ACTA CHEMICA SCANDINAVICA 13 (1959) 489—496

Ammonium Salts as Nitrogen Source in the Synthesis of Protein by the Ruminant

HARING LAND and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

Two cows have been fed on a diet containing an ammonium salt labelled with ^{15}N. Milk protein has been shown to contain this heavy isotope to an extent corresponding to incorporation of 17—25 % of the ammonium nitrogen into proteins. It is made plausible that the maximum incorporation into both milk and body proteins may be some 40 %. The milk collected about 14 hours after administration of the labelled salt shows the highest content of ^{15}N in the amides, followed by glutamic acid, aspartic acid and alanine. After 24 hours the labelling of most of the protein components has already become comparatively even. Histidine shows an especially low content of ^{15}N; this may be attributed to incapability of the rumen bacteria to synthesize the imidazole ring. Cystine, too, is relatively low in ^{15}N content.

An extensive literature has been published in the last three decades concerning the synthesis of protein by the ruminants, the role displayed in this synthesis by the rumen bacteria and the significance of non-protein nitrogen compounds as substitutes for proteins in the diet of the ruminants. A survey of this literature has been given by Chalmers and Synge [1]. The results of the experimental work show considerable divergence among the various authors as to the extent to which non-protein nitrogen, in the form of urea (in most of the cases), ammonium salts, ammoniated molasses, ammoniated straw, ammoniated sugarbeet pulp, dicyanamide, is effectively used in the process of protein synthesis. This difference of opinion arises mainly from the difficulty, inherent to all experimental work on nutrition problems, to start from welldefined uniform basic diets, and from the lack of a common standard of comparison. Little doubt, however, exists about the reality of the utilization of non-protein nitrogen by ruminants. A vast number of indirect experiments has been performed in which the extent of the utilization of the non-protein compound fed is inferred from either protein synthesis in an artificial rumen [2,3] or from changes in body weight or milk production of the animal under observation. Direct results, however, are rather rarely met in literature. To the

authors' knowledge the only direct evidence of protein synthesis from non-protein nitrogen by ruminants is presented in a paper by Watson *et al.*[4] They fed urea labelled with ^{15}N to sheep and were able to detect this isotope in proteins from blood, liver and kidneys in excess over the ^{15}N content of proteins of sheep fed with unlabelled urea.

The present paper gives the results of an investigation based on feeding experiments in which ammonium salt labelled with ^{15}N was fed to two cows. The experiments were conducted on the farm of one of the authors (A.I.V.). The cows received AIV-fodder, coarsely ground oats and some hay. According to the principle of the AIV preservation method the acidity of the fresh fodder is increased so that the pH of the ensilaged material does not exceed the critical value 4. In the preparation of the AIV-fodder used in these experiments, instead of strong mineral acids 1 kg of ammonium bisulphate had been added per 100 kg of fresh greenfodder in order to reach this acidity; the pH of the finished product was 3.8—3.9. The preserved material consisted of a mixture of about 70 % of red clover and 30 % of timothy, blown into the silo by means of a fan equipped with cutting blades.

When the AIV-method was developed in the years before 1930, some attempts were already made to reach the necessary acidity in the fodder mass by means of acid salts, but the quantity of salt required for non-chopped clover-rich fodder was so high (1.8—2.0 kg per 100 kg of fresh fodder) that the health of the cattle might be impaired. The amount of acid to be added decreased, however, when choppers came into use. In recent years (1953—1958) new series of preservation trials have been conducted in this laboratory by Virtanen and Kreula [5] and their results indicate that application of ammonium bisulphate or of mixtures of ammonium and sodium bisulphate is entirely feasible also in the preservation of clover. For fodderplants poorer in protein much smaller quantities of these salts suffice. Therewith the question of the utilization of ammonium nitrogen in the synthesis of amino acids and proteins in the rumen has become actual also when an effective method for preservation of fresh fodder precluding the formation of ammonia from the fodder proteins, is used.

