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Incorporation of ^{15}N and ^{14}C into amino acids of bacterial and protozoal protein in the rumen of the cow on urea-rich feed

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Abstract. The utilization of the non-protein nitrogen and carbon of feed by rumen microorganisms for the synthesis of protein was studied by administering [U-^{14}C] sucrose and $^{15}NH_4Cl$ to a cow on urea-rich, low-protein feed. By studying the labelling of the protozoa and bacteria and the amino acids isolated from them at intervals up to 48 hours afterwards, it was found that the bacteria synthesized amino acids from non-protein nitrogen much more rapidly and effectively than the protozoa. Also the labelling of the carbon in the amino acids of the bacteria was more rapid than in the protozoa. In both protozoa and bacteria there was intracellular storage of [^{14}C] sucrose.

Of the bacterial amino acids the most vigorous ^{14}C labelling was found in Glu, Arg, Lys, Val and Ala and the weakest labelling in Gly, His and Ser. Of the protozoal amino acids Ala, Asp, Glu, Leu and Lys had the highest labelling and Pro, Gly, His and Phe the lowest. In the bacterial protein the labelling of Pro and Arg was ten times that of the corresponding protozoal amino acids, and Asp, Ser and Ala four times.

After the $^{15}NH_4Cl$ dose the half-life of ^{15}N in the rumen fluid was estimated to be 3.3 h. Labelled ammonium nitrogen was about 11—15 % of the bacterial nitrogen and 2—3 % of the protozoal nitrogen after 1 h. Of the protozoal amino acids Ala, Glu, Val, Asp and Met had the most vigorous labelling, and of the bacterial amino acids Glu, Asp, Ser, Ile and Tyr. The slowest incorporation of ammonium nitrogen was into His, Pro, Arg and Gly in both bacteria and protozoa. The labelling of the bacterial amino acids was approximately 7—8 times more vigorous than that of the protozoal amino acids. The labelling of Ala was only 4 times, and that of Val, Met and Glu 5 times more vigorous than with protozoal protein. The pathway of histidine synthesis seemed to be restricted in both bacteria and protozoa and therefore may be a limiting factor in protein synthesis, particularly in cows fed urea as the sole source of nitrogen.

Of the ^{14}C and ^{15}N label given, 12.9 and 9 % respectively was secreted in the milk during the first 3 days; over the same period the ^{14}C and ^{15}N excreted in the faeces plus urine accounted for 16.9 and 44.3 % respectively of that administered.

Introduction

The annual milk yield of cows fed urea and ammonium salts as their sole source of nitrogen (0-cows) is 1 000—2 000 kg lower than that of cows fed large amounts of urea but which obtain part of their nitrogen requirement from natural feed (ULP-cows) (VIRTANEN 1966, VIRTANEN et al. 1972, ETTALA

and KREULA 1976). However, the composition of the protein of the milk produced by 0- and ULP-cows is similar to that of cows (NorP-cows) on normal protein-rich feed (SYVÄOJA 1971).

Since the rumina of 0-cows are almost entirely devoid of protozoa (VIRTANEN 1966, MÄKINEN 1972) and no exogenous protein is included in the feed, the protein synthesized by the rumen bacteria is the only protein source for these cows. The significance of protozoa in the nutrition of ruminants is still not clear; their absence may cause a deficiency of some nutritive factor, such as one or more amino acids, which reduces milk production.

The amino acid composition of rumen microorganisms was found to be very similar in cows which had been fed either urea-rich feed or normal feed (SYVÄOJA and KREULA 1979). The *in vitro* digestibility of protozoa was better than that of bacteria. However, a larger number of bacteria in the rumina of the cows fed urea partly compensated for the poorer digestibility of the bacteria.

The main purpose of the present study was to compare the rates and extents of incorporation of ammonia-N labelled with ^{15}N into amino acids of bacterial and protozoal protein and to find out whether there might be some amino acid which the protozoa could synthesize better from non-protein nitrogen than the bacteria in the rumen of a cow fed large amounts of urea.

