Utilization of the nucleic acids of *Escherichia coli* and rumen bacteria by sheep

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1. *Escherichia coli* and mixed cultures of rumen bacteria were grown with [8-^{14}C]adenine to label their nucleic acids specifically.

2. The labelled bacteria were injected into the rumen of sheep and the radioactivity incorporated into tissue nucleic acids and that excreted in the urine and faeces was determined.

3. The radioactivity was present in the cold trichloroacetic acid-soluble fraction and the RNA and DNA fractions of all tissues examined. Liver, kidney, spleen, and blood had the highest levels of radioactivity.

4. The radioactivity of the RNA was present only in adenosine monophosphate and guanosine monophosphate.

It has been suggested that the nucleic acids of rumen bacteria may be used for the synthesis of tissue nucleic acids by ruminants (Ellis & Bleichner, 1969 *b*). The amount of nucleic acid in digesta apparently decreases as the digesta moves from the duodenum to the ileum in calves and sheep (Ellis & Bleichner, 1969 *a*; Smith & McAllan, 1971). About 70 % of the purines absorbed from the intestinal tract are not immediately excreted in the urine (Ellis & Bleichner, 1969 *b*). Thus, these findings suggest that some of the purines may have been absorbed and used for the synthesis of nucleic acids in the tissues. This possibility is supported by the finding that adenine, uracil, and RNA infused into the abomasum are incorporated into the RNA and DNA of the tissues of lambs. In contrast, glycine, a normal precursor of purines, is not used for nucleic acid synthesis (Condon, Hall & Hatfield, 1970).

Recently we reported that the nucleic acids of *Streptococcus bovis* and mixed cultures of rumen bacteria could be labelled almost exclusively by growing the micro-organisms in a medium containing [8-^{14}C]adenine (Smith & Mathur, 1973). The present study was initiated to ascertain if the radioactivity of bacteria with labelled nucleic acids was incorporated into the tissue nucleic acids of sheep.

MATERIALS AND METHODS

Growth and labelling of the bacteria

Escherichia coli K12 was grown in a tris(hydroxymethyl)aminomethane (Tris) salts medium containing 2 g glucose/l (Smith & Salmon, 1965 *a*). An exponentially growing culture of *E. coli* was used to inoculate 2 l of Tris–glucose medium containing 100 μCi [8-^{14}C]adenine (6·7 μCi/μmol) obtained from New England Nuclear Corp., Boston,

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Mass., USA). Samples of the culture were removed every 30 min and filtered through a membrane filter (25 mm diameter, 0·45 μm pore size, Millipore Filter Corporation, Bedford, Mass., USA). A portion of the filtrate (0·2 ml) was removed, added to 10 ml of an aqueous scintillation fluid (Strength, Yu & Davis, 1965), and the sample counted using a Packard Tricarb liquid scintillation counter. Efficiencies were determined by addition of an internal standard. After 2·5 h, over 95 % of the radioactivity was incorporated into the cells. The culture was harvested by filtration on a membrane filter (265 mm diameter, 0·45 μm pore size, Gelman Instrument Co., Ann Arbor, Mich., USA). The cells were removed from the filter by washing three times with 70 ml Tris medium. The suspension of cells was centrifuged and the pellet resuspended in 10 ml Tris medium. After removal of a 0·2 ml sample for analysis, the suspension was transferred into a syringe fitted with a stainless steel needle (76 mm long, gauge 10) and it was injected through the wall into the rumen of a ewe.

The rumen bacteria were prepared by collecting rumen fluid from a sheep by stomach tube and filtering it through four layers of gauze. The filtrate was centrifuged at 130 g for 5 min (model B20A International centrifuge). The middle one-third of the supernatant fluid was removed with a pipette for use as an inoculum and as a source of clarified rumen fluid. The rumen bacteria were grown and labelled in the presence of 100 μCi [8-^{14}C]adenine as described previously (Smith & Mathur, 1973) until more than 95 % of the radioactivity was taken up by the cells. The whole of the culture medium was centrifuged and the bacterial pellet, containing about 95 μCi ^{14}C, resuspended in 10 ml fresh medium, and injected through the wall into the rumen of the same sheep from which the original sample was taken.

Treatment of the sheep

The sheep used in this study were Western Blackface ewes about 1 year old. The three sheep injected with *E. coli* were given a pelleted ration containing (g/kg): maize starch 650, cottonseed hulls 200, cane molasses 100, urea 20, maize oil 20, defluorinated phosphate 5, and trace mineralized salt 5; 6·8 mg retinoyl palmitate/kg diet was added. The two sheep injected with rumen bacteria were fed with a non-pelleted ration containing (g/kg): maize 670, grass hay 150, molasses 100, cottonseed meal 60, defluorinated phosphate 10 and salt 10. The sheep were fed *ad lib*.

