basal diet alone, the results do not support the view that urea is converted to protein in the rumen during the first 8 hr. following a meal.

The evidence upon which Wegner *et al.* [1941] appear to place most confidence is that on the basal diet alone the crude protein content of the rumen ingesta varied from 8·9 to 10·6 %, whereas with the addition of urea to the diet this value became much higher and varied between 12·0 and 12·7 %. In all probability, however, these values might be due to the fact that when 100 g. urea were added to the diet which supplied only 5 or 6 lb. dry matter per day, of which 3 lb. was starch, water consumption and also salivation would be much increased. This would cause the starch and the more soluble matter in the corn silage to be washed from the rumen much more rapidly than in the absence of urea. The proportion, in the dry matter of the rumen ingesta, of fibre and of the less soluble protein enmeshed in it would thus be increased.

These experiments of Wegner *et al.* [1941] therefore confirm the views held by the present authors that *in vivo* experiments of this type cannot be expected to yield any certain evidence of protein synthesis from urea until truly representative samples of the total ingesta can be obtained and analysed, and until more is known of the effect of urea on the passage of the various dietary constituents through the rumen.

SUMMARY

1. The relative merits of trichloroacetic acid, sodium tungstate with H_2SO_4, and alcohol have been compared as precipitants in the estimation of N.P.N. in rumen ingesta.

2. A description is given of the methods finally adopted for the estimation of N.P.N., urea and NH_3.

3. The heterogeneous nature of the rumen contents is described and illustrated by analyses.

4. The difficulties involved in the interpretation of the results obtained by *in vivo* experiments are discussed.

5. Owing to these difficulties, the results of *in vivo* experiments of this type cannot be regarded as supplying evidence either for or against the theory that urea is converted to protein in the rumen.

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The Utilization of Urea in the Bovine Rumen. 2. The Conversion of Urea to Ammonia

By R. M. PEARSON and J. A. B. SMITH, *From the Hannah Dairy Research Institute, Kirkhill, Ayr*

(*Received 14 October 1942*)

In the previous paper of this series [Pearson & Smith, 1943] evidence was advanced to show that the interpretation of experiments carried out *in vivo* on the utilization of N.P.N. by ruminants was so difficult as to render the work of little value and that more reliable results could probably be obtained by *in vitro* methods. A plan of investigation was therefore prepared in which samples of rumen ingesta were to be incubated in the laboratory with and without urea, and the N partition studied at intervals throughout the incubation period. At the outset of the work it appeared that even when 40 g. urea were fed per day to a steer with a rumen fistula, no urea could be detected in the rumen ingesta. In preliminary incubation experiments it was also found that 100 g. rumen contents were able to convert some 100 mg. urea to NH_3 in 1 hr. It is probable therefore that any utilization of urea by ruminants involves the conversion of urea to NH_3 as the first stage of the process. A detailed study of this conversion has therefore been made, with particular reference to the effect upon it of various factors such as temperature, *p*H, concentration of urea, nature of the gases present during incubation and certain inhibitory substances. The present paper consists of a brief description of these experiments.

METHODS

Samples of rumen contents were obtained as follows. 2–3 l. semi-solid rumen contents were transferred with an aluminium cup by way of the fistula to a large flask contained in a bucket of sawdust to minimize heat losses. The sample was strained through muslin and the residue pressed by hand. The 'filtrate' so obtained was rich in Protozoa and bacteria and was very similar to the rumen contents as a whole except that the small amount of solid vegetable matter which it contained was in a more finely divided form. The greater part of the solid ingesta of the rumen and reticulum is impregnated with this liquid which, in converting urea to NH_3 and NH_3 to protein, probably behaves very similarly to the more solid ingesta. Since no sampling difficulties arise with this liquid it was used throughout the present experiments and is referred to as the 'rumen liquor'.

Incubation of the rumen liquor was carried out in a water-bath thermostatically controlled at 39°, the body temperature of the bovine. Throughout the greater part of the experimental period the diet of the steer from which the samples were obtained consisted of 14 lb. hay, $2\frac{3}{4}$ lb. oats, 2 lb. bran, 1 lb. starch, 0·7 lb. molasses and 40 g. urea per day with water *ad lib.* The methods of analysis have already been described [Pearson & Smith, 1943].

