^{15}N enrichments of casein and plasma protein amino acids in cows ingesting ^{15}N-labelled ammonium sulphate

By FLORENCE CASSERON*, GUIDO RYCHEN†‡, XIMO RUBERT-ALEMAN†, GERARD JEAN MARTIN* and FRANÇOIS LAURENT†

**Laboratoire d'Analyse Isotopique et Électrochimique de Métabolismes (LAIEM), UPRES-A 6006, Université de Nantes, 2 rue de la Houssinière, BP 92208, F-44322 Nantes Cédex 03, France§*

†Laboratoire de Sciences Animales, INRA École Nationale Supérieure d'Agronomiques et des Industries Alimentaires (ENSAIA), 2 avenue de la Forêt de Haye, BP 172, F-54505 Vandoeuvre-lès-Nancy Cédex, France

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Summary. The aim of this work was to determine by ion-exchange liquid chromatography and isotope ratio mass spectrometry the specific ^{15}N enrichment of amino acids in casein and plasma proteins in cows receiving three successive daily oral doses (300, 150 and 150 g) of $(^{15}NH_4)_2SO_4$ (10 atom per cent isotopic enrichment) and to examine the ^{15}N enrichments obtained with regard to nitrogen transport and metabolism in the lactating cow. To investigate the ^{15}N distribution in amino acids in casein and in plasma proteins, samples of ^{15}N-labelled casein and plasma proteins were extracted either from a pool of several milkings (36–96 h after starting to administer the tracer) or from pooled venous blood (removed on the fourth day after the start of administration) from the four lactating cows. ^{15}N enrichments of the proteins studied, expressed as atoms percent excess, were 0·2509 for casein and 0·0577 for plasma protein. Chromatographic fractionation of the amino acid mixture (protein hydrolysates) resulted in nine groups containing between one and four amino acids: Asp, Ser and Thr; Glu; Pro; Gly, Ala, Val and Met; Ileu and Leu; Tyr; Phe; His and Lys; and Arg. High ^{15}N incorporation was demonstrated in all individual or groups of amino acids studied. In both proteins, Glu appeared to be the most enriched amino acid, Phe and Arg the least enriched. Most aliphatic molecules with a single amino group were highly enriched. The much lower (3·5–7·7-fold) enrichments in plasma protein compared with casein suggest considerable intracellular dilution at the site of liver protein synthesis. Finally, the amino acid separation methods are discussed and suggestions for improving them considered.

^{15}N labelling of milk proteins by ruminal or oral administration of $(^{15}NH_4)_2SO_4$ to lactating cows (Mahé *et al.* 1994*a*; Colin-Schoellen *et al.* 1997) has been considered as one of the most valuable techniques for studying N transport and metabolism within the ruminant animal or for nutritional investigations in human subjects. Recently, the use of ^{15}N-labelled milk proteins made it possible to distinguish exogenous from endogenous N fractions in the human intestine after the ingestion of ^{15}N-labelled

‡ For correspondence.

§ Formerly Laboratoire de RMN et Réactivité Chimique, URA-CNRS 472, Université de Nantes, 2 rue de la Houssinière, F-44072 Nantes Cédex 03, France.

milk, casein (Mahé *et al.* 1994*b*) or yogurt (Gaudichon *et al.* 1995). Studying the incorporation of ^{15}N from orally administered $(^{15}NH_4)_2SO_4$ into the amino acids of milk proteins should result in useful information on the metabolism of amino acids from gut absorption via liver metabolism to their incorporation into secreted milk proteins. Indeed, nitrogenous metabolism is complex and there is nitrogen isotopic fractionation during assimilation, synthesis, transport and excretion of nitrogenous compounds. The aim of this study was to determine by ion-exchange liquid chromatography (IELC) and isotope ratio mass spectrometry (IRMS) the specific ^{15}N enrichment of protein amino acids (casein and plasma proteins) in the cow receiving oral $(^{15}NH_4)_2SO_4$ and to discuss how these relate to the overall N transport and metabolism of absorbed amino acids in the lactating cow.

