Influences of dietary sucrose and urea on transfer of endogenous urea to the rumen of sheep and numbers of epithelial bacteria

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1. The rates of transfer of plasma urea to the rumen of six sheep given brome grass (*Bromus inermis*) pellets alone or with supplements of sucrose or urea were determined using [^{14}C]urea and ^{14}C-labelled sodium bicarbonate infusions during three periods.

2. The sheep were slaughtered after the third period and samples of rumen epithelium were taken for assessment of numbers of adherent bacteria.

3. Maximum transfer (0·31 g nitrogen/h) of urea to the rumen was observed for sheep given supplements of 150 g sucrose/d plus 20 g urea/d. Maximum clearance of plasma urea to the rumen (rate of urea transfer to the rumen per unit plasma urea concentration, 5·8 l/h) was observed for sheep given 300 g sucrose/d.

4. Urea clearance to the rumen was negatively related to rumen ammonia concentration; the slope of the relationship was increased with each addition of sucrose to the diet.

5. Numbers of facultative bacteria adherent to the rumen epithelium were increased by urea and sucrose supplements.

6. The results are discussed in relation to a hypothesis which relates the ureolytic capability of the bacteria adherent to the rumen epithelium to the control of the rate of transfer of urea into the rumen.

The quantitative importance of the transfer of endogenous urea from the blood to the rumen of sheep and cattle appears to vary with diet, with poor-quality roughages and lucerne (*Medicago sativa*) being associated with low transfer rates (Nolan & Leng, 1972; Norton *et al.* 1978; Norton *et al.* 1979; MacRae *et al.* 1979; Kennedy, 1980). However, the high urea transfer values obtained for sheep given brome grass (*Bromus inermis*) pellets (Kennedy & Milligan, 1978), molasses supplements (Nolan & Stachiw, 1979) and for steers given sucrose supplements (Kennedy, 1980) suggest that the permeability of the rumen wall to the movement of urea can be increased, with the concentration of rumen ammonia and rate of digestion of organic matter (OM) in the rumen exerting opposing effects on permeability (Engelhardt *et al.* 1978; Kennedy & Milligan, 1980).

Recent observations by Cheng and co-workers (Cheng *et al.* 1979; Cheng & Wallace, 1979; Wallace *et al.* 1979) that urease (*EC* 3.5.1.5) activity of facultatively anaerobic bacteria adherent to the rumen epithelium was inversely related to rumen ammonia concentration led to the extension by Cheng & Wallace (1979) of Houpt's (1970) theory of the facilitation of transfer of endogenous urea by urease activity in the rumen epithelium to encompass the role of the adherent bacteria. In this they postulated that bacterial urease activity serves to maintain a localized concentration gradient of urea across the rumen wall, and that the rate of urea transfer is inversely related to rumen ammonia concentration. However, there are no direct comparisons available between measurements of urea transfer to the rumen and bacterial numbers or urease production in the rumen epithelium. Accordingly, this paper describes an experiment in which the influence of rumen ammonia concentration and sucrose supplements on urea transfer and numbers of bacteria adherent to the rumen epithelium in sheep given brome grass pellets are examined.

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EXPERIMENTAL

Sheep, diets and experiments

Six mature Suffolk wethers, weighing 34–43 kg, were fitted with permanent cannulas in the rumen and maintained in individual metabolism cages. Brome grass pellets were offered at hourly intervals (50 g dry matter (DM)/h) by means of an automatic feeding device (Turner *et al.* 1980).

Six treatments were imposed during each of three periods of 2 weeks according to a randomized complete block design. Three levels of sucrose (0, 150, 300 g/d) with or without urea (20 g/d) were given orally twice daily (08.00 and 20.00 hours) for 8 d, followed by 6 d during which solutions of the supplements were pumped (750 ml/d) directly into the rumen.

Three other Suffolk wethers given brome grass pellets were used to obtain additional bacteriological information. One sheep was slaughtered to provide epithelial samples with which to assess the bacteriological methods. The others (one infused with 300 g sucrose and 40 g urea daily) provided samples for a more detailed analysis of the facultative ureolytic population.

