Note

Quantity and Quality of Microbial Protein Produced by an Agnotobiotic Culture Ecosystem of a Rumen Protozoon, *Entodinium caudatum*

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The rumen of ruminant animals has been known to be inhabited by ciliate protozoa since old times. The significance of the protozoa for the host animal has long been examined by many investigators, but no clear-cut conclusion has been obtained.¹⁾ With regard to microbial protein synthesis in the rumen, for example, Ørskov (1982)²⁾ assumed that the microbial yield of protein would be lower in faunated than defaunated animals as a result of the increase in rumen turnover of microbial protein, since the main source of nitrogen for protozoa had been demonstrated to be bacteria, and, before Ørskov, Hungate (1978)¹⁾ also proposed that the conversion of bacterial protein to protozoal protein in the rumen should entail a loss in quantity of microbial protein and that the net value of the protozoal protein should be the sum of its greater quality and lower quantity. But there is little clear evidence that a reduction in microbial yield occurs as a result of faunation,²⁾ instead there is a suggestion that the increase in bacterial nitrogen brought about by defaunation is not sufficient to compensate for the protozoal nitrogen loss from the total amount of microbial nitrogen in the rumen.³⁾ Because of this, research is strongly needed in this area.⁴⁾

The present paper reports the protozoal growth curve and the time courses of microbial production of protein and also lysine by an agnotobiotic culture of a rumen protozoon, *Entodinium caudatum*, as a basic and simplified example that will facilitate elucidation of the *in vivo* relationship between microbial protein production and ciliate protozoa in the rumen.

An agnotobiotic culture of *E. caudatum* as a stock culture has been maintained by us since 1979, we having previously isolated⁵⁾ the organism from a Japanese-Saenon goat. Prior to the growth test, the optimum agnotobiotic culture medium for the growth of *E. caudatum* was determined to have the following composition: 0.6 mg/ml of haycube powder (screened with a 80-mesh sieve), 10 µmol/ml of ammonium chloride and 0.4 mg/ml/day of

13% β -sitosterol-coated rice starch⁵⁾ in addition to 0.1 ml/ml of supernatant fluid of the stock culture (centrifuged at $540 \times g$ for 2 min) (SFSC). Six flasks containing the components mentioned above in 300 ml of MB9 buffer solution⁵⁾ were inoculated with 0.04 ml/ml of the stock culture, followed by incubation at 39°C for 12 days. In the growth test for *E. caudatum* (group BP), the medium was not replaced, as it was in the previous study⁵⁾ and the protozoon grew well in it. Another six flasks, into which 0.04 ml/ml of SFSC (only bacteria) was inoculated, instead of the inoculum of the stock culture of *E. caudatum*, and to which the other components were similarly added, were also incubated for comparison (group B). All results are presented as the average values for six cultures.

The protozoon density was determined by counting with a Fuchs-Rosenthal hemacytometer under an optical microscope ($\times 100$), after sampling 1 ml of culture medium with stirring and then mixing it with an equal amount of a MFS solution for fixation.⁶⁾ The doubling time of the protozoon was calculated with the following equations, which were derived from the equation of Warner:⁷⁾

$$t = 48/n, \quad n = \log(b/a)/\log 2$$

where t is the doubling time, n the frequency of division, a the initial density of the protozoon and b the density of the protozoon after a given time.

Microbial nitrogen was determined by calculating the difference in nitrogen content between the whole culture medium and the supernatant fluid of the medium after centrifugation at $27,000 \times g$ for 30 min. Nitrogen was analyzed by the micro-Kjeldahl method. Ammonia was determined by Conway's microdiffusion method.⁸⁾ Amino acids were analyzed as follows: a 5-ml portion of the culture medium was taken and put into a 100-ml oval-shaped flask with a joint and connector, and then mixed with 4.3 ml of conc HCl, the final normality as to HCl being 5.3 N. Air in the flask was removed *in vacuo* as completely as possible under acetone-dry ice conditions at 10 m Torr for 10 min. With this treatment, the HCl normality became 6.0 N. Then the mixture was heated at 110°C for 20 hr to hydrolyze the microbial protein. Amino acids in the hydrolyzate were analyzed with an automatic amino acid analyzer (AA-100; Sibata Chemical App. Mfg. Co., Ltd., Japan).

In the agnotobiotic culture medium used in the present experiment, *E. caudatum* grew well, showing a peak (9.2×10^4 /ml) at the 9th day (Fig. 1) in spite of no replacement of the medium, which was replaced in the previous study.⁵⁾ The average doubling time tended to increase with the culture time, and values of 24 and 27 hr were obtained at the 2nd and 4th day, respectively, the value becoming rapidly greater thereafter (44 and 144 hr at the 6th and 8th day, respectively). Warner⁷⁾ reported that the mean doubling time of *Entodinium* spp. varied within the range of 5.5 to 58 hr *in vivo* in the rumen of sheep receiving food once daily.

FIG. 1. Growth Curve for *E. caudatum* (Δ) and the Time Course for Microbial Nitrogen during the Growth of the Bacteria with ($\circ$) or without ($\bullet$) *E. caudatum*. The values are the means for six cultures. ** $p < 0.01$.

Ammonia nitrogen in group B was significantly lower than that in group BP after the 4th day; ammonia nitrogen should be more efficiently retained in the microbial cells in group B (bacteria only). Thus the microbial nitrogen in group B was significantly higher than that in group BP after the 4th day (Fig. 1). Similarly, the total amino acid concentration and total essential amino acid (TEAA) concentration in group B were significantly higher than those in group BP after the 6th day. The lysine concentration in group B was also higher than that in group BP after the 6th day (Fig. 2). After the 4th day, a considerable amount of the amino acid nitrogen in the medium for group BP might be used for maintenance of the protozoon, as indicated by the elongation of the doubling time, so the total microbial nitrogen in the medium for group BP might tend to decrease with increased recycling of nitrogen through engulfment and digestion of bacteria by the protozoon.

Before the 4th day, however, no obvious differences in microbial nitrogen, total amino acid concentration or lysine concentration were apparent between groups B and BP. This may mean that there is no significant difference in the production of microbial protein between the ecosystems of the agnotobiotic culture of *E. caudatum* and the culture of only bacteria, as long as the doubling time of the protozoon is shorter than 27 hr.

It is interesting that the relative content of lysine (mol lysine/mol TEAA, %) in group BP was significantly higher than that in group B after the 4th day (Fig. 2). This should be due to the ability to synthesize lysine⁹⁻¹¹) and the higher content of lysine of the protozoon.¹²⁾

As might be expected, it was shown that there were quantitative and qualitative differences in microbial protein between the agnotobiotic culture of the protozoon

FIG. 2. Changes in the Lysine Concentration (a) and the Relative Content of Lysine (b) (mol lysine/mol TEAA, %, Where TEAA Denotes Total Essential Amino Acids) during the Growth of the Bacteria with ($\circ$) or without ($\bullet$) *E. caudatum*.

* $p < 0.05$ ** $p < 0.01$.

and the culture of only bacteria. In particular, it was noteworthy that the doubling time (growth rate) of the protozoon had an appreciable effect on the amount of microbial protein in the agnotobiotic culture, i.e., the shorter (less than 27 hr) the doubling time, the greater the production of microbial protein (which almost reached the level in the case of the culture of only bacteria). These data may partially support the speculations of Hungate¹⁾ and Ørskov²⁾ mentioned in the introduction.

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