Glycosyl ureides in ruminant nutrition

2. In vitro studies on the metabolism of glycosyl ureides and their free component molecules in rumen contents

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1. The fate of glucosyl urea (GU), lactosyl urea (LU) and corresponding mixtures of the free sugars and urea and their degradation products were examined during in vitro incubation of the compounds with rumen contents taken from donor sheep and steers at various stages of adaptation to these compounds.

2. The sugar–urea bond was virtually unattacked in rumen contents from unadapted sheep and steers but generally a slow release of the galactose moiety occurred. After feeding LU or GU to animals for a period of approximately 10 d, the rates of disappearance of both bound urea and sugar had increased, but were still markedly slower than those of the corresponding free sugars and urea. In vitro rates of degradation of both free lactose and urea also increased in response to the feeding of lactose and urea to rumen content donor animals.

3. Ammonia accumulation in rument contents when GU or LU were the substrates was notably lower than when equivalent amounts of glucose and urea or lactose and urea were the substrates.

4. Bacterial growth was estimated using an vitro method based on incorporation of ^{32}P into bacterial nucleic acids. Markedly different patterns of bacterial growth were observed depending on whether LU or lactose and urea were the substrates.

Methods for preparing glycosyl ureides on a laboratory scale and in rather poor yields have been known for many years (Goodman, 1958), but little attention has been given to their use as dietary non-protein-nitrogen (NPN) supplements for ruminants. A brief report by Milligan *et al.* (1972) indicated that even after adaptation, glucosyl urea (GU) was more slowly metabolized in the rumen than glucose and urea in the free forms. It seems possible that simple chemical modification of sugars to form ureides such as lactosyl urea (LU) and GU could not only serve the purpose of reducing the potential toxicity of urea but also alter the rate of degradation of soluble sugars to be more like that of starch. It has been argued that starch, preferably that which had been cooked, is the most suitable carbohydrate source for supporting rumen microbial growth (Bartley & Deyoe, 1977; Smith, 1979), and some experimental evidence certainly indicates that starch is superior to sucrose in this respect (Al Attar *et al.* 1976; Oldham *et al.* 1977).

Some workers have investigated the use in ruminant feeding of ill-defined mixtures prepared from molasses (Galyamin, 1975; Demeyer & Van Nevel, 1978), which are claimed to contain ureides, but for a proper understanding of the processes involved and the true potential of such compounds it is necessary to use well-characterized products. The preparation of such materials and particularly of LU have been described previously (Merry *et al.* 1982*a*) and an examination of the metabolism of GU, LU and also their free-component molecules under in vitro conditions with rumen contents, form the subject of the present investigation. A brief account of some of this work has already been published elsewhere (Merry *et al.* 1979).

Table 1. *Daily intake (kg dry matter (DM)) of major components of diets given to steers and sheep, together with total nitrogen and metabolizable energy (ME) contents*

Diets	Daily amounts (kg DM) given in the diets			
	Steers		Sheep	
	A	B	C	D
Rolled malting barley–straw (1:1, w/w) cubes	3·41	—	0·810	—
Chopped straw	—	—	—	0·405
Alkali-treated straw cubes	—	1·72	—	—
Rolled malting barley	—	1·72	—	0·405
Urea	—	0·025	0·006	0·006
N (g/d)	51·2	49·8	16·7	11·3
ME (MJ/d)	34·4	34·7	8·2	8·2

Table 2. *Composition (g/kg dry matter) of whey preparations* containing lactosyl urea*

	Preparation		
	1	2	3
Total nitrogen	57·7	62·8	65·7
Lactosyl urea	313·4	333·9	281·0
Glucosyl urea	4·8	6·6	2·8
Galactosyl urea	3·2	13·3	5·0
Unbound lactose	137·7	164·0	122·7
Unbound urea	34·3	34·2	40·7

* Prepared by Ewos AB, Södertälje, Sweden.

EXPERIMENTAL

Animal management and feeding

Four Suffolk × Scottish Blackface mature wether sheep (50–70 kg) were fitted with rumen cannulas and housed in individual pens on expanded metal floors. The cannulas used were as described by Smith & McAllan (1970). Four Friesian steers (140–160 kg) equipped with similar rumen cannulas were also used. During introductory periods of at least 3 weeks before experiments, and for some experimental periods, sheep were given diets with sufficient energy for maintenance (diets C and D, Table 1) and steers were given diets providing intakes of metabolizable energy (ME) sufficient for a body-weight gain of approximately 0·4 kg/d (diets A and B, Table 1).

During experimental periods, ureide supplements replaced part of the basal diets A–D (Table 1) on an isoenergetic basis. Diet A + GU contained 200 g pure GU which replaced part of the barley and straw cubes in basal diet A (Table 1) to give an N intake of 71·6 g/d. Diet B + LU_1 included 0·492 kg dry matter (DM) of whey preparation 1 (Table 2) containing 154 g LU, which replaced the urea and some of the barley in basal diet B (Table 1) giving an N intake of 57·7 g/d. Diet C + LU contained 160 g pure LU, and diet C + LU_2 included 0·175 kg DM of whey preparation 2 (Table 2) containing 58 g LU. These supplements replaced urea and part of the barley and straw cubes in diet C (Table 1) to give respective

N intakes of 22·1 and 21·6 g/d. Diet D + LU_3 included 0·150 kg DM of whey preparation 3 (Table 2) containing 42 g LU, which replaced the urea and part of the barley in basal diet D (Table 1) to give an N intake of 15·4 g/d.

All diets were offered in equal amounts twice daily at 09.00 and 17.00 hours. Dietary ingredients were rolled, chopped or cubed or both as shown in Table 1 in order to make the rumen contents relatively homogeneous so that reasonably representative samples could be taken which could be handled easily for in vitro experiments.

Animals that had previously only received introductory diets containing barley, straw and urea will subsequently be referred to as 'unadapted'. The effects of adding ureide to the diet for different periods (adaptation) and of subsequently withdrawing it (de-adaptation) were studied.

Collection of digesta samples

Samples of rumen contents were taken by gentle suction using a perforated tube (i.d. 20 mm), connected to a vacuum pump. Care was taken to move the sampling tube around the rumen to obtain a fairly representative sample of total rumen contents. Contents were immediately transferred to a pre-warmed Thermos flask fitted with a Bunsen valve and tube through which gas could be passed. The gas space above the contents was flushed for 2 min with oxygen-free carbon dioxide, or in some experiments an N_2:CO_2 (90:10, v/v) mixture depending on which gas was to be used for the succeeding in vitro experiments. Samples were taken to the laboratory and used for incubations within 30 min of collection. Animals were allowed at least 2 weeks between each experiment for re-establishment of normal rumen conditions.

All experiments to be described were made with whole rumen contents collected in the way outlined previously. Preliminary experiments (Merry, 1980) showed that fermentative activity in whole rumen contents was considerably greater than for the corresponding strained material; this was particularly marked when LU was the substrate.