Experimental procedures

The six wethers in the main experiment were prepared on day 11 of each period with catheters in each jugular vein and the rumen, and infused intraruminally with $NaH^{14}CO_3$, (750 ml/d, 0·14 μCi/ml, 1 mg $NaHCO_3$/ml) for 11 h. Blood (6 ml) and rumen fluid (10 ml) were taken at 1 h intervals for the final 6 h of infusion (day 12). On day 14, following a priming dose (18 μCi in 14 ml saline (9 g sodium chloride/l)), [^{14}C]urea was infused (170 ml/d, 1·3 μCi/ml, 1 mg urea/ml saline) for 11 h. Blood and rumen fluid were taken hourly.

After the final period, supplement infusions were continued for 13 d until the sheep were serially slaughtered. The rumen was exposed and a piece of rumen wall approximately 100 × 100 mm removed from approximately 20 mm below the left lateral pillar. The tissue was rinsed by agitation for 10 s in two changes of anaerobic buffer (Hungate, 1969) containing $Na_2S.9H_20$ (200 mg/l) and resazurin (1 mg/l). Papillae were then rapidly excised with scissors and dropped into ice-cold anaerobic buffer before being transferred to weighed tubes of anaerobic buffer which were then closed with an atmosphere of carbon dioxide (Hungate, 1969) and placed in ice.

Analytical methods

Urea in plasma, and specific raioactivities of urea-C in plasma and of bicarbonate-C in blood and rumen fluid were determined as described previously (Kennedy, 1980). Ammonia concentration in rumen fluid was estimated using a specific ion electrode (Model ISE 10-10-00; HNU Systems Inc., Newton MA 02164).

Calculations

The transfer of plasma urea to the rumen was calculated from the appearance of $H^{14}CO_3^-$ in the rumen during [^{14}C]urea infusion into the jugular vein as described previously (Kennedy, 1980), based on the method of Norton *et al.* (1978). Clearance of plasma urea to the rumen (l/h) was calculated as:

$$\frac{\text{Transfer of urea (mg nitrogen/h)}}{\text{Plasma urea concentration (mg nitrogen/l)}}.$$

Bacterial methods

The tube containing papillae was weighed and the papillae homogenized in the tube using a Polytron blender in an anaerobic glove box with a CO_2 atmosphere. The homogenate was used for preparing serial tenfold dilutions in anaerobic buffer in stoppered tubes by the method of Hungate (1969). For aerobic counts, 0·5 ml of appropriate dilutions were spread on three agar plates. The agar medium (TBY) consisted of (g/l) Bacto-Tryptone 3.0, Bacto-Beef Extract 1·5, Bacto-Yeast Extract 1·5, sucrose, glucose, fructose each 1·0, agar 13·0. Rumen fluid, clarified by centrifugation, was sometimes added (100 ml/l) for post-isolation studies, as were Bacto-Casamino Acids (2 g/l). For anaerobic counts 0·5 ml was added to each of three anaerobic roll tubes containing 98–5 agar (Bryant & Robinson, 1961). All incubations were at 39°.

For purification, isolated colonies were picked onto new agar plates or roll tubes. Anaerobic growth of aerobes was tested by inoculation of isolated colonies into anaerobic tubes of TBY liquid medium with cysteine hydrochloride (250 mg/l) and resazurin (10 mg/l).

Bacteria capable of both aerobic and anaerobic growth (facultative) were tested for their ability to produce urease using a thick suspension of cells washed from an agar plate with the buffer of Gorin & Chin (1966). The cell suspensions were incubated with 0·17 mM-urea for 3 h at 39°, with appropriate controls, and ammonia production was tested for with the specific ion electrode, or Nessler (Umbreit *et al.* 1964) or phenate (Cook, 1976) reagent.

Bacteria growing aerobically were identified according to the criteria of Bergey (Buchanan & Gibbons, 1974), Skerman (Buchanan & Gibbons, 1974), Baird-Parker (1966), and Harrigan & McCance (1966).