RESULTS

Influence of CO_2 and N_2 on incubation. One of the objects of the present experiments was to imitate as far as possible in the laboratory the conditions in the intact rumen. In the rumen there are large amounts of carbohydrates such as cellulose, starch, and their breakdown products, and the gas formed in the decomposition of some of these substances usually contains about 62 % CO_2. A few experiments were therefore made to discover whether the nature of the gas and the presence or absence of starch affected the urea-splitting power of the rumen contents. Three 650 ml. aliquots from the same sample were incubated with 100 ml. of a solution containing 1 g. urea, 4 g. K_2HPO_4 and 10 g. glucose. A constant stream of CO_2 was passed through the 1st flask, air through the 2nd and N_2 through the 3rd. Urea was estimated every 10 min. for 2 hr. The results illustrated in Fig. 1 show that the rate of urea conversion was much the same with all three gases. It appeared, however, in the final stages to be slightly more efficient with CO_2 than with air or N_2.

In a 2nd experiment of this type with a much less active rumen liquor, aliquots of the same sample were incubated with air and CO_2, both with and without starch. KH_2PO_4 was present as a buffer but glucose was not included. The results shown in Fig. 2 suggest that CO_2 in the presence of starch accelerates the rate of conversion of urea slightly; otherwise no very significant differences were observed.

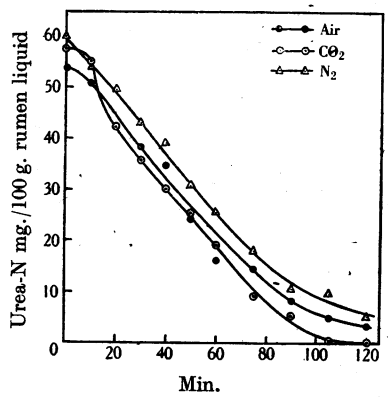

Fig. 1. The conversion of urea in the presence of different gases.

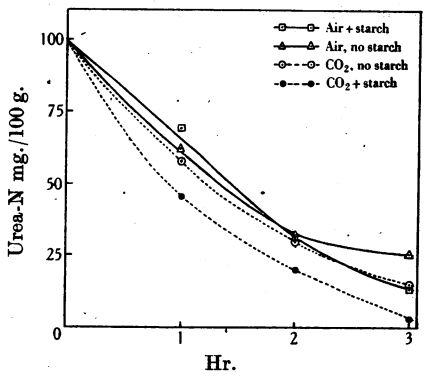

Fig. 2. The effect of CO_2 and starch on the rate of urea conversion.

The effect of starch and phosphate was also tested by incubating them with two different samples of rumen contents in air under strictly comparable conditions. The results are recorded in Table 1.

Table 1. *Ammonia-N produced from urea in 1 hr. at 39°*

(mg./100 g.)

Substances added	Sample 1	Sample 2
Urea alone	27·1	36·7
Starch + urea	31·2	36·5
Starch + urea + phosphate	21·5	30·1
Phosphate + urea	—	30·1

Starch appeared to have a slight accelerating action on the rate of urea conversion with the 1st sample but no effect whatever with the 2nd, which

was the more active of the two. Phosphate obviously had a retarding effect with both samples.

Influence of temperature. 5 ml. of a solution containing 3 % KH_2PO_4 and 3 % starch were placed in each of ten 200 ml. flasks which were kept in water baths at temperatures ranging from 4 to 89°.

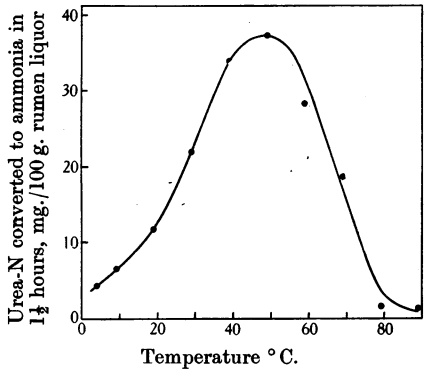

Fig. 3. The effect of temperature on the rate of conversion of urea to ammonia by the rumen liquor.