Incubation and processing of samples

Metabolism of ureides and corresponding mixtures of free sugars and urea

Three in vitro incubation procedures were adopted to study mechanisms and rates of fermentation of ureides and mixtures of their free component sugars and urea. These procedures were used in Expts 1–4, more details of which are given on pp. 292–297.

Incubation procedure A. Samples (400 g) of rumen contents were taken from a steer 2 h after feeding, before and during adaptation to GU, and incubated with 35 ml of the appropriate substrates or 0·15 M-sodium chloride as a control.

Incubation was carried out with shaking for suitable periods at 39° in 750-ml wide-necked conical glass flasks which were stoppered with rubber bungs fitted with Bunsen valves. To maintain anaerobic conditions, the gas space above the rumen contents was flushed at the start of the incubation and thereafter at 30 min intervals (for 1 min) with an N_2 : CO_2 (90 : 10, v/v) mixture. Samples (25 g) were withdrawn at zero time and at intervals after the start of the incubation period and acidified with 5 ml 2 M-hydrochloric acid to stop fermentative activity. The acidified samples were strained through four layers of surgical gauze, ultrafiltered (Gregory, 1954) and the ultrafiltrates stored at −20° to await analysis for urea, GU, glucose or ammonia.

Incubation procedure B. Samples (400 g) of sheep rumen contents taken 2 h after feeding were added to 100 ml substrate solution or 0·15 M-NaCl as a control. Incubation was carried out as for procedure A. Samples of the incubation mixture (50 g) were removed at intervals and acidified with 10 ml 3 M-HCl (to give an approximate final acid concentration of 0·6 M). The acidified samples were strained through four layers of surgical gauze and the fluid obtained centrifuged at 35000 ***g*** for 15 min and the supernatant fractions stored at

−20°. Free or bound urea, glucose or lactose and ammonia were determined in the supernatant fractions.

Incubation procedure C. With procedures A and B the pH of the incubation mixture tended to decrease with the period of incubation. In procedure C a buffer was included to maintain a pH value of between 6·4 and 6·8. Each sample (400 g) of rumen contents, taken before a morning feed from sheep or steers at different times before and during adaptation to LU was incubated with 100 ml buffer (0·174 M-sodium bicarbonate equilibrated to pH 6·8 by gassing with oxygen-free CO_2), with or without an appropriate substrate. Incubations were carried out as described for procedure A but oxygen-free CO_2 was used for flushing. For experiments with sheep, 30 g samples of the incubates were removed at intervals and strained through four layers of surgical gauze. To 25 g strained fluid was added 2·5 ml mercuric chloride (50 g/l) to stop microbial fermentation, and supernatant fractions (35000 *g*) were prepared. Analyses for ureides, urea and lactose were carried out on these supernatant fractions. In experiments with steers where free urea and lactose analyses were required, strained samples of the incubation mixture were treated with $HgCl_2$ in the same way as described previously and supernatant fractions (35000 *g*) prepared. Portions (10 ml) of the $HgCl_2$-treated supernatant fractions were acidified with 2 ml 3 M-HCl in preparation for estimation of bound urea and sugars after acid-hydrolysis. For ammonia determination, 15 g samples of rumen contents were treated with 3 ml 2 M-HCl and supernatant fractions (35000 *g*) prepared. All samples from these experiments were stored at −20°.

Estimation of bacterial protein synthesis

A method was developed based on measurement of ^{32}P incorporation, but bacterial growth was estimated from the extent of incorporation of ^{32}P-labelled inorganic phosphate into bacterial nucleic acids, rather than into total microbial P as used by Van Nevel & Demeyer (1977). We have considered such incorporation to provide an index of bacterial rather than total microbial growth as protozoa use preformed units to synthesize a large amount of their nucleic acids (Coleman, 1968, 1975). There is no evidence to show that protozoa make use of appreciable amounts of free inorganic phosphate in this synthesis and it seems very likely that the P which they incorporate comes in large part from the bacteria that they ingest.

Samples of rumen contents were obtained from three sheep receiving either diet D (Table 1) or the same diet supplemented with LU (D + LU_3, see p. 288) or LU + ground, cooked starch (Starea). To study ^{32}P incorporation, 40 g rumen contents were transferred to flasks fitted with Bunsen valves, containing 10 ml 0·174 M-$NaHCO_3$ (equilibrated to pH 6·8 with oxygen-free CO_2), together with the appropriate substrate. Approximately 4 μCi (in 0·2 ml) of ^{32}P-labelled sodium orthophosphate (Radiochemical Centre, Amersham) in 0·01 M-H_2PO_4 were added to each flask. Part of the contents of one flask (25 g) was immediately treated with 25 ml trichloroacetic acid (TCA; 180 g/l) in ethanol (step 1 in the procedure for extraction of nucleic acids from digesta samples; McAllen & Smith, 1969) to stop microbial activity. The remaining flasks were flushed with oxygen-free CO_2 for 1 min and incubated at 39° in a shaking water-bath. Further gas was passed into the flasks for 1 min, every 30 min of the incubation period. At appropriate intervals, 25 g samples were removed and treated with TCA and ethanol as described previously. The mixtures were then subjected to the series of acid- and lipid-solvent extractions (steps 1–7) described by McAllan & Smith (1969). This left a dry residue from which virtually all inorganic and organically-bound P had been removed, except that in the nucleic acid fraction (see p. 298). ^{32}P was counted in this residue and total nucleic acid-P (NA-P) incorporation estimated from this activity and the specific activity of the inorganic phosphate in an ultrafiltrate (Gregory, 1954) prepared from the rumen contents used in each incubation.

Study of total N:NA-P values of mixed bacteria

To prepare large enough samples of rumen bacteria to make it possible to determine total N:NA-P values it was necessary to incubate larger samples under similar conditions to those used in studying ^{32}P incorporation. Rumen contents (700 g) were incubated for up to 7 h with 175 ml of the appropriate substrate dissolved in bicarbonate buffer (pH 6·8) but without addition of ^{32}P-labelled phosphate. After incubation the samples were cooled in an ice bath and strained through four layers of surgical gauze. Mixed bacteria were separated from the strained, incubated rumen digesta by a method involving differential centrifugation, which has been described by Smith & McAllan (1974). Total N, P and dry matter were determined in whole bacteria, and NA-P and in some experiments RNA and DNA were determined in the dry residue obtained after extraction of bacteria by the method of McAllan & Smith (1969).

