Degradation of the nucleic acids in mixed rumen bacteria on incubation with different media

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(*Received* 11 *June* 1976 – *Accepted* 1 *October* 1976)

1. Mixed rumen bacteria were grown in vitro in a medium containing [U-^{14}C]adenine. Radioactivity was incorporated into the purine bases of the nucleic acids.

2. Labelled bacteria were incubated with a nitrogen-deficient cell-free medium (medium A), a protozoa-free supernatant suspension from a slow-speed centrifuging of a steer's rumen contents (medium B) and whole rumen contents of a steer (medium C). Mean (six experiments) proportional rates of release of radioactivity(/h) were significantly greater for medium A (0·140) than for media B or C (0·055 and 0·043 respectively). Some of the radioactivity accumulated in the cell-free media; some was lost, partially as carbon dioxide.

3. Calculation from this and other information indicated that about 30% of bacterial nucleic acids may turn over in the rumen even in the absence of protozoa.

Dietary nucleic acids entering the rumen are extensively and rapidly degraded (McAllan & Smith, 1973*a*) and it is mainly nucleic acids of bacterial origin which are digested in the small intestine to yield components which may be incorporated into the tissues of the host (Smith, Moussa & Hawkins, 1974). Bacterial nucleic acids may, however, also turn over in the rumen although quantitative estimates of the extent of turnover have not been reported. Turnover of both nucleic acid and protein occurs intracellularly in pure bacterial cultures, particularly in those starved of an essential nutrient (Mandelstam, 1960; Pine, 1972) but may also occur when some cells in a culture are degraded and act as nutrients for others (e.g., see Coleman, 1972). Nolan & Leng (1972) calculated that for a sheep given infusions of ^{15}N-labelled ammonia or urea into the rumen about 20% of microbial N re-entered the peptide–amino acid–ammonia pools as a result of cell degradation and Jarvis (1968) showed appreciable liberation of radioactivity from strains of *Streptococcus bovis* and *Butyrivibrio* sp. which had been labelled with tritiated thymidine and then incubated with rumen contents.

In the present study an examination was made of the stability of the nucleic acid fraction of mixed rumen bacteria labelled with [U-^{14}C]adenine when the bacteria were incubated in different media.

METHODS

Source of rumen contents

Samples were taken from the ventral sac of the rumen of a Guernsey steer, 13–15 months of age, receiving a diet of equal weights of flaked maize and hay, with suitable mineral and vitamin supplements, twice/d.

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Labelling and subsequent examination of rumen bacteria

Rumen contents were removed 1–2 h after a morning feed, strained through one layer of gauze and centrifuged at 650 *g* for 1 min. The supernatant suspension of bacteria was removed, a portion was added to a 100 ml flat-bottomed flask, and a N_2–carbon dioxide (9:1, v/v) mixture was passed into the flask for 2 min. In this, and in subsequent similar treatments, the gas mixture was introduced about 10 mm above the surface of the culture and the mixture shaken. The flask was sealed and incubated at 39° for not more than 40 min, until the suspension was used to inoculate a growth medium (see below). The remainder of the bacterial suspension was centrifuged at 12000 *g* for 20 min at 4°. The clear, yellow, supernatant solution was used to prepare the growth medium described by Smith & Mathur (1973). [U-^{14}C]adenine (Radiochemical Centre, Amersham, Bucks.) (4 μCi) was added to 27 ml of this medium with sufficient unlabelled adenine to give a final concentration of 5 μg/ml, and N_2–CO_2 (9:1, v/v) was passed for 10 min. The mixture was inoculated with 3 ml bacterial suspension, a sample was removed for fractionation and examination, and N_2–CO_2 (9:1, v/v) was passed for 2 min before the flask was sealed with a Bunsen valve. It was then shaken in a water-bath at 39° for 3 h at approximately 80 strokes/min, a sample of the culture was removed for fractionation and the remainder of the culture was poured into a centrifuge tube in which N_2–CO_2 (9:1, v/v) was passed for 1 min. The tube was stoppered, and centrifuged at 12000 *g* for 10 min. The supernatant solution was removed, the cells were suspended in 30 ml growth medium with 5 μg unlabelled adenine/ml but without urea or hydrolysed casein, and the suspension was centrifuged. The pellet was resuspended in 6·5 ml of the same medium and 2 ml suspension was used to inoculate 28 ml of: the growth medium rendered N-deficient by omitting urea and hydrolysed casein (medium A), the supernatant suspension obtained by centrifuging fresh rumen contents at 650 *g* for 1 min (medium B) and fresh rumen contents (medium C). Medium C contained about $0{\cdot}8–2{\cdot}0 \times 10^6$ ciliate protozoa/ml; medium B lacked these as well as fine food particles and probably some larger bacteria. A sample (1 or 2 ml) was taken from each culture immediately after inoculation. The remaining cultures in 100 ml flasks, usually sealed with Bunsen valves, were incubated at 39° with shaking after N_2–CO_2 (9:1, v/v) had been passed through the flasks for 2 min. Further samples were taken 2, 4, 6 and 20 h after inoculation with further passage of N_2–CO_2 (9:1 v/v) into the culture flask after each sample had been taken. Experiments were carried out on six occasions with different samples of rumen contents over a period of about 7 weeks. In one experiment with medium C the flasks were fitted with gas inlet and outlet tubes which were closed with a screw clamp during incubation. Before flasks were opened to sample the contents N_2–CO_2 (9:1, v/v) was passed for 3 min to flush any $^{14}CO_2$ into a trap containing 10 ml hyamine hydroxide.