The purpose of the present investigation was to establish the value of ammonium salts in the synthesis of proteins in the ruminant. Apart from this directly important information, knowledge of more details of the actual synthetic process might be obtained by tracing the heavy isotope in the various fractions and different amino acids of the milk proteins.

EXPERIMENTAL

Two experiments have been performed so far. During the first one, started on March 21 at approximately 4.30 P.M., the test cow Latsi with a production of about 10 kg of milk (4.3 % fat) per day was fed on a diet administered in two portions (in the morning and in the evening) each consisting of 3.4 kg of coarsely ground oats, 9.2 kg of AIV-fodder and 1 kg of hay. This corresponds to 10 Scandinavian fodder units and 1 040 g of digestible raw protein (NH_4-N subtracted) per day. The overfeeding both in fodder units and in protein was fairly high, because the food requirement corresponding to the milk production was about 8 fodder units and 850 g of digestible protein. This diet was given for 2 1/2 days before the beginning of the experiment proper; then 0.9993 g of ammonium nitrate containing 61 atom % of ^{15}N in the ammonium part was added as a fairly concentrated solution in a small volume of water to the oats. For another 5 days after the addition of the labelled ammonium salt the cow received the same diet as mentioned, and after this period returned to her normal diet. Analytical details of the diet are given in Table 1.

Table 1. Analyses of fodders used in the experiments.

	% total N	% dry matter	% NH_4—N	kg fed	g total N	g NH_4—N
	1.54	87.5	0.020			
Oats	1.53	87.6	0.020	3.4	52	0.7
	1.53	87.6	0.019			
	0.70	25.5	0.131			
AIV-fodder	0.63	26.1	0.135	9.2	60	12.1
	0.61	25.4	0.131			
	1.10	91.1	0.042			
Hay	1.22	90.9	0.044	1.0	12	0.4
	—	91.6	0.043			

According to Table 1 the NH_4-N was 10.7 % of the total N. 0.9993 g of NH_4NO_3 with 61 atom % ^{15}N in the ammonium group contains 0.181 g of NH_4-N, that is, 1.37 % of the total NH_4-N of the fodders. The amount of excess ^{15}N in it is 7.52 mg-atoms.

In the second experiment beginning on May 14 at about 4.00 P.M., another cow (Hieno) with a daily production of about 16 kg of milk (4.2 % fat) was fed on substantially the same diet; this could provide her with the required food units (10 f.u.) per day. The digestible protein in the diet was already somewhat lower than the need (1 040 g and 1 100 g, respectively). After the same preparatory feeding period as in the first experiment, 1.8953 g of ammonium sulphate containing 90.1 atom % ^{15}N was added to the food, *i.e.* 0.443 g of NH_4-N which amounts to about 3.5 % of the total NH_4-N of the diet. The excess ^{15}N administered in this case is 25.42 mg-atoms. After 5 days on the diet as mentioned before, the cow received again the customary rations, and on May 23 was brought out to pasture.

Table 4. Excess ^{15}N in milk protein and in its amino acids in the second experiment (cow Hieno).

Milk a: collected 15.5.58, at 7.10 A.M.
b: » 15.5.58, » 5.15 P.M.

Protein precipitated with 15 % trichloroacetic acid; precipitate washed with water, dialyzed; lyophilized; extracted with ether; hydrolyzed with 6 N HCl.

Substance	At % excess ^{15}N in: (a)	(b)
Total protein	0.039	0.032
Amide-N	0.071	0.039
Glu	0.054	0.030
Asp	0.047	0.037
Tyr	0.042	0.036
Ala	0.047	0.038
Ser + Thr	0.038	0.031
Gly	0.044	0.033
Val	0.032	0.032
Pro	0.026	0.023
Lys	0.029	0.031
Met + Leu + Ileu	0.033	0.029
His	0.008	0.009
Phe	0.027	0.027
Arg	0.022	0.022
Cys	—	0.018

The cows were milked twice daily. In the first experiment the milk was weighed for 12 days, in the second for 20 days. Urine and feces were not collected quantitatively. From samples of milk the ^{15}N content was determined in the total N, in the protein and the non-protein fractions separated according to Rowland [6], and in the amide-N fraction split off from the precipitated protein fraction [7]. The total-N content of the milk of the first experiment, estimated on a number of samples from various days, had an average value of 0.468 %, and a non-protein N content of 0.035 %. In addition, the ^{15}N content was estimated in samples of urine and feces, and in a few cases in the urine ammonia distilled according to Vickery *et al.* [7]; no analyses were performed on urine and feces to assess the total amount of nitrogen excreted.