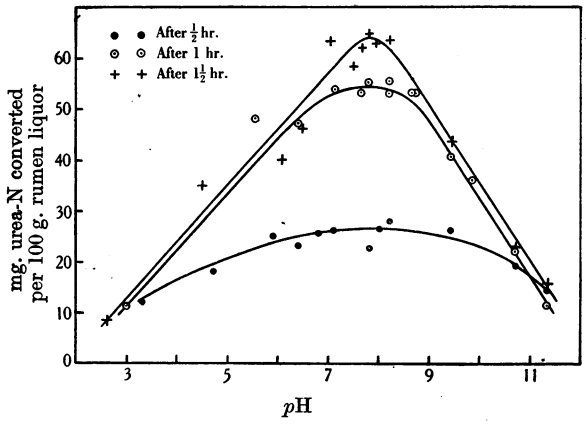

Fig. 4. The effect of *p*H on the urease activity of the rumen contents.

A 50 ml. aliquot from one sample of rumen contents was added to each flask and the contents of the flask were allowed 10 min. in which to reach the temperature of the surrounding bath. Exactly 5 ml. of a solution containing 1 g. urea-N/100 ml. were then added to each flask, and the mixture was incubated for 2 hr. At 10-min. intervals a slow stream of CO_2 was passed through the contents in each flask. At the end of the incubation, urea and NH_3 were estimated. KH_2PO_4 was added in this instance to minimize any loss of NH_3 which might occur at the higher temperatures. The results (Fig. 3) show that urea conversion is insignificant at 4°, but that it increases with increase in temperature and reaches a maximum at 49°. Thereafter it steadily decreases till at 79° there is almost complete inactivity. In two other incubations lasting 1½ and 2 hr. respectively with entirely different samples of rumen liquor, in which no starch was present and through which no CO_2 was passed, the optimum temperature was again 49°.

Influence of p*H*. The technique was similar to that used in the above experiments but the temperature remained constant at 39° while the *p*H in the various flasks ranged from 3 to 11. Three series of incubations were carried out, one for 30 min., another for 1 hr. and a third for 1½ hr. It is probable that during the longer incubations, the salts in the buffer used to obtain a certain *p*H had a more marked effect in retarding urea conversion than the *p*H itself. This may be the cause of anomalous results (see Fig. 4), particularly on the acid side of neutrality. The urease activity of the rumen liquor appears to be very marked over quite a large *p*H range. The optimum probably lies between 7 and 9 and there is little activity below 3 or above 9·5.

The *p*H of the material in the rumen probably varies slightly with a number of factors, but it is rarely below 6·5, and only after a long fast does it approach 7·4. Acid products formed in the rumen are partially neutralized by the incoming saliva, but the buffering capacity of the rumen liquor itself is very high, particularly in view of the fact that its total content of solids is only 2–3 %. This is illustrated by the fact that when 5 ml. of approximately *N* HCl were added to 50 ml. rumen liquor the *p*H was 4·7, whereas when 50 ml. of water were used instead of rumen liquor the corresponding value was 1·6.

Influence of urea concentration. In some enzymic reactions the concentration of the substrate has a marked effect on the enzyme activity. To ascertain whether the concentration of urea influenced the urea-converting activity of rumen liquor, various aliquots of the same sample of ingesta were incubated for 1 hr. with different amounts of urea and the liberated NH_3 was estimated. The NH_3 in the tungstate filtrate was not determined by distillation in steam, for with higher concentrations of urea small but significant amounts were converted to NH_3 during the actual

Table 2. *The amount of urea converted to ammonia in varying concentrations of urea*

	(mg. N/100 g.)				
Urea-N in the rumen liquor	30·8	50·0	116	216	414
NH_3-N produced in 1 hr.	17·9	19·2	20·1	20·6	22·2
Urea-N in the rumen liquor	808	1302	1646	3330	
NH_3-N produced in 1 hr.	24·8	25·1	25·4	25·1	

distillation, however accurately the filtrate was neutralized. Prolonged aeration at room temperature in the presence of K_2CO_3 was therefore adopted. The results recorded in Table 2 show that there was a slight increase in the conversion of urea with increased substrate concentration. The effect, however, was not great.