One of the sheep injected with *E. coli* was used for collection of urine and faeces over a long period. The other two sheep and the two sheep injected with rumen bacteria were used for slaughter and tissue analysis; faeces and urine were collected for radiological safety purposes only.

Just before each sheep was injected with the labelled bacteria, it was placed in a metal metabolism cage designed to collect separately the urine and faeces. The radioactivity in samples of urine was estimated direct; the faeces were extracted with cold and then hot trichloroacetic acid (50 g/l) and these extracts analysed. The sheep were kept in the metabolism cages with food and water until slaughtered, when samples of liver, kidney, spleen, blood, lung, heart, brain, rumen wall, large intestine, small intestine, tongue and muscle of the thorax and hind-limbs were removed for analysis.

Analysis of the nucleic acids

The RNA and DNA of the bacteria were extracted and the radioactivity estimated as described previously (Smith & Mathur, 1973). The RNA and DNA from the tissues were extracted either by the method of Kirby (1956) for column chromatography using phenol or by the method of Schmidt & Thannhauser (1945) for tissue analysis using trichloroacetic acid. After alkaline hydrolysis the nucleotides from the RNA were chromatographed either on Whatman No. 3 MM filter paper using a propan-2-ol–concentrated HCl–water (170:41:39, by vol.) solvent system (Wyatt, 1951), or an isobutyric acid–concentrated ammonium hydroxide–water (66:1:33, by vol.) solvent system (Magasanik, Vischer, Doniger, Elson & Chargaff, 1950) or on a Dowex-1 (Dow Chemical Co.) column with 4 M-formic acid as the eluent (Smith & Salmon, 1965*b*). The nucleotides on the paper chromatograms were located with an ultraviolet lamp and eluted by extraction with 5 ml distilled water. The extinction at 250, 260, 280, and 300 nm was determined with a Beckman DU spectrophotometer (Beckman Instruments Inc., Fullerton, California). A portion of the sample was dried in a counting vial with a stream of air, 10 ml scintillation fluid added, and the amount of radioactivity determined. The nucleotides were identified by the ratios of peak heights at 250, 260 and 280 nm, i.e. 250:260 and 280:260, and by co-chromatography with known compounds. Extinction of fractions of column effluent was determined at 260 and 280 nm. A portion of each fraction was evaporated to dryness in a counting vial, 10 ml scintillation fluid added, and the amount of radioactivity determined. The concentration of the nucleotides was calculated using molar extinction coefficients (Beaven, Holiday & Johnson, 1955).

RESULTS

Analysis of the bacteria

Since the primary objective of the research reported here was to determine whether the nucleic acids of bacteria are used for the synthesis of tissue nucleic acids of sheep, it was important to know that only the nucleic acids were labelled by the [8-^{14}C]-adenine. When *E. coli* or the rumen bacteria were fractionated by the method of Schmidt & Thannhauser (1945), it was found that over 95 % of the radioactivity taken up by the bacteria was in the nucleic acid fraction. The radioactivity was present only in the adenine and guanine nucleotides. The amount of radioactivity in adenosine monophosphate (AMP) was two to three times that of the guanosine monophosphate (GMP). From these results it was assumed that most of the radioactivity found in the urine, faeces or tissues of the sheep after injection of the radioactive bacteria would be derived from the nucleic acids of the bacteria.

Analysis of the sheep tissues injected with E. coli

One sheep injected with ^{14}C-labelled *E. coli* excreted about 15 % of the radioactivity in the urine during the first 10 d (Fig. 1). Nearly half of this radioactivity was excreted in the first 2 d. Samples of the urine (24 h) were chromatographed on paper in several solvent systems. Several radioactive compounds were present on the chromatograms,
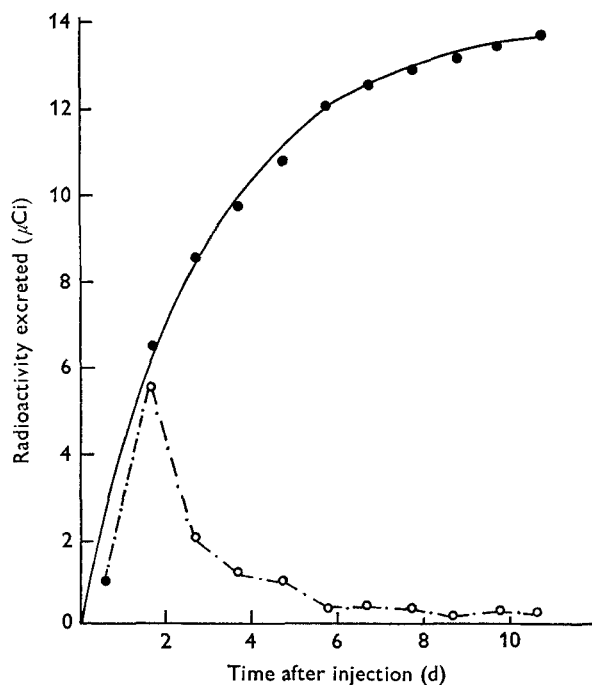
Fig. 1. Cumulative (●—●) and daily (○—·—○) excretion of radioactivity in the urine of sheep injected with ^{14}C-labelled *Escherichia coli* into the rumen.