From samples of milk, urine and feces collected during the second experiment similar estimations were made; the total-N content of the milk was here 0.430 % (average of 8 determinations); the non-protein N content was not determined. The amide-N content was estimated according to Vickery [7] in the protein of the milk collected on May 15 at 7.10 A.M. and at 5.15 P.M.; it amounted to 8.9 % of the total protein-N content in both cases.

From these same milk samples the protein fractions obtained by precipitation with 15 % trichloroacetic acid, were dialyzed and lyophilized. After extraction with ether to remove fatty substances the dry protein was hydrolyzed with 6 N HCl in a sealed tube at 105° for 24 h, and the hydrolysate was separated into various amino acid fractions according to Hirs, Moore and Stein [8]; their procedure was in so far modified that it permitted the separation of rather large quantities of the amino acids. Details of the separation have been published elsewhere [9]. A rigorous separation of all amino acids was not aimed at; hence methionine, *iso*leucine and leucine have been taken together, so have serine and threonine. The yield of cystine from the first protein sample was too low for further treatment. Phenylalanine and arginine, emerging together from the ion exchange column, have been separated on a cellulose column in order to keep a distinction between basic, acidic and neutral amino acids.

The various fractions of the purified amino acids were subjected to Kjeldahl digestion and the ^{15}N content was estimated in the ammonia obtained. The measurements were performed with a CEC mass spectrometer model 21−401, at an ionizing current of 90 μA and an auxiliary amplifier output of 20 V. Corrections for the presence of air have not been applied, since in no nitrogen sample the quantity of air present as indicated by the argon peak, exceeded 0.1 %. Measurements were performed on either three or four samples, each sample allowing at least four readings; the cystine sample from the second protein fraction only allowed four measurements altogether to be made. The standard deviation did not exceed 0.1 % in the most unfavourable case and was usually of the order of 0.05 %.

The results of the measurements are given in Tables 2, 3 and 4.

In Tables 2 and 3 the time given in column 2 is the time of milking; the samples of urine and feces mentioned behind the same time, were collected within one hour after that time.

DISCUSSION

From Tables 2 and 3 the high rate of uptake of ammonia into the milk becomes evident: within one hour the milk is labelled, although in the first experiment the protein fraction does not yet contain any ^{15}N, and in the second experiment the labelling of the proteins is very weak. The non-protein fraction has not been subjected to a closer investigation whether the ^{15}N occurs in the form of the ammonium ion or as amide or low-molecular peptide. Analysis of the milk collected after about 14 h after the feeding of the labelled fodder shows a high content of ^{15}N in the amide-N (0.071 atom % excess in the second experiment), whereas the total-protein N contains 0.039 atom %. Since amide-N makes up 8.9 % of the protein-N, its ^{15}N content accounts for only 15 % of the total amount of ^{15}N present in the total N. This percentage

Table 2. First experiment started on March 21 at approximately 4.30 P.M. (cow Latsi). Administered 7.52 mg-atom excess of ^{15}N as NH_4NO_3 in the NH_4; total quantity of NH_4-N in diet 13.4 g. Total N in milk: 0.468 %; non-protein N: 0.035 %.