The influence of time of sampling. When urea-feeding is adopted the question arises whether it should be given twice a day with the other concentrates or ingested at more frequent intervals. The following experiments were therefore made to see whether the urea-converting activity of the rumen liquor varied throughout the day.

A sample of rumen liquor removed 1 hr. after a meal was found to convert urea to NH_3 at the rate of 13·4 mg. N/100 g. rumen liquor in 20 min. After a 16 hr. fast the corresponding value was 14·8 mg. There was therefore no significant difference. In a 2nd test a sample taken 17 hr. after the previous meal converted 27·6 mg. urea-N to NH_3 in 1 hr. A meal was then given and samples taken at 2-hourly intervals for 8 hr. During this time the conversion rate remained unchanged at 23·4–23·8 mg. N/100 g. In a 3rd test the value 17 hr. after a meal was 46·3 mg. urea-N/100 g./hr. Samples taken during the next meal and subsequently at hourly intervals gave conversion rates which varied irregularly between 35·4 and 52·3. If the weight of the rumen contents be estimated at 75 kg. these results mean that 40–80 g. of urea could be converted to NH_3 in the rumen in 1 hr. It therefore follows that during urea-feeding, when as much as 100 or 120 g. urea may be ingested in one day, it matters little so far as the urea conversion to NH_3 is concerned whether this be given twice a day in two equal amounts with other concentrates or at more frequent intervals.

Rumen urease

Since the constituents of the diet of the steer during these experiments showed only insignificant amounts of urease activity, and since there is no active enzyme secretion into the rumen except the saliva, the rumen urease must be generated by some of the micro-organisms present in the paunch. From the amounts of urea which can be converted by the rumen liquor in 1 hr. or even in a few minutes at any time throughout the day, it may be assumed that the enzyme is generated in large amounts compared with the rate of growth of the bacteria producing it. Preliminary attempts were made to prepare a sterile but active filtrate of rumen liquor. The filtrate from Berkfeld filters was sterile but without urease activity. With urease extract from jack bean meal also Berkfeld filtration removed the greater part of the urease activity. Since the molecular weight of crystalline urease from soya beans has been found by Sumner, Gralen & Eriksson-Quensel [1938] to be 483,000 it is not surprising that in the present work no filter has been found which will allow the enzyme to pass without the bacteria.

An attempt was made to concentrate the urease activity, and to effect at least a partial separation of the enzyme from the bacteria, by centrifuging. 2 l. of rumen contents were centrifuged for 5 min. at 1000 r.p.m. and 200 ml. of the top portions of the supernatant liquid collected. 200 ml. of the sludge at the bottom of the centrifuge cups, which was extremely rich in Protozoa and bacteria, were also collected. The top layers, the sludge and also some of the original rumen contents which had been centrifuged and then remixed, were each incubated with about 260 mg. urea, and samples were analysed every 30 min. for 2½ hr. The results shown in Fig. 5

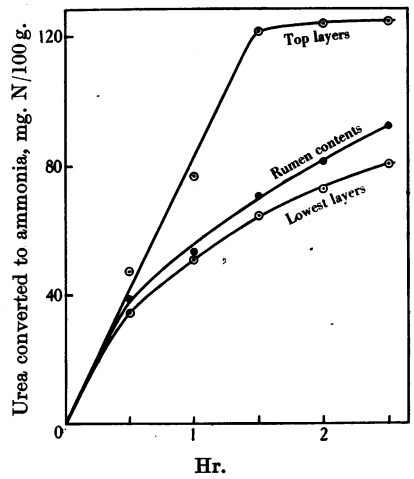

Fig. 5. The rate of urea conversion in the rumen liquor compared with that in the top and bottom layers of the centrifuged rumen liquor.

suggest that for the first ½ hr. or, until some 40 mg. urea-N had been converted to NH_3, the rate of conversion in the lower layers was slightly less than in the rumen liquor, while in the top layers it was slightly greater. At the end of 1 and 1½ hr. the increased rate in the top layers was still more apparent compared with that in the other two. In other experiments in which very much less urea-N was present, and in which conversion was complete in 30–60 min., this difference was by no means clear. The less rapid rate in the rumen liquor and lower layers in the first experiment may have been due less to a smaller content of urease than to a greater concentration of inhibitory substances capable of reducing their urease activity, particularly after the first ½ hr. The results shown in Fig. 5 therefore need not indicate a concentration of urease in the upper layers on centrifuging.