MATERIAL AND METHODS

Animals and experimental protocol

The animal protocol was in accordance with the French Animal Care Guidelines. Four lactating cows of Prim'Holstein breed from the Domaine Expérimental de la Bouzule (INRA-ENSAIA, F-54505 Nancy, France) were chosen for their milk yield (average 24 ± 3 l/d during the 2 weeks before treatment). They were housed in free stalls and were fed *ad lib.* once a day in the morning a total mixed ration comprising (g dry matter/kg) maize silage 681, chopped straw 35, barley 177, formaldehyde treated soyabean and rapeseed meal (50:50) 89, ammonium sulphate 300 and two mineral supplements (6 g $CaCO_3$/kg and 5 g/kg of a mineral containing 140 g Ca/kg and 140 g P/kg). For the average dry matter intake (20·4 kg/cow), the total mixed ration provided 1·58 Mcal net energy for lactation and 140 g crude protein/kg dry matter. After 3 weeks adaptation to the total mixed ration, the cows received three successive daily doses of 300, 150 and 150 g $(^{15}NH_4)_2SO_4$ (10 atom per cent isotopic enrichment; Euriso-Top CEA, F-91194 Saint-Aubin, France), replacing equal amounts of unlabelled ammonium sulphate. Labelled and unlabelled ammonium sulphate were mixed together and added to the total mixed ration (Table 1). For each animal, samples of milk (M0–M11, Table 1) and jugular venous blood (B0–B11, Table 1) were taken simultaneously during milking to establish the total ^{15}N enrichment levels of whole milk and venous plasma as a function of time. To investigate the ^{15}N distribution in amino acids in casein and in plasma proteins after oral administration of the three doses of $(^{15}NH_4)_2SO_4$, a representative 100 g sample of ^{15}N-labelled casein (15NCA) was extracted from a milk mixture from several milkings (M3–M8, Table 1) and a representative 100 g sample of ^{15}N-labelled plasma proteins (15NPP) was extracted from pooled venous blood removed on the fourth day after the start of $(^{15}NH_4)_2SO_4$ administration (B6, Table 1) from the four lactating cows. Previously, to perfect the isolation and analysis methods of casein amino acids, an initial sample of ^{15}N-labelled casein had been prepared as described below from ^{15}N-labelled milk obtained 36 h after oral administration of a single dose of 150 g $(^{15}NH_4)_2SO_4$ (10 atom per cent isotopic enrichment) to a lactating cow (Colin *et al.* 1993).

Sample preparations

Milk samples. Milk samples (M0–M11, Table 1) were collected, stored at -20 °C and freeze dried before total ^{15}N enrichment measures by IRMS.

Casein extraction. Milkings M3–M8 (Table 1) were pooled in order to extract 15NCA. The milk mixture was centrifuged at 1000 ***g*** and 4 °C for 20 min, the cream

Table 1. *Experimental design used for each cow in this study*

	Day					
	1	2	3	4	5	6
Ammonium sulphate supply, g						
Unlabelled	0	150	150	300	300	300
Labelled	300	150	150	0	0	0
Samplings of milk (M) and blood (B)						
Morning	M0, B0	M2, B2	M4, B4	M6, B6	M8, B8	M10, B10
Evening	M1, B1	M3, B3	M5, B5	M7, B7	M9, B9	M11, B11

discarded and the skim milk processed to separate casein by a procedure based on that of Oddy *et al.* (1988). The skim milk was first acidified to pH 4·6 with 1 M-HCl and centrifuged (1000 ***g***, 4 °C, 15 min). The resulting pellet was resuspended in an equal volume of water and dissolved by addition of 1 M-NaOH to pH 7. The casein was precipitated again by addition of 1 M-HCl to pH 4·6 as described above, recentrifuged and freeze dried. Milk caseins were defatted by adding methanol–chloroform (1 : 1, v/v) at 75 g/l in order to promote acid hydrolysis of proteins and avoid contamination of the chromatographic systems. The whole 15NCA, extracted by centrifugation (1000 ***g***, 4 °C, 10 min) from the supernatant liquid containing soluble lipids, was dried at 40 °C.