Analytical methods

LU and GU in rumen contents were determined chromatographically as described by Merry *et al.* (1982*a*). Estimations of bound urea and reducing sugar in ureides after their release by acid-hydrolysis and of free urea in digesta samples were made by procedures described by Merry *et al.* (1982*a*). Lactose and glucose were determined after ion-exchange chromatography as described by Smith & McAllan (1971). Total reducing sugars in hydrolyzed rumen content samples were determined by an automated method (Coombe, 1972) in which a cupric-neocuproine chelate was reduced by reducing sugar to form a coloured cuprous–neocuproine complex. Total N was estimated by the procedure of Smith & McAllan (1970) and RNA and DNA by the methods of McAllan & Smith (1969). Inorganic phosphate in ultrafiltered (Gregory, 1954) rumen contents was estimated as described by Smith *et al.* (1978). Total P in rumen bacteria was measured by the following procedure. Nitric acid (specific gravity 1·42; 0·8 ml) and perchloric acid (specific gravity 1·54; 0·8 ml) were added sequentially with mixing to 75–100 mg freeze-dried or extracted rumen bacteria. Digestion was carried out until the solution cleared and white $HClO_4$ fumes began to appear. The samples were cooled and made to a volume of 10 ml with distilled water. P in the resulting samples was estimated as described by Smith *et al.* (1978). Dry matter was determined by heating approximately 10–20 g digesta or mixed bacteria at 105° for 24 h in a forced-draught oven. Ammonia ($NH_3 + NH_4^+$) was estimated in acidified rumen contents by an automated procedure described by Merry (1980).

To prepare samples for liquid scintillation counting, it was necessary to solubilize the residue. ^{32}P-labelled residues from whole rumen contents were not readily solubilized by commercially-available tissue solubilizers and were thus subjected to a wet-ashing procedure.

A sample of dry residue (250–300 mg) was digested with 7 ml nitric acid (specific gravity 1·42) until the volume was reduced to approximately 2 ml. The sample was cooled and 1 ml $HClO_4$ (specific gravity 1·54) added and further digestion carried out until white fumes of $HClO_4$ appeared. The digest was cooled and made up to 10 ml with distilled water. A portion (1 ml) of the diluted digest was pipetted into a screw-capped glass counting vial and 10 ml Instagel (Packard Instruments Co. Ltd, Illinois) added. An internal standard was added to counted samples, and the mixture recounted to enable correction for quenching in calculations of ^{32}P activity.

Statistical analysis

Tests of significance were carried out by paired *t* tests (Snedecor & Cochran, 1972).

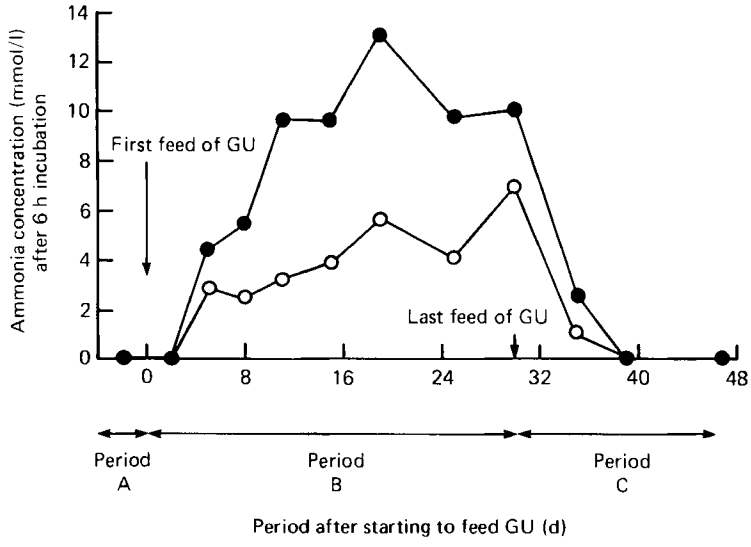

Fig. 1. Expt 1. Ammonia concentration after 6 h of incubation of glucosyl urea (GU) (●) (initial concentration 11 mM) or lactosyl urea (○) (initial concentration 16 mM) with rumen contents from a steer which for period A had received a basal diet of barley and straw cubes, for period B had been given the same basal diet supplemented with GU and for period C had been returned to the basal diet without GU. Results were corrected by subtracting values obtained for incubations where no substrate was added.

RESULTS

Degradation of ureides

Samples of rumen contents were taken from animals before and during adaptation to LU or GU. The fate of these compounds and the products formed were followed.

Expt 1. Adaptation when glucosyl urea was included in the diet. In a preliminary experiment, samples of rumen contents from an unadapted steer receiving diet A (Table 1) were incubated with GU (initial concentration in the mixture 9 mM) for periods up to 4 h using incubation procedure A. Ammonia concentrations remained below 1 mmol/l but when equivalent amounts of free glucose and urea were incubated with another sample of the same rumen contents, ammonia concentration rose rapidly to 10 mmol/l and remained there until the end of the incubation.

In another experiment samples of rumen contents from a steer given diet A+GU (100 g/feed) were taken on different days during a period of adaptation to GU. The samples were incubated in one experimental series with LU (initial concentration in the mixture 16 mM) and in another with GU (initial concentration in the mixture 11 mM) using procedure A. Ammonia concentrations after 6 h of incubation are shown in Fig. 1. When either GU or LU were the substrates, values did not change greatly after approximately 8 d of adaptation but with LU ammonia accumulation was less than with GU. De-adaptation occurred rapidly after stopping GU feeding. Recoveries of added GU, measured chromatographically after 6 h of incubation in this experiment were 0·92, 0·87, 0·43, 0·01, 0·00, 0·00, 0·00 and 0·01 before and at 2, 5, 8, 11, 15, 19, 25 and 30 d respectively of adaptation (period B in Fig. 1) and 0·99, 0·81 and 0·93 after 3, 7 and 15 d respectively of de-adaptation (period C in Fig. 1).

Expt. 2. Adaptation when lactosyl urea was included in the diet. An experiment was done in which a sheep was kept on a basal diet of barley and straw cubes (diet C, Table 1), and then given pure LU (80 g) as a supplement with each feed (diet C+LU, see p. 288). Samples

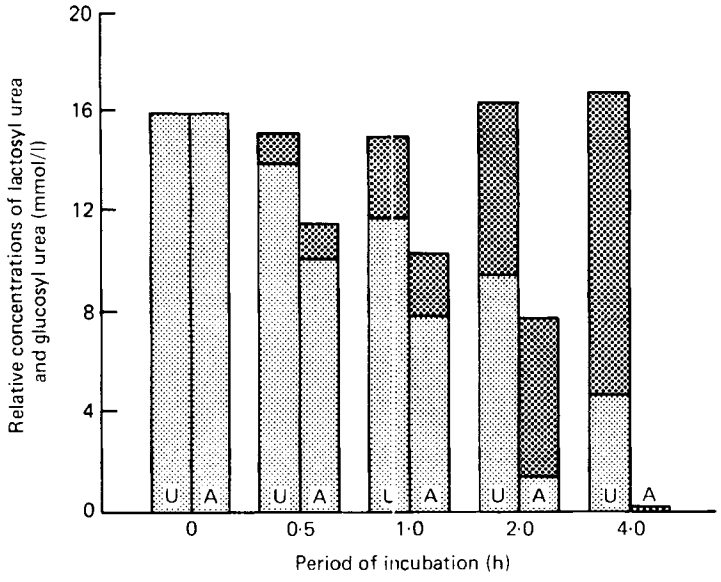

Fig. 2. Expt. 3. Changes in relative concentrations of lactosyl urea (▨), and glucosyl urea (▩) during incubation of lactosyl urea (initial concentration 16 mM), with rumen contents from an unadapted sheep (U) receiving a basal diet of barley and straw cubes, or the same sheep after 21 d of adaptation to the basal diet supplemented with lactosyl urea (A).

of rumen contents were taken before and at different times after starting to add LU and each was incubated (procedure B) in one set of experiments with LU (initial concentration in the mixture 46 mM) and in another set of experiments with GU at the same concentration.