Fractionation of samples

Duplicate samples (1 or 2 ml) were removed at each interval after inoculation, immediately put into a centrifuge tube cooled in an ice-bath, and centrifuged within 5 min of sampling at 12000 *g* at 4° for 10 min. The supernatant solution was removed

and radioactivity in 0·2 ml estimated (supernatant fraction). The pellet was suspended in 10 ml ice-cold trichloroacetic acid (TCA) (50 g/l). After 10 min the suspension was centrifuged at 12000 *g* for 5 min. The supernatant solution was removed and radioactivity in 0·2 ml estimated (cold TCA-soluble fraction). The pellet was suspended in 10 ml TCA solution (50 g/l) and heated in a boiling water-bath for 30 min. The extract as centrifuged at 12000 *g* for 5 min and radioactivity in 0·2 ml supernatant solution was estimated (hot TCA-soluble fraction). The residue was dissolved in 10 ml 0·1 M-sodium hydroxide by heating the samples at 60° for 2 h. Radioactivity in a sample of the solution (0·2 ml) was estimated.

Estimation of radioactivity

Samples were added to 10 ml Polyphosphor (600 ml 2-methoxyethanol, 400 ml anisole, 80 g naphthalene, 7 g 2,5-diphenyloxazole and 0·3 g 1,4-di-2(5-phenyloxazolyl) benzene and counted in an automatic liquid-scintillation analyser (N. V. Philips, Eindhoven, Netherlands).

Comparison of results

Six replicate experiments were carried out with each of the media A, B and C. For each set of replicates the mean regression of log radioactivity in hot TCA-soluble fraction (expressed as a proportion of the initial inoculum total radioactivity) *v.* period of incubation (h) was approximately linear up to 4 h (Fig. 1). The significances of differences between regression coefficients for the different media during the period of incubation were estimated by a *t* test.

RESULTS

Cultures of mixed rumen bacteria growing in the medium containing [U-^{14}C]adenine rapidly incorporated ^{14}C into the nucleic acid fraction (hot TCA-soluble fraction) and after 3 h, in a typical experiment, the proportions of the activity in the supernatant, cold TCA-soluble and hot TCA-soluble fractions were 0·16, 0·06 and 0·78 respectively, with virtually none in the residual alkali-soluble fraction. In one experiment the bases present in the hot TCA-soluble fraction were separated by chromatography on Whatman No. 3MM paper using a propan-2-ol–concentrated hydrochloric acid-water (170:41:39, by vol.) developing-solvent system. Bases were located under ultraviolet light and each base was eluted with 6 ml water. The solutions were filtered and 3 ml portions were scanned, using a spectrophotometer (Model 25; Beckman-RIIC Ltd, Glenrothes, Scotland), from 220 to 300 nm and then dried, taken up in Polyphosphor and the radioactivity estimated. Radioactivity was shown to reside entirely in the adenine and guanine fractions, with a value for the ratio, radioactivity in adenine (disintegrations/min): radioactivity in guanine (disintegrations/min) of 3·4.