Venous plasma samples. Blood samples (B0–B11, 10 ml/cow, Table 1) were removed from an external jugular vein, collected in heparinized tubes and immediately centrifuged (1000 ***g***, 4 °C, 10 min) to separate the plasma. Venous plasma samples were freeze dried before total ^{15}N enrichment measurements by IRMS.

Plasma protein extraction. After milking M6 (Table 1), large quantities of venous blood (B6, 250 ml/cow, Table 1) were removed and pooled to extract 15NPP. The blood samples were collected in heparinized tubes, immediately centrifuged (1000 ***g***, 4 °C, 10 min) and the resulting plasma stored at −20 °C until treatment. Plasma proteins, obtained by addition to the plasma of trichloroacetic acid (200 g/l) at 200 g/l and centrifugation (1000 ***g***, 4 °C, 10 min), were freeze dried before analysis.

Hydrolysis procedure. Amino acids were obtained by acid hydrolysis of proteins (15NCA and 15NPP) as follows (Zumwalt *et al.* 1987): 6 M-HCl (50 ml) was added to 400 mg purified proteins, and the mixture was refluxed at 110 °C for 24 h. Following acid hydrolysis, the mixture was evaporated to dryness under reduced pressure at 40 °C and the residue dissolved in 50 ml distilled water. The resulting solution was microfiltered (0·4 μm filter; Millipore, F-78054 Saint-Quentin-Yvelines, France) for complete removal of the residual insoluble substances and was evaporated to dryness under reduced pressure at 40 °C. The residue was dissolved in 500 μl 66·7 mM-sodium citrate buffer containing 70 ml alcohol/l adjusted to pH 2·80 with perchloric acid before use in chromatographic analysis.

Analytical methods

Amino acid isolation using IELC. For the chromatographic fractionation of mixtures of amino acids we applied the traditional method of ion-exchange separation of amino acids developed by Moore *et al.* (1958). The amino acids were

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separated by elution on a strongly acidic cation-exchange resin (8 g) which was a cross-linked polystyrene polymer (100 Å, 12 μm, divinylbenzene (100 g/kg)) packed into a stainless steel column (150 × 7·5 mm, no. COMU4190644-0001; Merck-Clévenot (Mitsubishi), F-94130 Nogent-sur-Marne, France). This resin, developed specifically for semi-preparative amino acid isolation using the sodium cycle, exhibited high stability to both acids and bases and high chromatographic efficiency. The exchange capacity was 2 mequiv./g moist resin. The amino acids were eluted on the column using a gradient system (L-6200 Intelligent Pump, Merck-Clévenot) programming a mobile phase of eluents: A, 66·7 mM-sodium citrate buffer containing 70 ml absolute alcohol/l adjusted to pH 2·80 with perchloric acid; B, 66·7 mM-sodium citrate buffer–6·67 mM-boric acid, adjusted to pH 6·40 with perchloric acid; C, 0·2 M-sodium hydroxide, pH 14·0. A discontinuous linear gradient was run from pure A to pure C over 210 min at a flow rate of 1 ml/min (Fig. 2). A concentrated hydrolysate (500 μl containing 400 mg protein hydrolysate, ~ 2 mmol total amino acids) was injected on the top of the equilibrated column (temperature controlled at 60 °C) at pH 2·80, and the elution was carried out with the series of buffers of progressively increasing pH. A fraction collector (model 203; Gilson Medical Electronics, F-95400 Villiers-le-Bel, France) was utilized to collect fractions of the effluent (3 ml/tube). An additional step with pure C was applied to wash the column prior to returning to initial conditions for the resin regeneration. To preserve the column and its resolving power, the pressure in the system was maintained below 3·45 MPa. The amino acid composition of a 1 μl sample from each fraction eluted was determined by specific amino acid reaction with ninhydrin after separation by thin layer chromatography (Kirchner, 1967). The chromatographic development used silica gel sheets (Si 60 F_{254}, 0·25 mm, Merck) and *n*-butanol–acetic acid (990 ml/l)–distilled water (4:1:1, by vol.) as eluent. For each hydrolysate sample, four IELC separation procedures were carried out to obtain sufficient nitrogen compounds for the ^{15}N measurements. Fractions containing the same amino acid or group of amino acids (characterized by similar migration distances) from a given hydrolysate were pooled and carefully evaporated to dryness under slightly reduced pressure.