When LU was the substrate, the ammonia concentration after 6 h of incubation was less than 1 mmol/l before adaptation and reached a fairly steady level of between 10 and 16 mmol/l during seven measurements made between 10 and 29 d after starting to give LU. It appeared that the microbial population was fully adapted to LU after approximately 6 d. After 29 d LU was omitted from further feeds; ammonia concentration at 6 h then dropped to approximately 6 mM only 4 d later. In the series of incubation experiments in which GU was used as substrate, 6 h ammonia concentration reached a fairly steady level of between 48 and 92 mmol/l, 6 d after starting to add LU to the diet. When GU was incubated with rumen contents from the adapted animal, bound urea disappeared almost completely after 6 h, but for seven experiments done days 6, 11, 15, 18, 22, 25 and 29, bound urea in LU incubated similarly only disappeared to a mean ($\pm$SE) extent of 24·6$\pm$1·3%.

In these experiments the incubation mixture was not buffered (procedure B). It was later found that lack of buffering under these conditions led to a drop in pH from approximately 6·8 at the start of the incubation to less than 6·0 after 3 h of incubation. In subsequent experiments carried out in a similar way but in which pH was maintained at 6·4–6·8 (procedure C), the disappearance of LU from samples obtained from adapted animals (i.e. those which had received LU for more than 10 d) was complete after 4 h of incubation (see, for example, Fig. 2).

Expts 3 and 4. Pattern and rate of degradation of lactosyl urea. In Expt. 2 chromatographic separation of a degradation product of LU, suspected to be GU, was not at that time possible, therefore the sum of urea bound in the form of GU and LU was estimated after acid-hydrolysis. Subsequently a chromatographic technique was developed which separated GU from LU and allowed quantification of both compounds when in a mixture. This technique enabled closer studies of the pattern of degradation of LU.

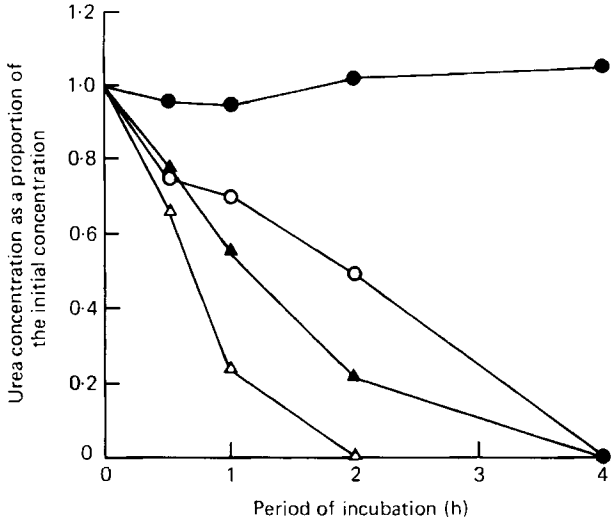

Fig. 3. Expt 3. Changes in proportions of urea remaining at different times after incubation of lactosyl urea (initial concentration 16 mM), with rumen contents from an unadapted sheep (●), receiving a basal diet of barley and straw cubes, or the same sheep after 21 d of adaptation (○) to the basal diet supplemented with lactosyl urea, lactose and urea. Also given are corresponding results for incubations where equivalent amounts of lactose and urea were incubated with rumen contents from the unadapted (▲) and adapted (△) sheep.

In Expt 3 a sheep received a diet of barley, straw and urea (diet C, Table 1) and was then given a supplement prepared from whey containing LU (29 g/feed, diet C+LU_2, see p. 288). Samples of rumen contents were taken before and 21 d after starting to give LU in the diet and incubated using procedure C with LU (initial concentration in the total mixture 16 mM).

It was apparent that even before adaptation, liberation of galactose from LU occurred to some extent, so that although free galactose was not detected in the incubation mixture, conversion of LU to GU occurred (Fig. 2). In common with other experiments (three with calves, four with sheep) virtually no degradation of bound urea occurred when LU was incubated with rumen contents from unadapted animals (Fig. 3). After 21 d of adaptation, release of galactose occurred at an accelerated rate and the GU so formed was further metabolized (Fig. 2), bound urea having disappeared completely after 4 h of incubation (Fig. 3).

Another experiment (Expt 4) was carried out in which three steers were offered diet B (Table 1) containing urea, barley and straw which was later replaced by diet B+LU (77 g/feed), the LU being supplied as a preparation from whey. Samples of rumen contents taken before, and 7 and 21 d after, starting to feed LU were incubated with LU (initial concentration in the mixture, 16 mM) using procedure C. The results for these experiments are shown in Figs. 4(*a*) and 5(*a*).

When LU was incubated with rumen contents from the unadapted steers, there was virtually no disappearance of bound urea or sugar during 4 h of incubation. This contrasted with Expt 3 where liberation and fermentation of the galactose moiety and hence disappearance of bound sugar occurred even before adaptation. After 7 and 21 d of adaptation to LU, bound urea and sugars were slowly degraded with little difference between mean rates of degradation (for three steers) occurring on either day, although there was some variation between animals. The mean differences between amounts of bound urea