Materials and methods

A ULP-cow was given 220 μCi [U-^{14}C] sucrose in 500 g 5 % sucrose solution and 1.26 g ^{15}N in excess as $^{15}NH_4Cl$ (54.65 atom % ^{15}N) in 250 g water through a fistula. The compounds were mixed well by hand with the rumen contents. The daily ration of urea given to the cow was 204.7 g and the total nitrogen 373 g. Complete details of the feeding have been published (VIRTANEN 1966, VIRTANEN et al. 1972, ETTALA and KREULA 1976).

The rumen samples were taken just before the administration of the labelled preparations and 0.5, 1, 5, 24 and 48 h afterwards. Neither feed nor water was given for 5 h after the administration Bacteria and protozoa were isolated as before (SYVÄOJA and KREULA 1979), except that in order to remove the unbound activity we used additional washes: once with unlabelled sucrose-containing phosphate buffer (0.1 M, pH 7.0) and once with water. The proteins were precipitated from the lyophilized bacterial and protozoal preparations with 10 % trichloracetic acid (TCA). Fat and residual TCA were removed with ether.

The nitrogen determinations were performed by the Kjeldahl method using selenium as the catalyst. For the preparative isolation of amino acids the samples were hydrolyzed and then purified with Amberlite IR-120 cation exchanger (in H^+ form) (SYVÄOJA 1971). The isolation was performed by a modified method of HIRS et al. (1954). Using a Dowex 1 × 8 anion exchange column (in acetate form, 55 × 3.2 cm), Phe, Tyr, Asp and Glu were separated by eluting with 0.05 and 0.5 M acetic acid buffer. The rest of the amino acids were isolated from a Dowex 50W cation exchange resin column (in H^+ form, 7 × 120 cm).

For the elution of the amino acids a gradient run of 1 N, 2 N and 4 N hydrochloric acid (4 litres each) was used. The amino acids were obtained in general in pure form, separated completely from one another. Only valine and proline as well as methionine and isoleucine were eluted as mixtures; these were separated on a cellulose column (Whatman CF 11) using butanol-acetic acid-water eluant (12: 3: 5).

With the exception of the aromatic amino acids, the rest of the amino acids were purified further by active carbon treatment and crystallized from concentrated aqueous solution by adding warm acetone. Tyrosine was crystallized from the concentrated acetic acid eluate at 0° C for 1—2 days. The purity of the preparatively isolated amino acids was determined with an amino acid analyzer. With the exception of methionine from the 5 h protozoa sample and cystine from the bacteria and protozoa all amino acids were isolated in sufficient quantity for the ^{14}C and ^{15}N determinations.

The milk was collected quantitatively for 3 days, and the faeces and urine together for 5 days. The samples were dried *in vacuo* at 60° C and ground to homegeneity.

The radioactivity in the samples was determined by combusting the sample, using CuO as the catalyst, and measuring the activity of the CO_2 formed with an ionisation chamber. Most of the ^{15}N measurements (0.5, 1, 5 h) were performed with a CEC 21—401 mass spectrometer; with some of the samples (24, 48 h) an emission spectrometer was used, after the nitrogen of the sample had been converted to ammonium chloride.

Results and discussion

In Fig. 1 the ^{14}C labelling of the amino acids of the rumen bacterial and protozoal protein is presented. All the amino acids of both bacterial and protozoal protein were labelled only half an hour after the administration of the ^{14}C. In the rumen bacteria the following radioactivities were found: 30.8, 28.4, 23.7, 2.1 and 0.8 nCi/g dry matter and in the protozoa 35.6, 24.6, 19.4, 2.1 and 2.0 nCi/g dry matter in the samples taken at 0.5, 1, 5, 24 and 48 hours. This showed that the protozoa had a similar capacity to store sugar intracellularly and to transport it to the abomasum to that of the bacteria, and that the bacteria were able to use sucrose approximately 7 times more effectively for the synthesis of their cell protein than the protozoa. After one or two days the high labelling of the amino acids of the protozoa compared with that of the bacteria could be expected, considering the rate of growth of bacteria.