Spectrometric technique. The overall $^{15}N:^{14}N$ isotope ratios of bovine samples (milk and plasma proteins) were determined by IRMS. Dry samples (10–40 mg depending on total nitrogen content) were burned in the combustion unit of an elemental analyser (Fisons Instruments model NA1500; Thermo-Fi, Crawley RH10 2QQ, UK). In the presence of pure oxygen, the organic compounds were converted into CO_2, N_2, N_xO_y and H_2O during passage through an oxidation oven. Passage through a reduction reactor reduced N_xO_y to N_2, any O_2 released from the oxidation reactor being removed. The combustion water was trapped by an anhydrone column and the CO_2 removed by passage through sodium hydroxide. The analyser was coupled to a chromato Porapack QS and, via a splitter valve, to an isotope ratio mass spectrometer (Delta E, Finnigan Mat, D-28088 Bremen, Germany). The N_2 content of the helium stream was analysed for relative contributions at masses 28, 29 and 30. The $^{15}N:^{14}N$ ratio was determined using a secondary laboratory standard (gaseous nitrogen) calibrated with reference to the international standard, atmospheric nitrogen ($R_{N_2} = 0{\cdot}36765$ atoms per cent ^{15}N at natural abundance). The nitrogen content of an amino acid is low compared with that of a protein such as casein, so the $^{15}N:^{14}N$ ratios of individuals or groups of amino acids were measured using another isotope ratio mass spectrometer (Optima, Fisons Instruments), which was more sensitive than a Delta E mass spectrometer. All the values were then recalculated in atoms percent excess (APE) relative to atmospheric nitrogen.

RESULTS

Total ^{15}N enrichment kinetics of whole milk and venous plasma

Fig. 1 presents the ^{15}N enrichment kinetics (means $\pm$ SEM, $n = 4$, one measurement per cow) of whole milk (M0–M11, Table 1) and venous plasma (B0–B11, Table 1) samples taken from four lactating cows receiving three successive daily doses of 300, 150 and 150 g $(^{15}NH_4)_2SO_4$ (10 atom % isotopic enrichment). The natural ^{15}N level of whole milk and venous plasma, determined before $(^{15}NH_4)_2SO_4$ administration, was $0{\cdot}0021 \pm 0{\cdot}0002$ APE ($n = 4$). In both milk and plasma, ^{15}N enrichments increased markedly after the first $(^{15}NH_4)_2SO_4$ dose. In milk, ^{15}N enrichment reached nearly 2-fold (0·6829 atom %) the natural ^{15}N abundance, with a maximal enrichment of 0·3151 APE 36 h after the first tracer administration (Fig. 1). The repeated supply of $(^{15}NH_4)_2SO_4$ also made it possible to maintain a plateau of $\sim 0{\cdot}3066$ APE for milk within 1 d (between 36 and 60 h). The ^{15}N enrichments of milk samples were $> 0{\cdot}1657$ APE up to 96 h, and then decreased rapidly to an average enrichment level of 0·0471 APE at 192 h after tracer administration (Fig. 1). Thus, the milk mixture (M3–M8, Table 1) used for extracting 15NCA was composed of the most highly ^{15}N-labelled milks. The ^{15}N recovered in the milk collected in the 8 d after the first administration of tracer represented 17 % of the total ^{15}N administered. No labelling peak was found for ^{15}N enrichment of plasma (Fig. 1), but there was a steady increase of ^{15}N enrichment up to 84 h (0·0637 APE) after tracer administration, followed by a very gradual decrease of the enrichment level up to 192 h (0·0522 APE). The plasma samples were relatively poorly labelled in ^{15}N (nearly five times less enriched than whole milk samples measured during the 24–84 h period). The venous blood pooled for 15NPP extraction was composed of the most highly ^{15}N-labelled blood samples (B6, Table 1).

