MICROBIOLOGY

Influence of Time of Addition of Antibiotic on the *in vitro* Life of Rumen Holotrich Protozoa

Extended maintenance *in vitro* of rumen oligotrich protozoa has been reported by Coleman[1]. Similar results have not been achieved with the rumen holotrich protozoa[2]. Coleman[1] suggested that to accomplish maintenance of the oligotrich protozoa satisfactorily, it was desirable to control the numbers of bacteria present rather than to eliminate them. One of the principal differences between oligotrich and holotrich protozoa is that the former ingests and then digests starch granules, while holotrichs utilize mainly soluble carbohydrates[3]. In view of this fact, it would seem possible that competition between bacteria and protozoa for available substrate may be greater in the case of holotrichs than with oligotrichs.

Recent experiments in this laboratory have shown that when streptomycin plus penicillin are added to a suspension of rumen organisms prior to the addition of nutrients a marked inhibition of gas production occurs over that observed when the two are added simultaneously[4]. Consequently, it seemed possible that adding antibiotics to holotrich cultures prior to the time nutrients are added to the cultures may provide more favourable growth conditions for the holotrichs. An experiment to test such a possible effect was carried out.

Rumen contents were collected from four sheep which had been fed a basal pelleted ration plus corn. Inoculum was obtained by a method similar to that described by Oxford[5]. The volume used was sufficient to provide a concentration of 20×10^3 holotrichs per ml. of culture media. *Isotricha* spp. were predominant. Cultures were maintained both in 16 mm × 150 mm culture tubes and in tissue culture flasks having an area of 15 cm². Seven different media were used (Table 1); the final volume for each culture was 2 ml. The incubation temperature was 39° C. Depleted media were removed each day by withdrawing the supernatant fluid after the cultures had been placed in an ice-bath for 3 min. Fresh media containing nutrients were then added. The cultures were gassed at all times with oxygen-free carbon dioxide. Streptomycin, at a final concentration of 20 μg/ml. of media, was added to half the cultures 1 h before the media was changed. As this streptomycin was removed with the depleted media, streptomycin (20 μg/ml.) was added to all cultures when the media were changed.

The cultures were observed each day under a stereoscopic microscope and the maximum life of the culture recorded. The results are shown in Table 2. Analysis of these results showed that the addition of antibiotic prior to the addition of nutrients significantly ($P < 0.001$) extended the life of holotrichs *in vitro*.

In this experiment particulate material was not removed from the cultures, nor were they divided. The maximum life obtained was 26 days (media 6). It will be of interest to determine whether division and/or removal of particulate material from the cultures will extend culture life longer. It has been found in this laboratory that oligotrich cultures maintained for extended periods on media similar to that described by Coleman[1] have a maximum life of approximately 30 days if debris is not removed and the culture is not divided.

Table 1. Composition of Media*

	1 /100 ml.	2 /100 ml.	3 /100 ml.	4 /100 ml.	5 /100 ml.	6 /100 ml.	7 /100 ml.
Basal media	†	†	†	†	†	†	‡
Glucose	0·05 g	0·05 g	0·05 g	0·05 g	0·05 g	0·05 g	0·05 g
Casein hydrolysate§	2 ml.	2 ml.	2 ml.	2 ml.	—	—	—
Clarified rumen fluid¶	—	—	20 ml.	20 ml.	—	20 ml.	—
Distilled water	40	40	20	20	40	20	—
Solka floc‖	—	50 mg	—	50 mg	—	—	—
Grass**	—	—	—	—	50 mg	50 mg	—

* Prepared under sterile conditions as described by Bryant and Robinson (ref. 6).
† Based on the media described by Bryant *et al.* (ref. 8).
‡ Media described by Coleman (ref. 1).
§ Enzymatic 'vitamin free' casein hydrolysate (NBC).
¶ Clarified rumen fluid as described by Bryant and Robinson (ref. 6), but autoclaved 20 min.
‖ Ball-milled 3 days.
** Ground in a Wiley mill using a 60-mesh screen.

Table 2. Maximum Culture Life (Days)

Treatment		Media 1	2	3	4	5	6	7
Culture flasks	Antibiotic prior to nutrients	13	12	16	16	12	26	4
	Antibiotic with nutrients	8	11	6	10	12	18	3
Culture tubes	Antibiotic prior to nutrients	7	11	11	11	9	20	4
	Antibiotic with nutrients	7	7	6	6	7	11	3

The results presented here suggest that the inability to maintain holotrichs *in vitro* may be due, in part at least, to ineffective control of bacterial competition for available substrate. It will be of interest to determine whether the rumen oligotrich protozoa can be more readily maintained using a similar technique.

Viable bacterial counts[6] were made on the 17th day, from culture flasks containing media 6, and these indicated that the concentration of bacteria was not markedly affected by the different antibiotic treatments. However, Gram stains and motility tests on bacteria isolated from the foregoing cultures suggested that the composition of the bacterial population was markedly affected; for example, organisms isolated from roll tubes containing approximately 30 colonies showed that 9 out of 9 of the organisms from the culture receiving antibiotic before nutrients were non-motile while 7 out of 10 of the organisms from the cultures not receiving antibiotic early were motile[7].

Work is now in progress to identify presumptively the isolated bacteria and to maintain holotrichs *in vitro* for extended time periods. It will be of interest to determine whether the relationships indicated here are cause or effect relationships.

D. B. Purser
H. H. Weiser

Institute of Nutrition and Food Technology,
Ohio State University, Columbus.

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Proteolytic Activity of Growth Media Filtrates from Non-pathogenic Species of *Verticillium*

The production of proteolytic enzymes by microorganisms is widely recognized, and several have been obtained from fungi. In an attempt to identify the enzymatic activities of the filtrates of the growth media of members of the genus *Verticillium*, it was noted that filtrates of some non-pathogenic species of this group of fungi are able to lyse purified proteins and ovalbumin blocks. The number of investigations dealing with the production of proteases in micro-organisms is low[1]. Recently our interest has centred on the genus *Verticillium* as a part of our investigations on the lytic activity of a non-pathogenic strain of *Verticillium hemileiae* on uredospores of various rusts[2]. The results reported here are a consequence of some studies on the effectiveness of certain species of *Verticillium* controlling rust, as well as their *in vivo* effect on the pathogenicity of these organisms. To our knowledge, no results have been reported of the quantitative or qualitative aspects of proteolytic enzyme production by *Verticillium*.

Cultures of *Verticillium* species were obtained from Estaçao Agronomica National of Oeiras (Portugal), University College of Swansea, Oregon State University and some were isolated by us. Forty strains of the follow-