Date	Time	kg milk	tot.N in milk (mg-at)	At% excess ^{15}N in milk			urine		feces	Excess ^{15}N in milk (mg-at)	% ^{15}N recovered from milk
				tot.N	prot.N	N.P.N	tot.N	NH_4-N			
21.3	5.30 P.M.	—	—	0.004	0.000	0.036	—	—	—	—	—
22.3	7.00 A.M.	5.0	1670	0.012	0.012	0.023	0.019	0.044	0.021	0.200	2.66
22.3	5.30 P.M.	4.2	1400	0.011	0.010	0.016	0.016	0.031	0.018	0.154	2.05
23.3	7.00 A.M.	5.4	1810	0.008	0.009	0.012	0.011	0.020	0.013	0.145	1.93
23.3	5.30 P.M.	4.4	1470	0.007	0.007	0.010	0.005	—	0.009	0.103	1.37
24.3	7.00 A.M.	5.9	1970	0.006	0.006	0.008	0.003	0.021	0.008	0.118	1.57
24.3	6.15 P.M.	4.4	1470	0.005	0.005	0.007	—	—	0.004	0.074	0.99
25.3	6.50 A.M.	5.4	1810	0.003	—	—	0.001	—	0.004	0.054	0.72
25.3	6.00 P.M.	4.6	1540	0.001	—	—	—	—	—	0.015	0.20
26.3	7.00 A.M.	4.3	1440	0.002	—	—	0.001	—	—	0.029	0.39
26.3	6.00 P.M.	4.3	1440	0.002	—	—	—	—	—	0.029	0.39
27.3	6.45 A.M.	5.6	1870	0.002	—	—	0.000	—	—	0.037	0.49
27.3	6.00 P.M.	4.4	1470	0.002	—	—	—	—	—	0.029	0.39
28.3	7.00 A.M.	3.7	1240	0.003	—	—	0.000	—	—	0.037	0.49
28.3	5.30 P.M.	4.3	1440	0.003	—	—	—	—	—	0.043	0.57
29.3	6.30 A.M.	4.8	1610	0.002						0.032	0.43
29.3	5.40 P.M.	4.2	1400	0.002						0.028	0.37
30.3	7.00 A.M.	5.0	1670	0.002						0.033	0.44
30.3	5.40 P.M.	3.8	1270	0.002						0.025	0.33
31.3	6.30 A.M.	5.2	1740	0.001						0.017	0.23
31.3	5.40 P.M.	4.0	1340	0.002						0.027	0.36
1.4	6.45 A.M.	5.0	1670	0.002						0.033	0.44
1.4	6.30 P.M.	4.0	1340	0.001						0.013	0.17
2.4	7.45 A.M.	5.0	1670	0.001						0.017	0.23
									Total:		17.21
13.4	7.00 A.M.			0.001							
13.4	6.40 P.M.			0.001							
14.4	7.00 A.M.			0.001							
6.5	7.00 A.M.			0.001							

Amide-N of milk protein of 22.3, 7.00 A.M.: 0.026 atom% excess ^{15}N.
» » » » » 22.3, 5.30 P.M.: 0.012 » » »

decreases quickly to 11 % in the following milk sample. After this it approaches the value found for the total-N. The incorporation of the ammonium-N into the milk proteins seems to proceed primarily *via* the amide, followed by glutamic acid (Table 4). Aspartic acid does not play a role comparable to that of glutamic acid, for the labelling of the latter compound decreases with 45 % within 10 h, of the former with 21 %; the decrease for the total N is 18 %. With the other amino acids there is a general downward trend in the ^{15}N content, more pronounced for the smaller molecules; the basic amino acids, however, show rather an increase. Especially histidine contains little ^{15}N; if labelling is assumed to occur in the amino-N only, the atom %

Table 3. Second experiment started on May 14 at approximately 4.00 P.M. (cow Hieno). Administered 25.42 mg-atom excess of ^{15}N as $(NH_4)_2SO_4$. Total N in milk: 0.430 %; amide-N 8.9 % of protein-N.