Toluene, which is known sometimes to destroy bacteria but to permit enzyme action, was found to be without effect on the urease activity of the rumen liquor, but here it is not certain whether the urea-converting bacteria were completely destroyed by that treatment. Reference will be made to this in a subsequent paper. 30 % alcohol extracts of the urease were inactive.

Urease activity was also inhibited by small quantities of substances such as quinone and NaF. Quastel [1933] has shown that the activity of ordinary urease preparations is readily inhibited by quinone, and that the inhibition can be prevented by thiol compounds such as H_2S and cysteine. Samples of rumen liquor containing urea were therefore incubated for 1 hr. with varying amounts of quinone and with quinone in the presence of H_2S and cysteine (Tables 3 and 4). With one sample of

Table 3. *The inhibitory effect of quinone on rumen urease and its neutralization by H_2S*

Urea-N converted to NH_3 (mg.)

Quinone %	Sample 1		Sample 2	
	Quinone	Quinone + H_2S	Quinone	Quinone + H_2S
0·0	71·1	71·4	61·2	58·6
0·005	26·8	69·3	13·1	50·9
0·0075	14·2	69·7	2·4	49·9
0·01	13·2	68·2	1·0	33·5
0·03	5·0	10·4	—	—
0·05	3·0	4·3	—	—

Table 4. *The influence of cysteine on the urease-inhibiting action of quinone*

0·01 % quinone + cysteine (%)	0·0	0·005	0·01	0·03
Urea-N converted to NH_3 in 1 hr. (mg.)	1·6	25·2	64·4	67·5
0·01 % quinone + cysteine (%)	0·05	0·07	0·10	
Urea-N converted to NH_3 in 1 hr. (mg.)	64·4	66·1	62·6	

0·05 % cysteine in the absence of quinone gave a value of 63·5.

rumen liquor concentrations of quinone as low as 5 in 100,000 substantially reduced the urease activity. At higher concentrations the degree of inhibition increased and ultimately approached completion at 3 parts in 10,000. With sample 1 H_2S neutralized the inhibitory action of quinone up to concentrations of 0·01 %. With sample 2 the neutralization of inhibition was marked but not quite complete. Cysteine at concentrations of 0·01 % or above also completely neutralized the inhibitory action of 0·01 % quinone (Table 4). Cysteine itself was found to have a very slight inhibitory effect. Thus 0·05 % cysteine in rumen liquor reduced the rate of urea conversion from 47 to 43 mg. N/100 g. rumen liquor/hr. These results for urease activity of rumen liquor are similar to those of Quastel [1933] for urease from jack bean meal, and show that the enzymes from these two very different sources have much in common and may be identical.

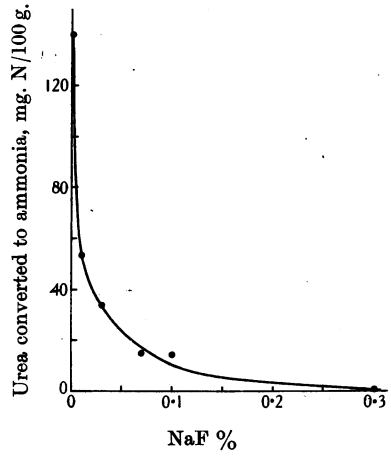

Fig. 6. The inhibition of urease activity in the rumen liquor by sodium fluoride.

Mystkowski [1928] has shown that NaF is a powerful inhibitor of ordinary urease. This was found to be true also for the urease of the rumen. Aliquots of a sample of rumen liquor containing urea were incubated for 2 hr. with various concentrations of NaF. At a concentration of 0·01 % the activity was reduced by about 50 % and at 0·3 % inhibition was complete (Fig. 6). This effect is due entirely to the fluoride radical, for in a control experiment with 0·5 % NaCl there was no inhibition. A similar experiment was carried out with boric acid and borax (Table 5). 0·05 % borax had

Table 5. *The inhibition of urease activity by boric acid and borax. Urea conversion to NH_3*

(mg. N/100 g.)