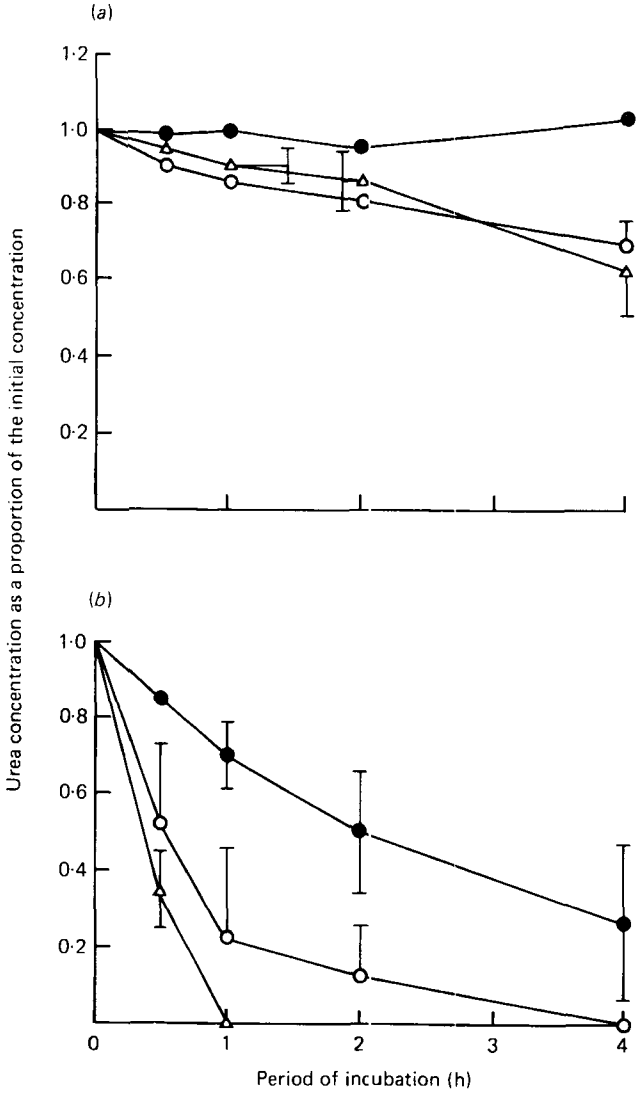

Fig. 4. Expt 4. Proportions of urea remaining at different times after incubation of (*a*) lactosyl urea (initial concentration 16 mM), or (*b*) equivalent amounts of lactose and urea with rumen contents from unadapted steers (●) receiving a basal diet of barley and alkali-treated straw cubes, or from the same steers after adaptation to the basal diet supplemented with lactosyl urea, lactose and urea for period of 7 (○) or 21 d (△). Mean values for three steers with their standard errors represented by vertical bars. For clarity, standard error values of less than 5% are not included.

or sugar remaining after 4 h of incubation of LU with adapted or unadapted rumen contents were significant after 7 d ($P \leqslant 0{\cdot}01$), but barely significant ($P \leqslant 0{\cdot}10$) after 21 d (Figs. 4*a* and 5*b*).

Degradation of free urea and sugars in mixtures

The sheep and steers in Expts 3 and 4 received diets $C+LU_2$ or $B+LU_1$ (see p. 288) which were supplemented with whey preparations containing LU. These preparations also contained some free lactose and urea and therefore in the experiments an examination

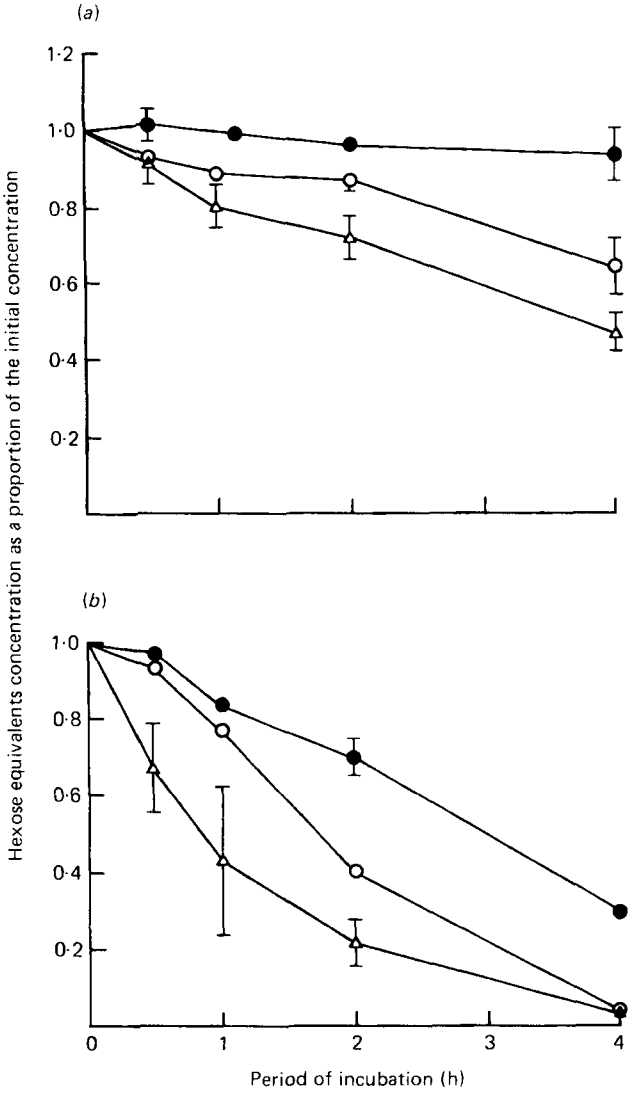

Fig. 5. Expt 4. Proportions of hexose equivalent remaining at different periods after incubation of (*a*) lactosyl urea (initial concentration, 16 mM), or (*b*) equivalent amounts of lactose and urea with rumen contents from unadapted steers (●) receiving a basal diet of barley and alkali-treated straw cubes, or from the same steers after adaptation to the basal diet supplemented with lactosyl urea, lactose and urea for periods of 7 (○) or 21 d (△). Mean values for three steers with their standard errors represented by vertical bars. For clarity, standard error values of less than 5% are not included.

was also made of the effects of adaptation to these compounds on their in vitro rates of degradation.

In both Expts 3 and 4 samples of rumen contents taken before and after periods of feeding of the supplement containing LU and lactose (L) and urea (U) were also incubated with L+U (initial concentration in the incubation mixture 16 mM).

It is apparent from the results shown in Fig. 3 that free urea was degraded much more rapidly than urea combined in LU. This was a general observation in twenty experiments made with rumen contents taken from sheep. A comparison of results for incubation with

Table 3. *Total nitrogen : nucleic acid-phosphorus (NA-P) in mixed bacteria separated from rumen contents after incubation for different times with different substrates*

(Substrate concentrations given are final concentrations in the incubation mixture)

Substrates	No. of samples	Period of incubation (h)	Total N:NA-P (as proportion of assigned starting values of 1·0)	
			Mean	SE
Lactosyl urea (20 mM)	2	2·0	1·04	0·02
	2	7·0	1·05	0·04
Lactose and urea (20 mM)	2	2·0	0·97	0·08
	2	7·0	1·07	0·01

LU or L + U as substrate (Fig. 4) shows that this was also true for three experiments with samples taken from steer rumens. The sugar part of the LU molecule was also degraded more slowly than the corresponding free sugar (Fig. 5).

When steers were given the diet supplemented with the whey product for 21 d not only did their rumen microbial population adapt in such a way that LU degradation increased, but adaptation also led to increased rates of degradation of free L + U (Figs. 4 and 5). Mean differences between 21 d adapted and unadapted steers in the proportions of urea disappearing after 2 h of incubation, were significant ($P \leqslant 0{\cdot}05$) and proportions of lactose disappearing were also significantly different ($P \leqslant 0{\cdot}01$). This adaptation leading to increased rates of urea degradation was also exhibited in experiments with a sheep (Fig. 2).

Ammonia concentrations in experiments with steers reflected the adaptive trend that was indicated by the rate of bound urea degradation. For steers adapted for 7 and 21 d respectively, ammonia concentrations were 2 and 6 mmol/l after 4 h in rumen contents incubated with LU, while after only 1 h with L + U addition for the same respective periods, concentrations of 16 and 28 mmol/l had been attained.