In each of the six groups of experiments in which cells were first labelled, as described previously, and then incubated with media A, B and C, the proportion of tracer initially taken up by the bacteria which was present in the hot TCA-soluble fraction was between 0·90 and 0·95. When labelled bacteria were added to media B and C and immediately sampled, this proportion was found to be virtually unchanged

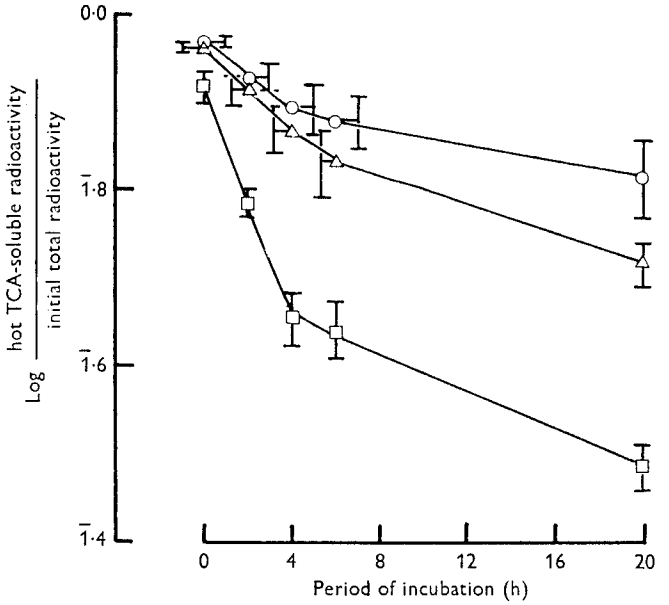

Fig. 1. Relationship between log amount of radioactivity in the hot trichloroacetic acid (TCA)-soluble fraction of rumen bacteria, expressed as a proportion of their initial total radioactivity, and period (h) of incubating the bacteria with a nitrogen-deficient, cell-free medium (□); the supernatant suspension after centrifuging a steer's rumen contents at 650 *g* (△) and whole rumen contents of a steer (○). Mean values are given for six experiments, with their standard errors represented by vertical bars. For details of fractions and their preparation, see p. 390.

(mean (± SE) values for the six experiments were 0·92 ± 0·01 and 0·93 ± 0·01 for media B and C respectively). When similar samples were examined immediately after an addition to medium A, however, the proportion was variable and sometimes appreciably lower (mean ± SE 0·82 ± 0·08). It appeared that even in the short interval between obtaining and centrifuging the sample (about 15 min, and for part of this interval the temperature was 4°) appreciable degradation of the bacterial nucleic acids had occurred in medium A.

For each experiment when a mixture containing labelled cells and one of the media was incubated there was a progressive decrease in the amount of radioactivity in the hot TCA-soluble fraction which was approximately exponential over the first 4 h of the incubation period (Fig. 1). After this period the rate became slower but in the first 4 h, mean regression coefficients for log radioactivity in the hot TCA-soluble fraction (expressed as a proportion of the initial inoculum total radioactivity) *v.* period of incubation (h) were −0·0657, −0·0245 and −0·0190 for media A, B and C respectively, with a pooled standard error of 0·0061. The value for medium A differed significantly ($P < 0·001$) from those for the other media, but the latter were not significantly different from each other. These results corresponded to mean proportions of the amount of radioactivity in the hot TCA-soluble fraction disappearing/h of 0·140, 0·055 and 0·043 for media A, B and C respectively.

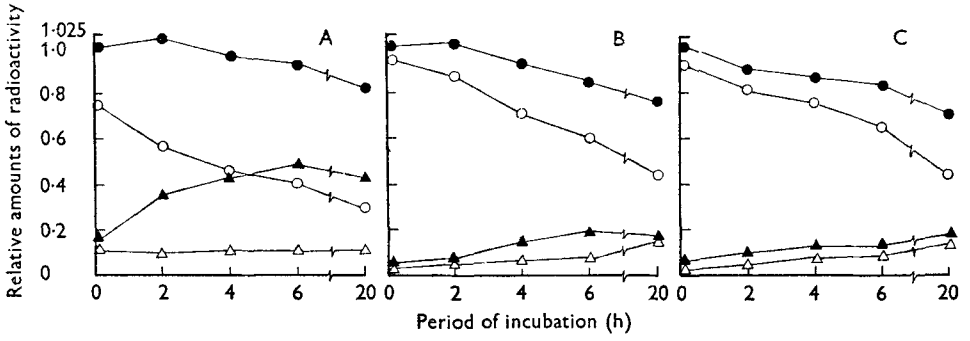

Fig. 2. Relative amounts of radioactivity present in total culture (●), hot trichloroacetic acid (TCA)-soluble fraction (○), supernatant fraction (▲) and cold TCA-soluble fraction (△) in a typical experiment at different periods after rumen bacteria labelled with [U^{14}C]adenine were incubated in a nitrogen-deficient, cell-free medium (A); the supernatant suspension after centrifuging a steer's rumen contents at 650 *g* (B), and whole rumen contents of a steer (C). For details of fractions and their preparation, see p. 390.