Date	Time	kg milk	tot. N in milk (mg-at)	At % excess ^{15}N in: milk: tot. N	prot.N	N.P.N	Amide-N	urine	feces	Excess % ^{15}N in milk (mg-at)	% ^{15}N recovered from milk
14.4	4.45 P.M.	6.4	1970	0.005	0.002	0.050	0.003	0.101	0.002	0.098	0.37
15.5	7.10 A.M.	9.1	2800	0.039	0.039	0.053	0.071	0.077	0.018	1.092	4.29
15.5	5.15 P.M.	7.2	2210	0.032	0.032	0.038	0.039	0.050	0.084	0.707	2.78
16.5	7.30 A.M.	8.8	2700	0.022	0.024	0.030	0.025	0.032	0.036	0.594	2.34
16.5	5.15 P.M.	6.9	2120	0.018	0.017	0.024	0.017	0.015	0.028	0.382	1.50
17.5	6.30 A.M.	9.0	2760	0.014	0.013	0.021	0.012	0.012	0.016	0.386	1.52
17.5	6.00 P.M.	7.9	2430	0.011	0.010	0.014	0.008	0.008	0.012	0.267	1.05
18.5	6.40 A.M.	8.7	2680	0.009	0.008	0.012	0.006	0.007	—	0.241	0.95
18.5	5.15 P.M.	7.0	2150	0.009	—	—	—	0.006	0.007	0.194	0.76
19.5	6.45 A.M.	8.5	2610	0.008	—	—	—	0.005	0.005	0.209	0.82
19.5	5.40 P.M.	6.2	1910	0.007	0.007	0.005	—	0.005	0.004	0.134	0.53
20.5	6.25 A.M.	7.8	2400	0.006	0.007	0.004	—	0.004	—	0.144	0.57
20.5	5.00 P.M.	6.1	1880	0.006	—	—	—	—	0.003	0.113	0.44
21.5	7.10 A.M.	6.7	2060	0.006	0.005	0.003	0.004	0.004	0.002	0.124	0.49
22.5	7.10 A.M.	6.0	1840	0.005	0.005	0.002	0.003	0.002	0.002	0.092	0.36
23.5	7.10 A.M.	5.7	1750	0.005				0.002		0.088	0.35
24.5	6.45 A.M.	5.5	1690	0.004				0.000		0.068	0.27
25.5	6.55 A.M.	6.5	2000	0.004						0.080	0.31
26.5	7.30 A.M.	7.0	2150	0.004				0.000		0.086	0.34
27.5	5.30 P.M.	6.2	1910	0.004						0.076	0.30
28.5	6.45 A.M.	7.6	2320	0.003						0.070	0.28
29.5	7.00 A.M.	8.4	2580	0.002					0.001	0.052	0.20
30.5	7.40 A.M.	8.5	2610	0.002						0.052	0.20
31.5	8.00 A.M.	9.0	2760	0.002						0.055	0.22
1.6	7.30 A.M.	9.3	2860	0.002						0.057	0.22
2.6	6.30 A.M.	8.8	2700	0.002						0.054	0.21
4.6	5.30 P.M.	7.5	2300	0.001						0.023	0.09

excess of ^{15}N in this group will be three times higher than the value observed. This new value (in histidine a and *b*, respectively, 0.0024 and 0.0027) is in fair agreement with the values found for the other amino acids. Thus the low content of ^{15}N in histidine may be interpreted as incapability of the rumen bacteria to synthesize the imidazole ring. The relatively low content in cystine is an interesting point, too.

The last columns in Tables 2 and 3 give the percentage of the initially added amount of ^{15}N that was recovered from the milk. No more labelling can be detected in urine and feces after about one week, but some six weeks after the start of Expt. 1 there was still a detectable trace of ^{15}N in the milk; in Expt. 2 the measurements have not been extended to so long a period. The first cow is seen to produce a recovery value of 17.2 % in ten days; the

trailing over another four weeks has not been considered since the excess of ^{15}N is so minute by that time that it approximates to the accuracy of the measurements. The recovery value for the second cow, having a considerably higher milk production (79.3 kg over a period of five days, compared with 47.9 kg over the same period for the first cow) with a slightly lower protein content (0.430 % *versus* 0.468 %), and being fed on approximately the same rations, is evidently higher. For its calculation it must be taken into account that the values after May 20 have to be doubled, since only one sample of milk per day was investigated. Thus the total recovery found over a period of 17 days amounts to 25.4 %, the yield after this period having again been neglected. The overfeeding with protein is probably the reason for the lower utilization of ammonium nitrogen in the first experiment. In the second experiment the protein in the diet was somewhat lower than the calculated requirement, and the ammonium nitrogen utilization is seen to be about 40 % higher than in the first experiment. More experimental material is needed to elucidate the influence of the composition of the food on the utilization of non-protein nitrogen.