Bacterial protein synthesis

In order to use measurement of NA-P incorporation into bacteria to calculate accretion of N it is necessary to use a value for total N:NA-P in mixed rumen bacteria during an experiment. The use of such a method of calculation relies on a constancy of the total N:NA-P value throughout a period of incubation in which bacterial growth is occurring.

To check whether this was so, experiments were done in which the total N:NA-P values of samples of mixed rumen bacteria, before incubation, were compared with those obtained after incubation for different times with different substrates. The results are shown in Table 3. It can be seen that total N:NA-P values in the residue prepared from the extracted bacteria in samples taken before incubation were similar to those for bacteria in samples which had been incubated with various substrates for different times up to 7 h. Results from thirteen similar experiments but where cellobiose, maltose and urea were incubated with strained rumen contents (rather than whole as in Table 3), supported these observations. The mean ($\pm$SE) total N:NA-P value in bacteria separated from incubated rumen contents at different times up to 4 h, as a proportion of the starting value, was $0{\cdot}98 \pm 0{\cdot}03$. However, although total N:NA-P values were reasonably constant within experiments, there were sometimes appreciable differences between one experiment and another. In different

experiments total N:NA-P values varied between 7·5 and 10·0. It was necessary, therefore, to determine the appropriate value in each experiment. To do this, an incubation flask was set up in which the conditions were similar to those in ^{32}P-containing flasks, but scaled up to contain 875 g rumen contents–buffer-substrate mixture, rather than 50 g. When ^{32}P incorporation was being examined this flask was incubated for half the total time of the experiment and the bacteria assumed to be representative of those in all samples taken during the experiment. The bacteria were separated and washed using differential centrifugation (McAllan & Smith, 1974) and analysed for total N and DM. A portion of the bacterial suspension was extracted with acid and lipid solvents (McAllan & Smith, 1969) and NA-P estimated in the dry residue.

In six experiments, P was determined in the same bacteria both before and after extraction. NA-P formed $78{\cdot}5 \pm 1{\cdot}5\%$ of the total P, similar to the value of 80% reported by Van Nevel & Demeyer (1977). An estimate was also made of NA-P content of bacteria by calculation from RNA and DNA measured in the same extracted bacterial residue. The estimated value was $96{\cdot}5 \pm 0{\cdot}5\%$ of that measured by direct determination of P in the extracted bacterial residue. These findings indicate that determination of P in the residue obtained after extraction gives a fairly accurate assessment of bacterial NA-P.

Extraction of bacteria in samples to which ^{32}P-labelled phosphate had been added but with no opportunity for bacterial incorporation of ^{32}P (i.e. bacterial activity terminated immediately by addition of TCA–ethanol), led to a low count in the residue. This indicated the possibility of non-enzymic binding similar to that encountered by Van Nevel & Demeyer (1977). Attempts to reduce this by more stringent washing procedures led to no improvement. The values (approximately 3% of normal values obtained for incorporation of radioactivity) were lower than comparable ones of approximately 5% obtained by Van Nevel & Demeyer (1977), and were deducted as blank values in the incorporation experiments.

Evaluation of lactosyl urea, lactose and urea, and other NPN sources as substrates for bacterial protein synthesis

Initial experiments showed very low rates of incorporation of ^{32}P when strained rumen contents were incubated with LU as substrate, even when using samples from fully-adapted animals. Incorporation rates were much greater when whole rumen contents were used. This is compatible with the observation that degradation of LU is adversely affected by using strained rumen contents (see p. 289). Thus, subsequent experiments were made using whole rumen contents from three sheep given a basal diet of unsupplemented barley and straw (diet D) or another in which part of the barley had been replaced with an LU-rich preparation from whey (diet $D + LU_3$, containing 21 g of LU/feed), for at least 21 d in order to induce complete adaptation to LU.

In a typical experiment to compare bacterial growth by ^{32}P incorporation with different substrates using the procedure described on p. 290, urea, LU or isonitrogenous and isoenergetic L+U were added to rumen contents from a 21-d-adapted sheep (Fig. 6). Results from a number of similar experiments but with different substrate concentrations and times of incubation are shown in Table 4. The latter results are expressed in terms of N incorporation and were derived using total N:NA-P values in mixed rumen bacteria determined in those experiments. In all experiments N was present in amounts greater than could reasonably be incorporated with the energy present.

Marked differences were observed in the pattern of growth and disappearance of sugar when lactose was provided in the bound or unbound form. Time of incubation and substrate concentration were varied but a consistent feature when L+U were added, was rapid initial growth followed by slower growth, even in the unadapted animal (Expts 1–5, Table 4). When LU was the substrate in incubations with rumen contents from an unadapted animal very

Table 4. *Bacterial growth and sugar disappearance from the medium (/kg rumen contents) and bacterial growth efficiency in terms of energy supply, during incubation of rumen contents from adapted or unadapted sheep with lactosyl urea (LU), equivalent amounts of lactose and urea (L+U) or ground, cooked starch (Starea)*

(Starea was supplied as an isoenergetic amount to lactosyl urea in terms of gross energy. Expt 1 was done with rumen contents from an unadapted sheep and Expts 2–6 were done with rumen contents taken from sheep which had been adapted for at least 3 weeks to the substrates used in the experiments)

Expt no.	Substrate concentration (mM) in incubation mixture	Incubation period (h)	LU			L+U			Starea
			N incorporation (mg)	Sugar disappearance (g)	Efficiency (mg N/g sugar fermented)	N incorporation (mg)	Sugar disappearance (g)	Efficiency (mg N/g sugar fermented)	N incorporation (mg)
1	20	0–2·5	18·0	0·28	64·3	84·0	4·70	17·9	—
		2·5–5·0	13·1	0·97	13·5	56·5	2·10	26·9	—
		5·0–7·5	1·0	0·44	2·3	1·6	Nil	∞	—
		0–7·5	24·1	1·69	14·3	142·1	6·8	21·0	—
2	20	0–2·5	75·5	4·08	18·5	114·1	6·70	17·0	—
		2·5–5·0	53·1	2·31	23·0	23·9	0·10	239·0	—
		5·0–7·5	45·6	0·27	169·0	11·0	Nil	∞	—
		0–7·5	174·2	6·66	26·2	149·0	6·80	21·9	—
3	13	0–1·0	28·9	1·07	27·0	44·4	2·98	14·9	—
		1·0–2·0	29·8	0·89	33·5	33·0	1·47	22·4	—
		2·0–4·0	31·0	1·07	29·0	8·6	Nil	∞	—
		0–4·0	89·7	3·03	29·6	86·0	4·45	19·3	—
4	20	0–1·7	38·4	2·11	18·2	100·9	5·50	18·3	—
		1·7–3·3	47·6	1·90	25·1	24·4	1·29	18·9	—
		3·3–5·0	53·0	1·70	31·2	26·9	Nil	∞	—
		0–5·0	138·9	5·71	24·3	152·1	6·80	22·4	—
5	6·5	0–1·0	25·1	1·50	16·7	31·0	2·09	14·8	—
		1·0–2·0	42·7	0·70	61·0	25·9	0·11	235·5	—
		0–2·0	67·8	2·20	30·8	56·9	2·20	25·9	—
6	20*	0–2·5	80·2	—	—	—	—	—	90·3
		2·5–5·0	113·1	—	—	—	—	—	92·6
		5·0–7·5	49·8	—	—	—	—	—	33·4
		0–7·5	243·1	—	—	—	—	—	216·3

* The concentration shown is for lactosyl urea.