but none were identified. They may include xanthine, hypoxanthine, uric acid, and allantoin, which were reported to be the major urinary excretion products of sheep treated with [8-^{14}C]adenine (Condon & Hatfield, 1970). Between 3 and 5 % of the radioactivity administered was excreted in the faeces during this period. Presumably the remainder of the radioactivity was incorporated into tissue components or expired as CO_2. Since very little of the radioactivity in purine bases is converted into CO_2 in animals, it seemed more likely that the radioactivity unaccounted by urine and faeces would be in the tissues.

Two sheep were killed 24 h after injection of the labelled bacteria. Radioactivity was present in both the cold trichloroacetic acid-soluble and the nucleic acid portion of all thirteen tissues examined. Liver, kidney, spleen, and blood contained higher levels of radioactivity than the other tissues. The radioactivity in these tissues is given in Table 1.

Liver and kidney had about the same amount of radioactivity in the cold trichloro-acetic acid-soluble pool when the results were expressed as disintegrations/min per g tissue, but the liver contained about seven times as much total radioactivity as the kidney. Spleen had about half the radioactivity of liver or kidney when expressed as disintegrations/min per g tissue. The radioactivity in the cold trichloroacetic acid-soluble fraction of liver accounted for about 3 % of the radioactivity injected. This radioactivity is probably present as low-molecular-weight nucleotides. When the bacteria are hydrolysed in the intestinal tract of sheep, the nucleic acids would be

Table 1. *Radioactivity in the tissues of two sheep 24 h after injection of*
¹⁴C-labelled Escherichia coli *into the rumen*

(Mean values for two sheep)

Tissue	Weight (g)	Radioactivity (Disintegrations/min per g tissue)	Radioactivity (Total disintegrations/min per tissue)[†]
Cold trichloroacetic acid-soluble pool			
Liver	468	12700	5943600
Kidney	76	10375	788500
Spleen	45	5075	228375
Blood	1325[*]	3140[*]	4160500
RNA fraction			
Liver		6030	2822000
Kidney		3330	253100
Spleen		1890	85050
DNA fraction			
Liver		300	140400
Kidney		390	29600
Spleen		1375	61875

[*] The volume of blood was estimated using a value of 47 ml/kg body-weight of the sheep (Altman & Dittmer, 1964). Results are expressed as disintegrations/min per ml blood.

[†] The total in all fractions was 14513000.

Table 2. *Radioactivity of the tissues of two sheep 24 h after injection of*
¹⁴C-labelled rumen bacteria into the rumen

(Mean values for two sheep)

Tissue	Weight (g)	Radioactivity (Disintegrations/min per g tissue)	Radioactivity (Total disintegrations/min per tissue)[†]
Cold trichloroacetic acid-soluble pool			
Liver	590	6375	3761250
Kidney	102	10350	1051560
Spleen	72	5650	405670
Blood	1672	1410[*]	2360000
RNA fraction			
Liver		6065	3578350
Kidney		2190	223380
Spleen		1935	139320
DNA fraction			
Liver		560	330400
Kidney		420	42840
Spleen		2265	163080

[*] The volume of blood was estimated using a value of 47 ml/kg body-weight of the sheep (Altman & Dittmer, 1964). Results are expressed as disintegrations/min per ml blood.