Of the bacterial amino acids the most vigorous labelling was found in Glu, Arg, Lys, Val and Ala and the lowest labelling in Gly, His and Ser. Of the protozoal amino acids Ala, Asp, Glu, Leu and Lys had the highest labelling and Pro, Gly, His and Phe the lowest. In the bacterial protein the labelling of Pro and Arg was ten times that of the corresponding protozoal amino acids, and Asp, Ser and Ala four times.

The pathways for the production of the C skeleton in the synthesis of the amino acids of rumen microorganisms have been studied by many investigators (Giese 1961, Allison et al. 1966, Somerville and Peel 1967, Harmeyer

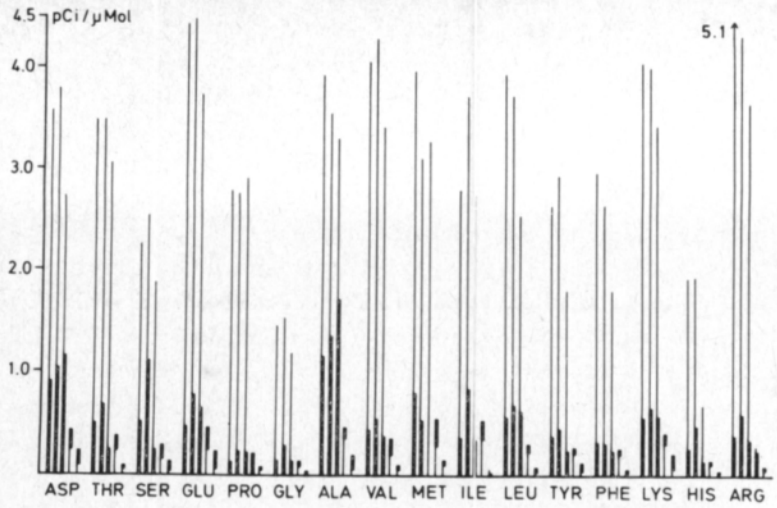

Fig. 1. Labelling of the amino acids of rumen bacteria (thin columns) and protozoa (thick columns) of a ULP-cow after administration of 220 μCi [U-^{14}C] sucrose. The five columns, from left to right, show the labelling of the isolated amino acids 0.5, 1,5, 24 and 48 h after the administration.

and HEKIMOGLY 1968). When several different carbon sources were used differences were found in the labelling of various amino acids and also in their labelling rate. In general Ala, Glu and Asp, however, were among those with the highest labelling.

ALLISON (1969) proposed that the carboxylation of acetate, isobutyrate, isovalerate, 2-methylbutyrate, phenylacetate and indolacetate to give the respective ketoacids, which are aminated to form Ala, Val, Leu, Ile, Phe and Try respectively, is accomplished by reductive reactions. He emphasized, however, that the carbon stem of these amino acids could be synthesized also by pathways other than *via* reductive carboxylation, and that these other pathways could be as important or even more important.

SAUER et al. (1975) found that mixed rumen microorganisms maintained in continuous culture readily incorporated $H^{14}CO_3^-$ and [1-^{14}C] acetate into all amino acids. [1-^{14}C] propionate, however, labelled the amino acids weakly, with the exception of Ile. The ^{14}C distribution in the amino acids showed that 2-oxoglutarate was not oxidized further by tricarboxylic acid-cycle enzymes. Instead, acetate was carboxylated to pyruvate and then to oxaloacetate. Of the amino acid precursors investigated, namely propionate, isovalerate, phenylacetate, succinate, acetate, 3-hydroxypyruvate, only the last one appeared to be synthesized *via* an oxidative step. Most 2-oxo precursors of amino acids in rumen microorganisms appeared to be formed *via* reductive carboxylation of the precursor acid.

Owing to the difficulty of cultivating protozoa the biosynthesis of their amino acids has been studied less than that of bacteria. The labelling of the amino acids varies with the species of protozoa (HARMEYER 1965, HARMEYER and HILL 1965). HARMEYER and HEGIMOGLY (1968) observed that [2-^{14}C]

acetate gave the higherst labelling in the same amino acids of oligotrichs and holotrichs as did [^{14}C] sucrose in our experiments.