RESULTS

Values for irreversible loss, transfer and clearance to the rumen of plasma urea, together with rumen ammonia and plasma urea concentrations, are given in Table 1. Addition of either level of sucrose to the diet, with or without urea supplementation, increased urea clearance and usually transfer to the rumen, while concentrations of plasma urea and rumen ammonia were reduced. Maximum transfer (0.308 g N/h) was observed for sheep given 150 g sucrose/d with urea. However, maximum clearance (5·83 l/h) of plasma urea to the rumen was observed for sheep given 300 g sucrose/d without urea. Urea supplements markedly depressed clearance at all sucrose levels, and a family of three curves relating clearance with ammonia concentration in the rumen was apparent (Fig. 1). Infusion of 0·39 g N/h as urea into the rumen increased the irreversible loss of plasma urea by 0·96 of the amount of urea infused for sheep without sucrose, but the values for sheep with 150 and 300 g sucrose/d were 0·75 and 0·85 respectively.

Preliminary studies on a sheep given brome grass showed that bacteria in numbers similar to those found by Wallace *et al.* (1979) (approximately 10^8/g epithelium) could be isolated aerobically on the TBY medium and it was used subsequently for all aerobic isolations even though colony numbers could be increased approximately 0·1 by the addition of sterile rumen fluid. Epithelial samples from this first animal were removed from four positions in the rumen; the left wall close to the midline, roof of the dorsal rumen, roof of the caudal rumen and floor of the caudoventral blind sac. Highest counts (6–30x) were obtained from the midline sample, and tissue samples from that area just below the left lateral pillar were used in studies on all other sheep.

Only five of the six sheep in the main experiment were available for slaughter. The numbers of bacteria grown aerobically from those five sheep and the other two examined are shown in Fig. 2. There was an increase in bacteria isolated with increasing supplementation

Table 1. *Transfer and clearance of plasma urea to the rumen, concentration and irreversible loss of plasma urea, and concentration of rumen ammonia in sheep given brome grass* (Bromus inermis) *pellets with three levels of sucrose and two levels of urea*

Supplement (g/d):							
Sucrose	—	150	300	—	150	300	SEM
Urea	—	—	—	20	20	20	
Urea transfer to the rumen (g nitrogen/h)	0·176	0·195	0·217	0·105	0·308	0·210	0·0436
Clearance of plasma urea to the rumen (l/h)	2·09^{ab}	3·76^{ab}	5·83^{b}	0·55^{a}	2·01^{ab}	2·76^{ab}	0·881
Irreversible loss of plasma urea (g N/h)	0·556^{ab}	0·585^{ab}	0·393^{a}	0·928^{b}	0·878^{b}	0·728^{ab}	0·0861
Concentration of plasma urea (mg N/l)	92^{ab}	65^{a}	38^{a}	195^{b}	153^{b}	100^{ab}	7·8
Concentration of rumen ammonia (mg N/l)	72^{ab}	48^{a}	28^{a}	258^{c}	161^{c}	178^{bc}	8·3

a, b, c, Means with different superscripts differ significantly ($P < 0·05$).