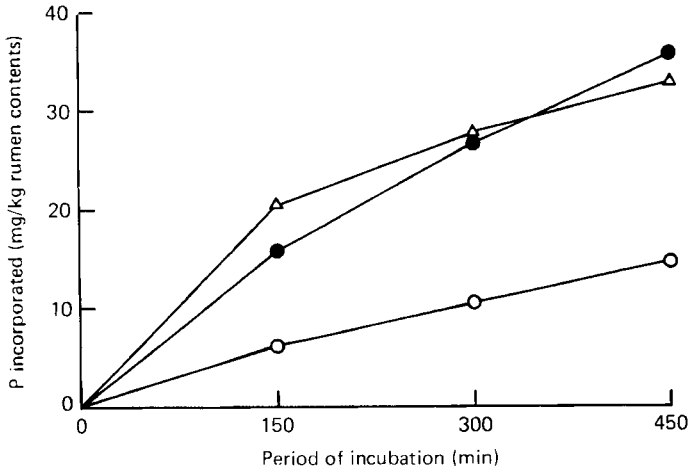

Fig. 6. Cumulative bacterial growth at different times after lactosyl urea (●) (initial concentration, 20 mM), equivalent amounts of lactose and urea (△) or an isonitrogenous amount of urea (○), were incubated with rumen contents from a sheep which had received a diet of barley, straw and lactosyl urea for 21 d and was adapted to lactosyl urea.

little growth was observed (Expt 1, Table 4), but after adaptation a more steady, uniform pattern of growth than with L + U ensued, which was associated with slower disappearance of bound sugar. Examples demonstrating the efficiency of bacterial growth in terms of sugar fermented were calculated and are also shown in Table 4. In instances where all sugar was metabolized during the period of incubation, steady growth generally continued with LU as substrate after that with L + U as substrate had nearly ended, until growth with the former substrate exceeded that with the latter (Table 4).

An experiment was also made to compare patterns of bacterial growth obtained with LU to those with an equivalent amount (in terms of gross energy content) of ground cooked starch (Starea; supplied by Lantmännen, Stockholm, Sweden). LU and Starea were incubated with rumen contents from a sheep receiving diet D + LU_3 but in which part of the barley had been replaced by an isoenenergetic amount of Starea. The pattern of growth obtained with LU as supplement was very similar to that observed with cooked starch (Expt 6, Table 4).

DISCUSSION

After a change of diet, ruminants require a period of adaptation in which the rumen microbial population adjusts to a different supply of nutrients (Kaufmann *et al.* 1980), and indeed this is so when a new NPN source is introduced into the diet (Nikolić *et al.* 1980). An example is when Biuret is introduced, for which adaptation times of approximately 2 weeks or sometimes much more have been reported (Fonnesbeck *et al.* 1975).

Milligan *et al.* (1972) found that adaptation, shown by increasing ammonia production, occurred when GU was fed for a period of approximately 7 d. The results of Demeyer & Van Nevel (1978) did not support this finding. These authors gave a sheep a diet containing a product prepared from molasses and urea for at least 1 month. Incubations of rumen contents from this animal with the preparation showed low accumulation of ammonia, compared with free urea. However, the authors themselves expressed doubt as to whether the product that they were studying in fact contained a ureide. The present studies confirmed and extended the observations of Milligan et al. (1972) and indicated that adaptation was complete by approximately 10 d. Cleavage of the sugar–urea bond (N-glycosidic link) in

LU also occurred only after a similar period of adaptation, although release of the galactose from LU occurred in the unadapted animal.

Attempts to isolate particular bacterial species responsible for such changes from rumen contents which had shown high in vitro LU-degradative activity (D. J. Jayne-Williams & R. J. Merry, unpublished results) were successful in respect of galactose liberation, but no organisms capable of cleaving the sugar-urea bond were found. This may have been because the organisms were sought in strained contents; subsequent work showed that much greater activity of this kind was associated with particulate material in the digesta.

It is of interest to note that during the feeding of either GU or LU, adaptation to both compounds occurs. This is not surprising as the sugar-urea bond is common to both compounds, and release of galactose from LU gives rise to GU. The rapid loss of degradative ability when ureide was withdrawn from a diet which had previously included the material was similar to that seen when the NPN source Biuret is removed from the diet of an adapted animal (Fonnesbeck *et al.* 1975). This finding shows the importance of maintaining a continuous dietary supply of ureide once an animal has been adapted, although it is possible that readaptation would be more rapid than initial adaptation, as was found for Biuret (Kondos, 1975).

It is not clear what mechanism is responsible for adaptation and de-adaptation to the cleavage of the sugar–urea bond in glycosyl ureides. For pure cultures of non-rumen bacteria it has been shown that an enzyme can be induced within a matter of hours in the presence of its specific substrate (e.g. lactose) and likewise lost after the removal of the substrate from the medium (Monod, 1947). Thus adaptation could be due to induction of an enzyme in a relatively abundant microbial species. Alternatively, variations in activity could occur as the result of proliferation or reduction in numbers of a minority population. At present there is no evidence to support either theory, although the latter seems more likely owing to the relatively extended period required for adaptation to occur.

Variations in in vitro rates of LU degradation between different experiments (e.g. Expts 2 and 3) may have been due in part to the fact that some cultures were not buffered. However, even when pH was maintained between 6·4 and 6·8, degradation rates observed with rumen contents from steers were much lower than from sheep, and varied to some extent from one animal to another. This could have been caused by non-representative sampling leading to varying amounts of particulate matter in the rumen contents. Steers tend to be less efficient than sheep in reducing the particle size of feedstuffs (Church, 1966) and stratification of rumen contents often occurs (i.e. a solid mat of plant material is lifted to the top of the rumen by trapped gas), making representative sampling of total rumen contents very difficult. Consequently, whole rumen contents from cattle are less homogeneous than those from sheep. As many microbes adhere to the solid material (Cheng *et al.* 1977) inadequate sampling of this fraction can lead to much reduced microbial fermentative activity; it was found that whole rumen contents from adapted sheep were much more active in degrading LU than were strained contents (Merry, 1980). Sampling problems in cattle may partly explain the slower rate of degradation of both LU and L+U found in vitro as opposed to those found in vivo with the same steers (Merry *et al.* 1982*b*).

In one experiment where a sheep was adapted to LU, added GU was rapidly degraded, whilst LU was only very slowly attacked. This is not easy to explain but may have been due to a more marked fall in pH with the fermentation of the extra molecule of sugar in lactose or possibly by the enzyme responsible for the liberation of galactose being adversely affected by lowered pH.