COLEMAN (1967 a, b) found that *Entodinium caudatum* cells grown *in vitro* assimilated free amino acids. When bacterial cells labelled with individual amino acids were used the protozoa incorporated the labelled amino acids intact into protein. The incorporated amino acid carbon skeletons were not used for the biosynthesis of other amino acids by the protozoa.

Table 1 shows the ^{15}N contents of the bacteria and protozoa isolated from the rumen, and that of the proteins, precipitated from them by 10 % TCA, and soluble fractions at different intervals after the administration of the ^{15}N. The utilization of ammonium nitrogen was very rapid. After only half an hour the bacteria in particular were labelled vigorously. The labelling of the bacteria was 5—6 times higher than that of the protozoa, reaching a maximum after 1—5 h.

Table 1. Atom % excess ^{15}N in rumen protozoa and bacteria and in their 10 % TCA-soluble and -insoluble fractions.

Time after administration h	Atom % excess ^{15}N in					
	Protozoa			Bacteria		
	Cells	TCA-precipitate	TCA-soluble fraction	Cells	TCA-precipitate	TCA-soluble fraction
0.5	0.131	0.123	0.310	0.755	0.746	0.943
1	0.176	0.159	0.286	0.832	0.827	0.969
5	0.108	0.105	0.155	0.664	0.656	0.910
24	0.061	0.060	0.045	0.051	0.058	0.045
48	0.026	0.026	0.021	0.011	0.031	0.010

The ^{15}N in one litre of rumen fluid, as a proportion of the total amount fed, was 1.11, 0.67, 0.26, 0.13 and 0.03 % in the samples taken at 0.5, 1, 5, 24 and 48 h. Extrapolation of these figures gave a value of 1.25 % at 0 min. Thus the half-life of the ^{15}N in the rumen fluid was approximately 3.3 h. This is shorter than that found by ABE and KANDATSU (1968), who reported that after the administration of [^{15}N] ammonium citrate and [^{15}N] urea the labelling in bacteria peaked at 6 h and that in protozoa at 9 h. In the same experiments bacteria assimilated only 2—3 times more ^{15}N than protozoa. Evidently the half-life depends largely on the time interval after feeding and on the resulting rise in the ammonia level, and also on the intake of energy and the utilization of ammonia in the rumen. The proportion of labelled ammonium nitrogen after the first hour was about 11—15 % of the bacteria nitrogen and 2—3 % of the protozoal nitrogen. ULBRICH and SCHOLZ (1966) obtained similar results: after giving ^{15}N urea the proportion of urea nitrogen of the bacterial nitrogen during the first few hours was 7.8—9.2 % and that of the protozoal nitrogen 1.0—1.8 %. Repeated feeding of labelled urea increased the labelling of both bacteria and protozoa, being 16—17 % in bacteria and 12—13 % in protozoa after 5 days.

The ^{15}N labelling of the amino acids of the bacterial and protozoal proteins is presented in Fig. 2. The rumen microorganisms of cows on urea-rich feed are able to utilize ammonium nitrogen rapidly and effectively. Of the protozoal amino acids Ala, Glu, Val, Asp and Met had the most vigorous labelling, and of the bacterial amino acids Glu, Asp, Ser, Ile and Tyr. The slowest incorporation of ammonium nitrogen was into His, Pro, Arg, and Gly with both bacteria and protozoa. The labelling of the bacterial amino acids was approximately 7—8 times more vigorous than that of the protozoal amino acids. The labelling of Ala was only 4 times more vigorous, and that of Val, Met and Glu 5 times more vigorous than with protozoal protein.

In the faeces of 0- and ULP-cows the content of those amino acids which had the lowest labelling in the rumen bacteria and protozoa (His, Pro, Arg and Gly) was smaller than in the faeces of NorP-cows (Ettala and Kreula 1979). However, the amino acid compositions of the rumen microorganisms, with the exception of the proportion of diaminopimelic acid, were very similar, being independent of the type of feed (Syväoja and Kreula 1979). The low level of the above amino acids in the faeces is probably an indication of the capacity of the cow to adjust its absorption of amino acids.