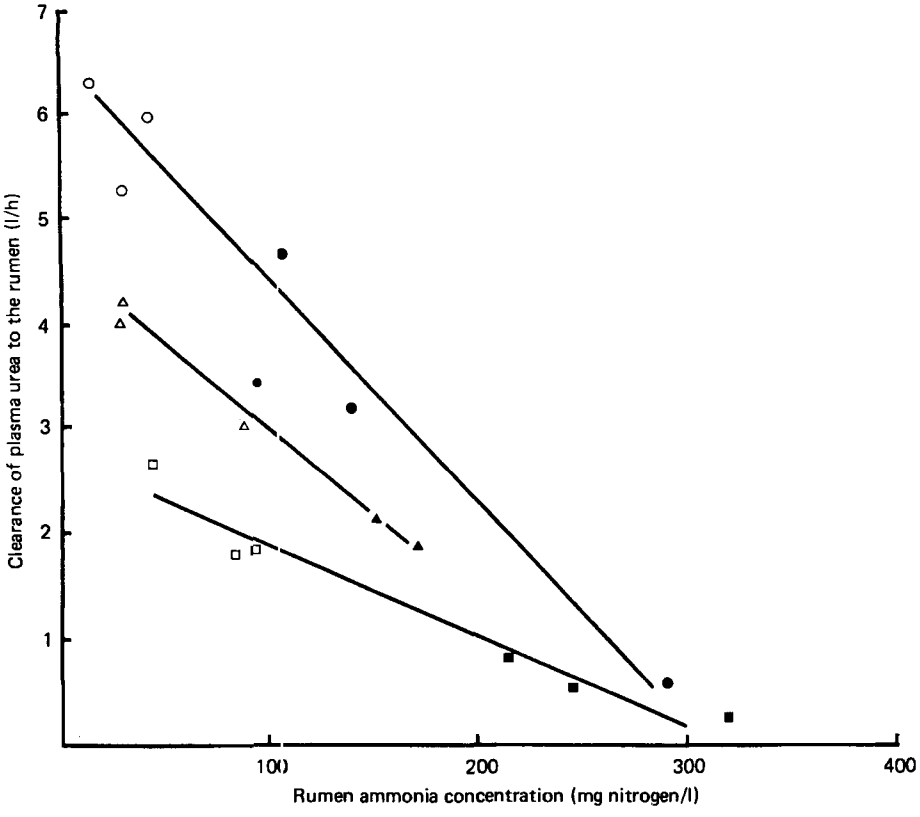

Fig. 1. Relationships between clearance of plasma urea to the rumen with rumen ammonia concentration in sheep given pelleted brome grass (*Bromus inermis*) alone or with sucrose and urea; no urea, (□, △, ○); urea supplemented, (■, ▲, ●). (□, ■) Sheep with no sucrose ($y = -0{\cdot}0082x + 2{\cdot}67$, r 0·97); (△, ▲) sheep with 150 g sucrose/d ($y = -0{\cdot}0159x + 4{\cdot}51$, r 0·99); (○, ●) sheep with 300 g sucrose/d ($y = -0{\cdot}0205x + 6{\cdot}41$, r 0·98).

with sucrose and urea. Because of difficulties in keeping the samples anaerobic during blending, counts of anaerobes were obtained from only three sheep; these were 11, 7 and 290×10^7/g wet tissue for brome grass + urea, brome grass + 150 g sucrose and brome grass + 300 g sucrose + urea respectively.

The number of aerobic isolates found to be capable of anaerobic (facultative) growth depended on the medium on which they were tested. Of thirty-nine aerobic isolates from TBY, twenty-four grew on anaerobic TBY in roll tubes, but none grew anaerobically on 98–5 medium. It was therefore difficult to assess accurately the numbers of facultative bacteria present. Over all, of approximately fifty aerobic colonies from each sheep tested on anaerobic TBY medium, the numbers growing anaerobically were 0·39–0·62 of those tested.

Indications were that ureolytic bacteria increased in proportion with aerobes and facultative bacteria but this was not verified even though specific attempts were made on two of the sheep, one given brome grass only and one given brome grass with sucrose and urea. Some of the difference in colony numbers between these two sheep resulted from an increase in pinpoint colonies of *Streptococcus bovis*.

The ureolytic facultative bacteria isolated were divided into groups according to cell

