to *B. ruminicola* adapted to tetronasin by growth in the presence of 0.1 μg tetronasin/ml was decreased in rate and extent by about one-half (figure 1).

B. ruminicola stripped of its outer membrane by EDTA accumulated [^{14}C] tetronasin in the same way as untreated cells (figure 1). However, tetronasin-adapted cells did not show the same marked decrease in binding compared to the non-adapted bacteria when the outer membrane was removed. Thus the decreased accessibility to tetronasin shown by adapted bacteria seems likely to have been mediated by a decreased permeability of the outer membrane.

Adapting *B. ruminicola* to grow in the presence of tetronasin had no effect on the uptake of phe_2 or phe_3 (table 1). Only the uptake of phe_4 was decreased (by 40%). Adding tetronasin to non-adapted cells had no influence on the rate of breakdown of any of the peptides (not shown).

It is well documented that the outer membrane affords protection from antibiotics in many Gram-negative bacteria (Nikaido, 1985), and a similar mechanism has been implicated in determining how sensitive different species of rumen bacteria are to ionophores (Russell and Strobel, 1989). This paper presents preliminary evidence that even resistant bacteria may modify the properties

TABLE 1. METABOLISM OF PHENYLALANINE OLIGOPEPTIDES BY *B. RUMINICOLA* GROWN AND INCUBATED IN THE PRESENCE OF 0.1 μg TETRONASIN/ML

	Rate of uptake (nmol/mg protein/min)	
Peptide	*B. ruminicola*	*B. ruminicola* grown in the presence of tetronasin
Phe_2	1.16 ± 0.14[a]	1.25 ± 0.20
Phe_3	1.63 ± 0.09	1.77 ± 0.20
Phe_4	4.23 ± 0.13	2.53 ± 0.19

[a]Mean ± SE, n = 4.

of their outer membrane during growth in presence of ionophores. Furthermore, adaptation may influence permeability not only to the ionophore (in this case tetronasin, M_r = 628), but to molecules to similar size, such as phe_4 (M_r = 667). Both of these molecules are close to the permeation limit of the outer membrane of about 600 daltons (Nikaido, 1985).

(**Key Words:** Ionophores, Tetronasin, *Bacteroides ruminicola*)

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