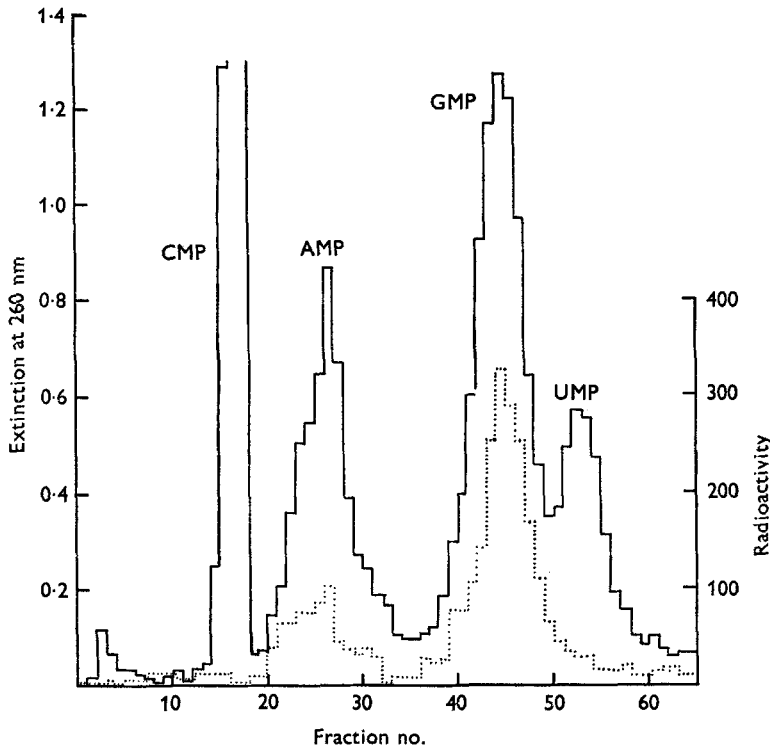[†] The total in all fractions was 12055740.

Fig. 2. Elution pattern during fractionation on a Dowex-1 column of ultraviolet-absorbing and radioactive compounds in alkaline hydrolysates of RNA from the liver of a sheep injected with ^{14}C-labelled rumen bacteria into the rumen. Fraction volume, 15 ml; (——) extinction at 260 nm; (····) radioactivity (disintegrations/min per fraction). CMP cytidine monophosphate, AMP adenosine monophosphate, GMP guanosine monophosphate, UMP uridine monophosphate.

released. Intestinal nucleases could then hydrolyse the RNA and DNA to low-molecular-weight fragments that would be absorbed from the intestine and transported to the tissues.

The RNA fraction of the liver contained about 1·5 % of the radioactivity injected into the sheep (Table 1). The RNA of kidney and spleen had less radioactivity than liver when the results were expressed as disintegrations/min per g tissue, disintegrations/min per mg RNA or disintegrations/min per tissue. The hydrolysate of a sample of RNA prepared from liver contained the four nucleotides of adenine, guanine, cytosine, and uracil. Only the adenine and guanine contained radioactivity. No other radioactivity was located on the chromatograms. These findings suggest that the radioactivity recovered in the tissue nucleic acids came from the purines of the bacterial nucleic acids through a salvage pathway (Murray, 1971) and not by degradation to CO_2 or acetate followed by resynthesis of the bases. The latter pathway would be expected to label both purines and pyrimidines. The DNA fraction of the spleen was labelled to a greater extent than that of liver or kidney on a disintegrations/min per g tissue basis, but had less total radioactivity than liver. This difference was
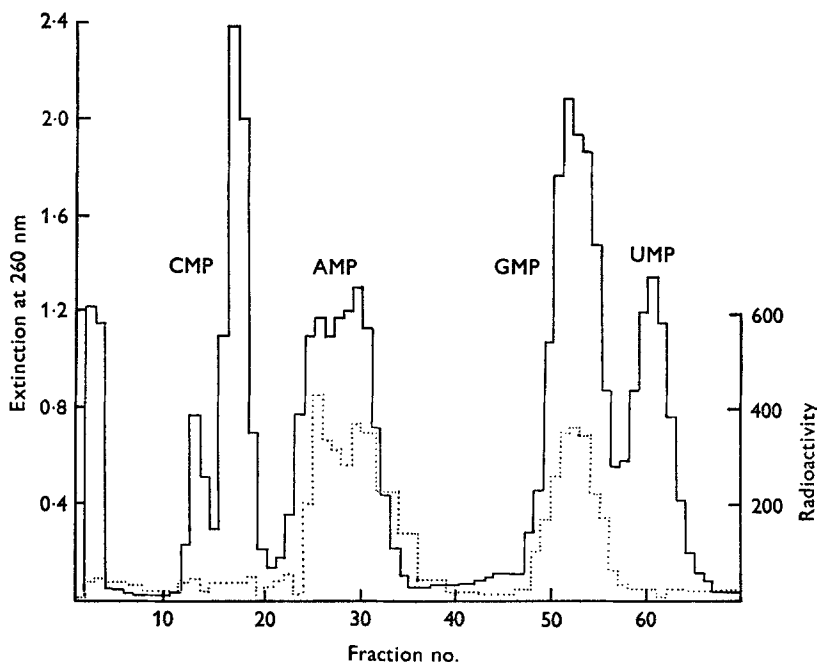
Fig. 3. Elution pattern during fractionation on a Dowex-1 column of ultraviolet-absorbing and radioactive compounds in alkaline hydrolysates of RNA from the kidneys of a sheep injected with ^{14}C-labelled rumen bacteria into the rumen. Fraction volume, 15 ml; (——) extinction at 260 nm; (····) radioactivity (disintegrations/min per fraction). CMP cytidine monophosphate, AMP adenosine monophosphate, GMP guanosine monophosphate, UMP uridine monophosphate.