For medium A, at least up to 6 h, decreases in the amount of radioactivity in the hot TCA-soluble fraction were accompanied by corresponding increases in that of the supernatant fraction but for the other media these increases were much less. For all media there were losses of total radioactivity between 0 and 6 h incubation and further losses between 6 and 20 h incubation. The results, given in Fig. 2 for a typical experiment, illustrate the changes observed. It has been shown that nucleic acids in rumen contents are rapidly degraded to free bases (McAllan & Smith, 1973*a*) and that anaerobic bacteria can degrade these bases to NH_3, CO_2 and acetate (Barker, 1961) so it seems probable that losses of total radioactivity were caused mainly by the metabolism of some of the labelled components to $^{14}CO_2$. This was demonstrated directly for the experiment with medium C in which evolved CO_2 was collected during the period 6–20 h incubation; it accounted for 75% of the observed decrease in total radioactivity.

DISCUSSION

It has been reported that appreciable intracellular turnover of bacterial nucleic acids occurs only in cultures starved of essential nutrients (Mandelstam, 1960). Such a process might help to account for the high rates of loss of activity in the hot TCA-soluble fraction in experiments with medium A, the N-deficient medium, but, in the growing cultures supported by media B and C, it seems probable that activity of bacteriophage, engulfment by protozoa or bacterial attack after death from other causes would be mainly responsible for release of radioactivity. Values observed for the rate of this release were a measure of net irreversible change and did not take account of the possible re-incorporation of liberated adenine or guanine. However, little or no adenine accumulates when nucleic acids, nucleotides or nucleosides containing it are incubated with rumen contents in vitro. In these compounds (although not when it is present as the free base) adenine is very rapidly deaminated to hypoxanthine and it is this base which accumulates in the mixture (McAllan & Smith,

1973*a*, *b*). Similarly guanine is replaced by xanthine. It appears unlikely, therefore, that direct re-incorporation of labelled bases into newly-synthesized bacterial nucleic acids would occur to an appreciable extent.

If the mean value observed for the proportional rate of release of radioactivity from the hot TCA-soluble fraction in medium C, whole rumen contents, (about 0·04/h) is assumed to represent the rate of bacterial nucleic acid breakdown within the rumen, then for a steer receiving a diet of flaked maize and hay, weighing 115 kg, with a rumen volume of 17·5 l (Smith & McAllan, 1970) and having a fairly constant concentration of microbial nucleic acid-N in rumen contents of approximately 0·11 g/l (Smith & McAllan, 1971), nucleic acid-N would be degraded at a rate of approximately 1·8 g/d. In other experiments (R. H. Smith & A. B. McAllan, unpublished results) for similarly fed steers of about this body-weight it was shown that nucleic acid-N (largely microbial) passed into the duodenum at a rate of approximately 4·4 g/d. Combining these results it can be estimated that total nucleic acid-N production would be 6·2 g/d, of which about 30% would turn over within the rumen. Such a turnover rate, which is in reasonable agreement with a value of about 20% for total microbial N turnover in the rumen of a sheep indicated by the results of Nolan & Leng (1972), would have a considerable effect on the energy required by the bacteria to achieve a given net synthesis of cell material. Although pure cultures of rumen protozoa have been shown to engulf bacteria and lead to the partial release of their nucleic acid constituents (Coleman, 1972), the quantitative importance of the process in the rumen is uncertain. The present results, showing little difference in degradation rate between experiments using media B and C, indicated that protozoa had little effect on nucleic acid turnover under our conditions. This was contrary to the findings of Jarvis (1968), perhaps in part because of differences in protozoal activity between the different samples of rumen contents used, but possibly also because protozoa show some selectivity in the species of bacteria that they engulf (Coleman, 1972), and Jarvis (1968) labelled only particular bacterial species.

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Printed in Great Britain