Boric acid or borax %	Boric acid		Borax	
	30 min.	60 min.	30 min.	60 min.
None	28·5	46·8	28·5	46·8
0·05	21·7	40·6	28·3	48·2
0·10	19·5	36·5	24·3	43·3
0·50	11·7	22·3	13·0	25·0

With further samples of rumen liquor 1 and 2 % boric acid and borax completely inhibited the urease activity.

no inhibitory effect and may even have stimulated the urease action slightly. Above this concentration, however, the amount of urea conversion was dimin-

ished significantly. At the lower concentrations inhibition with boric acid was greater than with borax.

SUMMARY

1. In the utilization of urea by dairy cattle the first stage is probably the conversion of urea to NH_3 in the rumen. Moreover, the urease activity of the rumen ingesta is so great at all times of the day, whatever the time of feeding, and remains so little affected by relatively large amounts of urea, that all the urea ever likely to be fed, even to a high yielding cow in full lactation, would readily be converted to NH_3 within 1 hr. This fact has been confirmed by Wegner, Booth, Bohstedt & Hart [1941], who found a marked increase in N.P.N. and NH_3 in the rumen after a urea meal but were unable to detect urea itself.

2. The urease of the rumen resembles, in activity, that from soya and jack beans. Changes in its activity with changes in temperature and *p*H, and its behaviour in the presence of such inhibitors as quinone and NaF, are typical of enzymes of the urease type. Preliminary attempts to obtain enzyme preparations free from bacteria have, however, proved unsuccessful.

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The Utilization of Urea in the Bovine Rumen. 3. The Synthesis and Breakdown of Protein in Rumen Ingesta

By R. M. PEARSON and J. A. B. SMITH, *From the Hannah Dairy Research Institute, Kirkhill, Ayr*

(*Received 14 October 1942*)

The urease activity of bovine rumen ingesta has been studied in detail by Pearson & Smith [1943*b*] who showed that the urease activity of the rumen is sufficient to convert rapidly to NH_3 all the urea ever likely to be included in the diet as a partial substitute for protein. The utilization of urea by the ruminant probably takes place in two stages, first the conversion of urea to NH_3 and second the conversion of NH_3 to protein. The present section of the work was undertaken to investigate this 2nd stage. Incubations of rumen contents were to be carried out *in vitro* under a variety of different conditions with and without urea, to obtain as much evidence as possible by chemical analysis either for or against the theory of protein synthesis in the rumen from non-protein N. Samples were to be taken for direct microbiological examination by Mr Frank Baker [Baker, 1942 *a*, *b*; 1943] and for plate counts by Dr C. Higginbottom to discover whether the protein synthesis, if it occurred, were accompanied by an increase in the numbers of micro-organisms present.

First, it had to be established by chemical means whether any protein synthesis could be detected, and if so, to what extent it was affected by the presence of ordinary dietary constituents such as carbohydrates, proteins and amino-acids, as well as by substances which might be of a toxic nature. The present paper is confined to a description of this particular aspect of the work.

METHODS

The method of obtaining samples of rumen liquor and the incubation and analytical procedures have already been described [Pearson & Smith, 1943 *a*, *b*].

Long-period incubations. It seemed possible that if any measurable change in N partition took place on incubating rumen contents *in vitro*, the changes would be more readily detected in fairly long incubations. Hence preliminary incubations with rumen contents were carried out, each lasting several days (Fig. 1). 900 ml. rumen liquor were incubated with 2·5 g. urea, 12 g. K_2HPO_4, 10 g. glucose and 0·1 g. $FeSO_4$ for 8 days. Note that while the total N remained constant throughout, the N.P.N. varied considerably. For the first 3–4 days it changed very slightly, but between the 3rd and 7th days it greatly decreased. Assuming that the difference between total N and N.P.N. is protein N, an assumption which is discussed later, Fig. 1 shows that the amount of protein in the medium changed little at first, but that during the last 5 days it increased