expected since spleen can synthesize DNA at a faster rate than liver or kidney (Parks & Smith, 1969).

Analysis of the tissues of sheep injected with rumen bacteria

Fractionation of the liver, kidney, spleen, and blood 24 h after the two sheep were injected with rumen bacteria showed that about 6 % of the radioactivity injected was present in these samples (Table 2).

The RNA extracts from the liver and kidney were chromatographed on Dowex-1 (Figs 2 and 3). In each sample the only radioactive areas of the chromatograms were associated with AMP and GMP peaks. The RNA prepared from liver had about twice as much GMP as it did AMP. The GMP peak contained about 2·5 times as much radioactivity as the AMP peak. The specific activities of AMP and GMP were 391 and 508 disintegrations/min per μmol, respectively. The RNA prepared from kidney had about 1·4 times as much GMP as AMP, but the AMP had about 1·35 times as much radioactivity as the GMP. The specific activities of AMP and GMP were 404 and 210 disintegrations/min per μmol, respectively. Although the nucleic acids of the bacteria injected into the sheep had three times more radioactivity in AMP than in GMP, the specific activity of the GMP from RNA of liver was about twice that of the AMP. In kidney the specific activity of AMP was about twice that of GMP.

The differences in specific activity of the AMP and GMP isolated could be due to differences in pool size of the free purine nucleotides, preferential utilization of one purine base over another by some tissues, or to a more rapid degradation of one purine over another.

In a further check on the purity of the compounds, the pooled fractions of AMP and GMP were evaporated to dryness under reduced pressure and chromatographed on paper in the propan-2-ol solvent system. In each instance only one radioactive compound was detected and it migrated with the same R_F value as either AMP or GMP. When all four of the nucleotides were rechromatographed in isobutyric acid–conc. ammonium hydroxide–water (66:1:33, by vol.) and the compounds eluted, only AMP and GMP contained radioactivity.

DISCUSSION

This is the first report of the utilization of radioactive nucleic acids of bacteria by ruminants, although previous reports suggested that the nucleic acids of rumen bacteria could be used for the synthesis of tissue nucleic acids by ruminants. The results obtained suggest that although some of the nucleic acids from bacteria are rapidly lost in the urine and faeces, a certain portion of the products from the digestion of bacterial nucleic acids can be used for the synthesis of tissue nucleic acids. Because of the large amounts of bacteria that pass through the intestinal tract of ruminants, these animals may have little need for a *de novo* pathway. This would explain why glycine, a normal precursor in purine biosynthesis (Condon *et al.* 1970), was not incorporated into tissue nucleic acids but the radioactivity of the nucleic acid bases and intact nucleic acids was incorporated. Smith (1969) concluded from his studies of nucleic acid metabolism that the nitrogen of microbial nucleic acids has only limited value to ruminants. The results presented in this report would indicate that at least 5 % of the bacterial nucleic acid was incorporated into the liver, spleen, and kidney alone. The values obtained for the liver, kidney and spleen are similar to those reported for rabbits given [14C]RNA (Barnard, 1969). Assuming that all of the muscle contained about the same amount of radioactivity as the samples assayed, muscle contained at least 20 % of the radioactivity injected.

Cells of *Bacillus subtilis* and *E. coli* labelled by growing them on [U-14C]glucose are extensively digested and absorbed by sheep (Hoogenraad, Hird, White & Leng, 1970). Less than 15 % of the radioactivity was recovered in the faeces. The authors suggested that some of the 14C was incorporated into body constituents such as protein and lipid. The results reported here would indicate that the bacterial nucleic acids are used for the synthesis of tissue nucleic acids. These results would support the idea of Barnard (1969) that ruminants have much higher levels of ribonucleases in the pancreas than other vertebrates because rumen bacterial RNA is degraded in the intestine to form nucleosides and nucleotides which are absorbed. He suggested that ribonuclease is essential only in ruminants and certain other herbivores, where it has a special function.

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