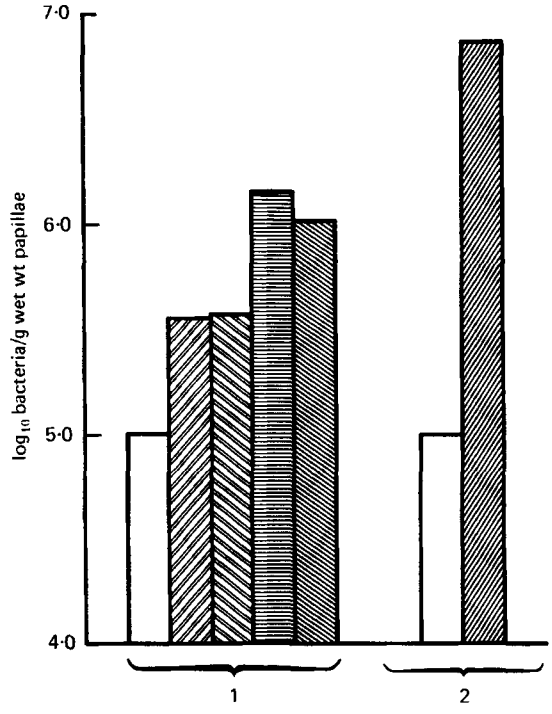

Fig. 2. Counts of aerobic bacteria from the left midline sample of rumen epithelium from sheep given pelleted brome grass (*Bromus inermis*) with sucrose and urea supplements. 1, Main experiment; 2, two additional sheep. Experiment 1 (□), no addition; (▨), +urea (20 g/d); (▧), +sucrose (150 g/d); (▤), +sucrose (300 g/d); (■), +sucrose (300 g/d); +urea (20 g/d). Experiment 2 (□), no addition (■), +sucrose (300 g/d) +urea (40 g/d).

morphology, Gram reaction and colonial characteristics. Twenty-two ureolytic isolates were thus selected for identification. Of these, seventeen were cocci and were identified as belonging to *Staphylococcus*. They were not a homogeneous group and included members of all six sub-groups of Baird-Parker (1966). Five isolates were bacilli, all different and belonging to *Propionibacterium* and *Corynebacterium*.

DISCUSSION

The amount (4·2 g N/d) of plasma urea that was transferred to the rumen of sheep given brome grass pellets without supplements was approximately 0·5 that observed previously (Kennedy & Milligan, 1978) for sheep given a similar diet, but because plasma urea concentrations were also lower in the present study, values for clearance of plasma urea to the rumen (approximately 2 l/h) were similar in both studies. In addition, the inverse relationship between urea transfer and ammonia concentration previously observed (Kennedy & Milligan, 1978) for sheep with relatively constant concentrations of plasma urea, appears to reflect the relationship between clearance of plasma urea to the rumen with rumen ammonia concentration (Fig. 1). At approximately 300 mg ammonia N/l rumen contents, urea clearance was reduced to a minimum of 0·5 l/h and did not differ with the level of sucrose supplementation. However, at rumen ammonia concentrations lower than 300 mg N/l, there was a higher urea clearance with increased sucrose intake, with differences in the slope of the inverse relationship of urea clearance and rumen ammonia being evident (Fig. 1). At the extrapolated intercept of zero ammonia, clearance was elevated

by 1·84 l/h by the first increment of sucrose and by a further 1·90 l/h by the second increment. These observations are in agreement with a previous suggestion (Kennedy & Milligan, 1980) that the inverse relationship between urea clearance to the rumen and ammonia concentration was described by a family of curves, with the *y* intercepts determined by the intake of OM or rate of OM fermentation in the rumen.

The increase in irreversible loss of plasma urea when urea was given to sheep receiving sucrose supplements was 0.037–0·079 g N/h less than for sheep without sucrose. This implies that a portion of the supplemented urea was utilized for microbial synthesis in the rumen of sheep given sucrose, and that ammonia-N from dietary and endogenous sources was limiting microbial growth in sheep given sucrose, as was found in cattle given sucrose (Kennedy, 1980).

A mechanism by which the transfer of plasma urea to the rumen is controlled was suggested by Cheng & Wallace (1979) to involve urease activity of the bacterial population adherent to the rumen epithelium, but simultaneous estimates of urease activity and urea transfer to the rumen were not made in their study. Efforts to derive relationships between urease activity in rumen epithelium and urea transfer or clearance in the present experiments (Kennedy, Clarke & Milligan, unpublished results) were not successful, perhaps due to the difficulty in obtaining samples of epithelium with urease activity truly representative of total epithelium. There is limited evidence from the present study, however, that sucrose supplements increased the numbers of adherent epithelial bacteria (Fig. 2). Both aerobic and anaerobic bacteria were shown to increase in numbers with sucrose and urea supplementation, but the actual numbers of bacteria that could be isolated aerobically were dependent on the medium used, and the results reported here relate to TBY and 98–5 media. Also, quantitatively different results may have been obtained had other rumen sites been examined. However, in the present study, the midline site was sampled as it showed moderately heavy bacterial colonization and high urease activity in the study of McCowan *et al.* (1980). It would be logical that bacteria having a regulatory role in urea transfer across the rumen epithelium would have a very intimate physical association with the epithelium. They would be expected in the 'numerically small, truly adherent bacterial population' (Cheng *et al.* 1979) that remains after washing. The presence of many epithelial cells that do not have adherent bacterial populations (Bauchop *et al.* 1975; McCowan *et al.* 1978, 1980) let alone adherent ureolytic bacteria suggests that even contiguous epithelial cells may have different capacities to allow the diffusion and transfer of urea. The proportions of facultative and ureolytic bacteria in the populations of the sheep in the present study were not precisely established.