No amino acid was found for which the protozoa had a better capacity for synthesis than the bacteria. It should be noted that in both protozoa and bacteria the rate of synthesis of histidine from non-protein nitrogen and sucrose was slower than any other amino acid. Therefore it is possible, particularly in cows on 0-feed, that histidine is a factor restricting milk production. Land and Virtanen (1959) suggested that the capacity of the rumen microorganisms to synthesize the imidazole ring is poor. They studied the labelling of the amino acids of milk after giving $(^{15}NH_4)_2SO_4$ and found that the histidine of the milk protein was labelled to only a very small extent. Histidine was still the most slowly-labelled essential protein amino acid even when the

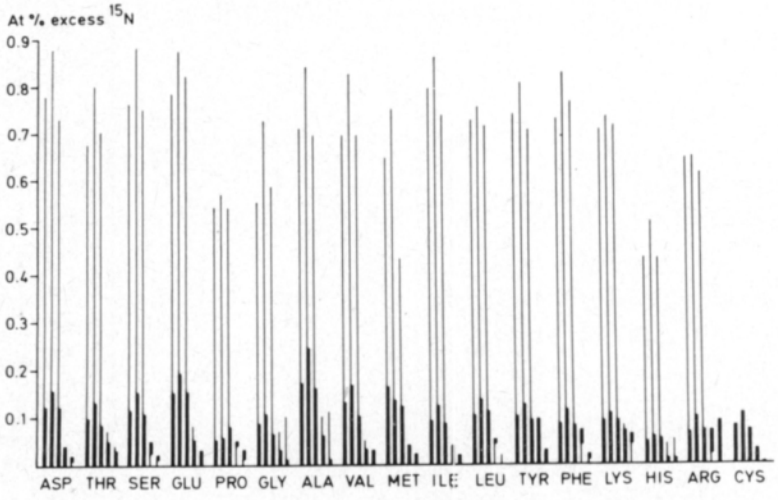

Fig. 2. Atom % excess of ^{15}N in the amino acids of rumen bacteria and protozoa of a ULP-cow after administration of 1.26 g excess ^{15}N as $^{15}NH_4Cl$. Columns and time intervals as in Fig. 1.

cow had been on 0-feed more than 2 years, but even so its labelling was much greater than that in the non-adapted cow (VIRTANEN 1967, KREULA 1979). An extremely slow labelling of histidine was also found by GRUHN et al. (1975) in milk protein, by PIVA and SILVA (1966) in rumen microorganisms after administration of a ^{15}N-labelled non-protein preparation, and by GIESE (1961) in bacterial and protozoal protein after giving [^{14}C] urea. In contrast, SALTER et al. (1979) found that although the labelling of histidine from ^{15}N urea was low when the nitrogen source of steers' feed consisted of decorticated groundnut meal, the plateau value was consistently higher when urea was the only N source in the feed. The present results parallel evidence obtained in *in vitro* and *in vivo* experiments that ammonia nitrogen is first incorporated into bacterial cells; the nitrogen in protozoal cells becomes labelled following the ingestion of the bacteria (CLARCE and HUNGATE 1966, ULBRICH and SCHOLZ 1966, ABE and KANDATSU 1968). ERFLE et al. (1977) studied enzymes which effect the incorporation of ammonia into amino acids (ammonia → glutamate → aspartic acid + alanine → other amino acids). They found that the activity of a number of these enzymes, as well as those involved in amino acid biosynthesis and utilization by rumen microorganisms, may be regulated by the concentration of NH_4^+ in the rumen. A low ammonia concentration caused a ten-fold increase in the specific activity of glutamine synthetase but had no consistent effect on the activity of asparagine synthetase or aspartate aminotransferase. Glutamate synthetase in conjunction with glutamine and asparagine synthetases may provide an efficient means of glutamate synthesis at low rumen ammonia concentrations. The amount of alanine too depended on the content of ammonia in the rumen, the formation of alanine being greatly increased at high ammonia concentrations.