On the basis of the milk yields and the content of ^{15}N one arrives, for this particular diet and these particular cows, at a utilization of about 20 % for the ammonium nitrogen in the formation of milk protein. This value presents a minimum for the total utilization because the uptake into body protein has been left out of consideration. Since in these experiments no quantitative data were collected regarding the amount of nitrogen excreted in the urine and the feces, a rigorous treatment of the total utilization of the ammonium-N cannot be given. It is, however, possible to derive an approximate value along the following lines.

On the assumption that the digestibility of the total protein of oats is 80 %, of AIV-fodder 70 % and of hay 60 %, the ration fed leads to an excretion of 33 g of nitrogen in the feces per 12 h period (*cf.* Table 1 for total N fed). The urine production may be estimated at 11 kg (containing 0.6 % total N) per 10 kg of milk; for the cow of the first experiment, producing slightly less than 10 kg of milk per day during the course of the experiment, the production of urine-N amounts to another 33 g of nitrogen, *i.e.* 2 360 mg-

Table 5. Estimation of the amount of ^{15}N excreted in urine and feces in Expt. 1. Administered 7.52 mg atoms of ^{15}N. Production (per 12 h period following the time mentioned) of urine-N and of feces-N: 2 360 mg-atoms.

Date	Time	Atom % excess in urine	Atom % excess in feces	Excess ^{15}N (mg-at.) in urine + feces	% ^{15}N recoveed
21.3	17.30	0.024	0.026	1.18	15.7
22.3	7.00	0.019	0.021	0.94	12.5
22.3	17.30	0.016	0.018	0.80	10.6
23.3	7.00	0.011	0.013	0.57	7.6
23.3	17.30	0.005	0.009	0.33	4.4
24.3	7.00	0.003	0.008	0.26	3.5
24.3	18.15	0.002	0.004	0.14	1.9
25.3	6.50	0.001	0.004	0.12	1.6
					57.8

atoms per 12 h. Extrapolation of the values for the atom % excess of ^{15}N in urine and feces to the first 12 h following the adminstration of the labelled ammonium salt gives the values 0.024 and 0.026, respectively, with a reasonable trustworthiness. In Table 5 the results are combined.

The further yield of ^{15}N after March 25 will still amount to a few percent, but has not been taken into consideration since measurements on urine and feces were only occasionally performed after that date. It has been found that the total percentage of ^{15}N found in urine and feces amounts to roughly 60 %. If loss of nitrogen through respiration is regarded as negligible, this would mean that from the ration fed, in which 10.8 % of the total N is present as NH_4-N, possibly 40 % of the NH_4-N is utilized for protein synthesis.

REFERENCES

1. Chalmers, M. I. and Synge, R. L. M. *Advances in Protein Chem.* **9** (1954) 93.
2. Pearson, R. M. and Smith, J. A. B. *Biochem. J.* **37** (1943) 153.
3. Bohstedt, G., Booth, A. N., Hart, E. B. and Wegner, M. I. *J. Dairy Sci.* **23** (1940) 1123.
4. Davidson, W. M., Kennedy, J. W., Muir, G. W., Robinson, C. H. and Watson, C. J. *Sci. Agr.* **29** (1949) 185.
5. *cf.* Virtanen, A. I. *Karjatalous* **34** (1958) 132.
6. Rowland, S. J. *J. Dairy Research* **9** (1938) 42.
7. Leavenworth, C. S., Pucher, G. W. and Vickery, H. B. *Ind. Eng. Chem. Anal. Ed.* **7** (1935) 152.
8. Hirs, C. H. W., Moore, S. and Stein, W. H. *J. Am. Chem. Soc.* **76** (1954) 6063.
9. Wahlroos, Ö. and Land, H. *Acta Chem. Scand.* **13** (1959) 595.

Received December 1, 1958.