One reason for the difficulty in assessing numbers of ureolytic bacteria is that they constitute only a small part of the population. There is no selective medium available for their identification so one has to test many hundreds of colonies at low dilutions to assess the population accurately. From the limited information obtained, the numbers of ureolytic bacteria seemed to increase proportionately with the aerobic isolates in response to the dietary supplementation. We recognize however that we did not enumerate the ureolytic anaerobes (Wozny *et al.* 1977). Much of the rest of the increase resulted from the growth of *S. bovis*. The role of this bacterium on the epithelium in unknown, but it is of special importance in lactic acidosis in cattle. It may be of significance that *S. bovis* on the epithelium could produce lactic acid in a situation where it would be available for immediate absorption by the host and not readily available for metabolism by lactic acid metabolizing bacteria. The numbers of *S. bovis* on the epithilium of the sheep receiving sucrose (300 g) and urea (40 g) were comparable to numbers often found in rumen fluid (Hungate 1966).

In spite of the complexity of the rumen epithelial populations in sheep (Bauchop *et al.* 1975) so far only staphylococci (Wallace *et al.* 1979) and some bacilli (the present study)

have been shown to have significant ureolytic activity. The distribution of these bacteria within the population on the epithelium is not known. Urease activity does vary considerably between different rumen sites (McCowan *et al.* 1980), and the variation is not consistent with differences in distribution of epithelial bacteria. The rumen epithelial discontinuity of area and sites available for bacterial colonization and urease formation as a result of differences in papillary size and spacing makes quantitative assessment, of both numbers of adherent bacteria and of total urease activity, a very difficult task.

Cheng & Wallace (1979) proposed that the ureolytic bacteria maintain a localized concentration gradient of urea across the rumen wall, and that urea transfer is controlled by the rumen ammonia concentration through a negative effect on urease activity. The experiment of Cheng & Wallace (1979) shows that the urease content of rumen fluid (presumed to reflect urease activity of rumen epithelium) required 24 h to adapt to a new steady-state level of ammonia. In the bacteria tested in vitro in the present study, the enzyme was synthesized in the absence of urea, and therefore its rate of synthesis did not appear to be very responsive, at least to substrate. Indeed if there was a direct ammonia inhibition of even some of the urease of the rumen wall, as contrasted to the insensitivity of urease in rumen fluid (Mahadevan *et al.* 1976), this could conceivably provide a measure of immediate control of urea transfer, but direct evidence is lacking. The theory will be clarified only when the over-all urease activity on the epithelium can be quantitated and the total number of ureolytic bacteria assessed. It is not even clear at the moment whether there are sufficient ureolytic bacteria present on the tissue to account for the urea hydrolysis that occurs in the epithelium.

The present work indicates that sucrose supplementation of a pelleted grass diet increased both numbers of bacteria adherent to the rumen epithelium and urea clearance to the rumen, an observation consistent with the hypothesis of Cheng & Wallace (1979). This raises interest concerning the influence of the rate of digestion and type of dietary OM on urea clearance and adherent bacterial activity and whether a limit of carbohydrate supplementation exists above which further increases in urea clearance and adherent bacterial numbers do not occur. In addition, an unequivocal and quantitative demonstration of the relationship between urease activity in the rumen epithelium and clearance of plasma urea to the rumen is still required.

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