Besides rumen microorganisms, the labelling of the milk, faeces and urine was also studied. In milk 12.9 % of the total ^{14}C activity fed was secreted in 3 days, 80 % of which in the first day. In a similar experiment with a 0-cow, 10.1 % of the ^{14}C was secreted in the milk in the first 3 days (KREULA et al. 1973, KREULA and RAURAMAA 1979). The activity excreted in the faeces and urine of the 0-cow (16.9 % of the amount fed) was lower than that excreted in the faeces and urine of the ULP-cow (23.3 %).

Of the ^{15}N isotope fed, 9.0 % was secreted in the milk during a period of 3 days, 60 % of which during the first day. Considering the volume of milk and its protein content, the amount of ^{15}N secreted in milk corresponded to the results obtained previously (LAND and VIRTANEN 1959, MIELKE 1966, VIRTANEN 1967, ABE and KANDATSU 1968, KREULA unpublished data). LAND and VIRTANEN (1959) found that the secretion of ^{15}N was very slow: even after 2 months labelling could be detected in the milk. In the present study, 42 % of the total ^{15}N fed was excreted in the faeces and urine in the first 3 days (44.3 % in days).

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SELOSTUS

^{15}N ja ^{14}C inkorporoituminen runsaalla urearuokinnalla olevan lehmän pötsin bakteeri- ja prototsoaproteiinin aminohappoihin

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Lehmän pötsimikrobien ei-proteiinitypen ja hiiliravinnon hyväksikäyttöä mikrobiproteiinin synteesissä tutkittiin syöttämällä runsaasti ureaa saaneelle lehmälle $^{15}NH_4Cl$ ja ^{14}C-sakkaroosia. Tutkimalla prototsoien ja bakteereiden sekä näistä preparatiivisesti eristettyjen aminohappojen leimaantuminen eri aikojen kuluttua todettiin, että bakteerit kykenivät nopeammin ja tehokkaammin kuin prototsoat syntetisoimaan aminohappoja ei-proteiinitypestä. Myös hiilen leimaantuminen oli bakteereiden aminohapoissa nopeampaa kuin prototsoien, vaikka prototsoat kykenivät yhtä hyvin kuin bakteerit varastoimaan ^{14}C-sakkaroosia intrasellulaarisesti. Eniten ^{14}C:llä leimaantuivat bakteeriproteiinin Glu, Arg, Lys, Val ja Ala sekä prototsoaproteiinin Ala, Asp, Glu, Leu ja Lys. Heikommin leimaantuivat Gly, His ja Ser bakteeriproteiinissa sekä Gly, His ja Phe prototsoaproteiinissa. Bakteeriproteiinin Pro ja Arg leimaantuivat 10 kertaa, Asp, Ser ja Ala 4 kertaa ja muut aminohapot keskimäärin 7 kertaa paremmin kuin prototsoien vastaavat aminohapot.

^{15}N puoliintumisaika pötsinesteessä arvioitiin olevan noin 3,3 h. Leimatun ammoniumtypen määrä oli 11—15 % bakteeritypestä ja 2—3 % prototsoatypestä yhden tunnin kuluttua ^{15}N syöttämisestä. Voimakkaammin leimaantuivat Ala, Glu, Val, Asp ja Met prototsoien ja Glu, Asp, Ser, Ile ja Tyr bakteerien aminohapoista. Heikoimmin leimaantuivat His, Pro, Arg ja Gly sekä bakteeri- että prototsoaproteiinissa. Bakteeriproteiinin aminohapot leimaantuivat keskimäärin 7—8 kertaa, mutta Ala 4 kertaa ja Val, Met ja Glu 5 kertaa paremmin kuin prototsoaproteiinin vastaavat aminohapot. Histidiinin synteesi saattaa olla urearuokinnalla olevalla lehmällä rajoittava tekijä mikrobiproteiinin muodostumisessa.

Syötetystä ^{14}C- ja ^{15}N-leimauksesta erittyi maidossa kolmen vuorokauden kuluessa 12,9 % ja 9 % vastaavasti. Lannassa ja virtsassa eritetty ^{14}C-aktiivisuus oli 16,9 % ja ^{15}N-leimaantuminen 44,3 % syötetystä kokonaisleimauksesta saman ajan kuluessa.