In general, however, despite variation between experiments in rates of degradation, which in any case seemed to affect both LU and L+U, it was clear that even after adaptation,

both the energetic and nitrogenous components of GU and LU were metabolized appreciably less rapidly than were sugars and urea in the free forms. The slower rate of degradation led to lower ammonia concentrations with LU than L+U which would imply a possible benefit in reduced chance of ammonia toxicity.

Although Walker & Lee (1961) incubated lactose with rumen contents from an unadapted sheep and noted a slow disappearance of the substrate, other early reports suggested that rumen bacteria do not readily ferment this sugar (Phillipson & McAnally, 1942; Hungate, 1966). These observations, however, were made either in experiments with pure cultures, or during incubation of lactose with strained rumen contents. It was apparent from our work using whole rumen contents that even when lactose was incubated with rumen contents from unadapted animals, its disappearance was fairly rapid. However, Walker & Lee (1961) also found an increase in the rate of lactose hydrolysis in contents from a sheep after adaptation to lactose had occurred. Adaptation, shown by an increase in lactose degradation rate, was a feature of the current experiments with steers given a diet supplemented with an LU-rich preparation from whey, containing appreciable amounts of free lactose and urea. It appeared that more than 7 d was needed for complete adaptation to occur. This adaptive response could be explained by an increase in the numbers of lactose-fermenting bacteria, similar to that found by Metzger *et al.* (1971) during the feeding of lactose-containing rations to cattle.

A similar type of adaptive response to urea degradation was noted with the same steers when given increased levels of urea in their diet. This was somewhat surprising, in view of the fact that these animals had previously been receiving urea, admittedly at a somewhat lower level, for at least 3 weeks. It has been suggested that ruminants respond to the continued feeding of urea with such effects as increased N retention, etc. and various theories have been put forward to explain this apparently adaptive phenomenon (Chalupa, 1968), including the possibility of suppression of protozoa (Smith, 1969). In the present experiments, however, protozoal numbers in the rumens of donor steers were very similar both before and after a period of adaptation (R. J. Merry, unpublished results). Preston (1972) intimated that adaptive responses to urea feeding are more likely to be related to adaptation to energetic components of the diet. It is thus possible that adaptation shown by increased rates of urea degradation could be linked in some way with changes in microbial population which occurred during the feeding of lactose.

In order to examine the effects of different forms of energy supply on microbial protein synthesis it was necessary to choose a method for assessing such synthesis. Walker & Nader (1968) used ^{35}S incorporation and Al-Rabbat *et al.* (1971) used ^{15}N for this purpose. These methods both suffer from the disadvantage that direct incorporation of amino acids can cause underestimation of growth. Methods using ^{32}P-labelled phosphate are not affected in this way. Incorporation of ^{32}P into total microbial P-containing compounds has been studied by Van Nevel & Demeyer (1977) as a means of measuring rumen microbial growth and Bucholtz & Bergen (1973) used incorporation of ^{32}P into microbial phospholipids for a similar purpose. Both procedures involve the use of values for P incorporated into a particular fraction of microbial matter: total microbial-N, to calculate N incorporation. The phospholipid-P: total-N value is very small and variable (Van Nevel & Demeyer, 1977) and as total bacterial-P may contain a variable proportion of phospholipid-P, interpretation of results can be difficult. On the other hand, bacterial nucleic acid-N: total N values are fairly constant (Smith, 1969) and, under the in vitro conditions studied in the present work, little variation in total N:NA-P was observed at different times during the incubation. It was considered, therefore, that incorporation of ^{32}P into bacterial nucleic acids would be less subject to variation than into total bacterial-P-containing compounds, as used by Van Nevel & Demeyer (1977).

On theoretical grounds it appears that the rate of release of energy from the carbohydrate

fraction of a diet is critical for determining microbial growth efficiency (Bartley & Deyoe, 1977; Smith, 1979). If energy is released too slowly, as from cellulose, bacterial growth will be slow, and maintenance requirements high (Stouthamer & Bettenhaussen, 1973). On the other hand, very rapid release of energy, as may occur when soluble sugars form the substrates, could lead to fermentation being uncoupled from the growth process, resulting in ATP being dispersed in ways other than for growth (Forrest & Walker, 1971). Excess ATP thus formed may be dissipated as heat after the action of ATPases (Lazdunski & Belaich, 1972). Inefficient use of ATP may partly explain why soluble sugars, although readily available, have been shown to be less effective than starch as dietary energy sources (Al-Attar *et al.* 1976; Oldham *et al.* 1977). Starch, particularly that which has been heated, is generally recognized as being the most suitable source of energy to support microbial growth (Smith, 1979).

Such arguments may apply to the soluble sugar lactose and there is an indication in the present work that formation of LU changes the pattern of energy release to make it more like that of heat-treated starch. The bacterial growth pattern when heat-treated starch or LU were the substrates were certainly very similar. These results are in sharp contrast to observations made with free lactose and urea where growth was extremely rapid during the initial stages of incubation when most of the sugar was being fermented, but slowed dramatically when supplies were exhausted. With LU, a steadier, more sustained pattern of growth was generaly seen which resulted in more efficient use of the energy derived from the slower fermentation of the bound sugar. The results support the view that rapid fermentation of soluble sugar is relatively inefficient in terms of use for supporting bacterial growth. The findings may have been in part related to the production of lactic acid when free lactose formed the substrate. This type of fermentation has previously been shown to coincide with lowered microbial growth effciencies (Van Nevel & Demeyer, 1977). However, lactic acid did not accumulate to any appreciable extent during lactose fermentation in the one experiment in which it was measured.

It seems more likely that adverse effects of very rapid metabolism of soluble sugars and the need to dispose of excess ATP may be partly offset by the use of some of this excess to synthesize storage polysaccharide (Walker, 1968; Thompson & Hobson, 1971). In the present experiments some apparent growth efficiencies in terms of sugar disappearance were abnormally high which illustrates clearly how in the situation of soluble sugars it is difficult to relate growth directly to exogenous energy supply. It is probable that bacteria made use of storage polysaccharide for growth during the later stages of incubation, particularly with soluble sugar, as marked growth often occurred when added sugar supply had already been exhausted. The conclusion was supported by observations made in one experiment of the marked accumulation and later disappearance of storage α-dextran, particularly when L+U were the substrates (R. J. Merry & R. H. Smith, unpublished results). Nevertheless, even with the buffering effect provided by accumulation and subsequent use of storage polysaccharide, it seems likely that the explanation for the relatively poor efficiency of use of free compared to bound sugar may lie in uncoupling of growth processes from fermentation.

The present in vitro studies clearly demonstrate that ureide formation from soluble sugars is potentially beneficial both in terms of reduction of toxicity of the NPN source and its efficiency of use for rumen microbial synthesis. Nevertheless, in vitro results may only act as a guide to processes occurring in vivo and although a reduction in the degradation rate of lactose by ureide formation may be potentially beneficial on efficiency of microbial synthesis, the value of a ureide-containing product could be reduced if slower degradation led to losses of undegraded bound urea and sugar occurring with the digesta flow. In vivo studies form the subject of a future publication.

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