Hydrolysis of bovine proteins and chromatographic fractionation of amino acids

Only minor ^{15}N isotopic fractionation was noted (< 1 % of total ^{15}N enrichment) following acid hydrolysis of 15NCA and 15NPP. We separated the fraction into nine groups containing between one and four amino acids (Fig. 2) using the chromatographic conditions described previously. The resolving power of the ion-exchange column used was not quite sufficient to accommodate the whole breadth of certain amino acid peaks and there were small overlapping areas. Therefore, the eluted fractions containing incompletely separated amino acids were pooled prior to ^{15}N analysis. We undertook a comparative study of the ^{15}N of unlabelled amino acid standards (as individuals or groups), measured by IRMS before and after IELC isolation. The linear correlation obtained ($r^2 = 0{\cdot}958$) demonstrated both the chromatographic integrity and the good repeatability of combustion mass spectrometry for ^{15}N measurements.

Specific ^{15}N enrichments of individual or groups of amino acids in ^{15}N-labelled caseins and plasma proteins

We found good repeatability of ^{15}N measurement by IRMS for the samples, indicating good homogeneity and a high nitrogen content for both proteins and hydrolysates ($\pm 0{\cdot}0002$ APE). The total ^{15}N enrichment of the labelled milk casein 15NCA was 0·2509 APE, whereas that of plasma protein (15NPP) was only 0·0577 APE. These enrichment levels were sufficiently high to make it possible to determine the ^{15}N enrichment and distribution within the nine separated amino acid groups derived from 15NCA and 15NPP obtained after oral administration of

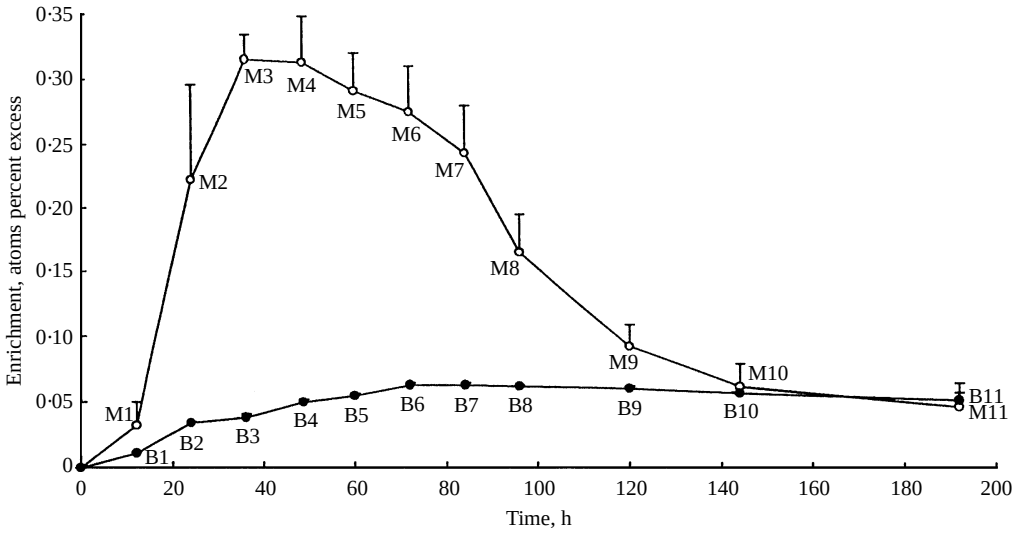
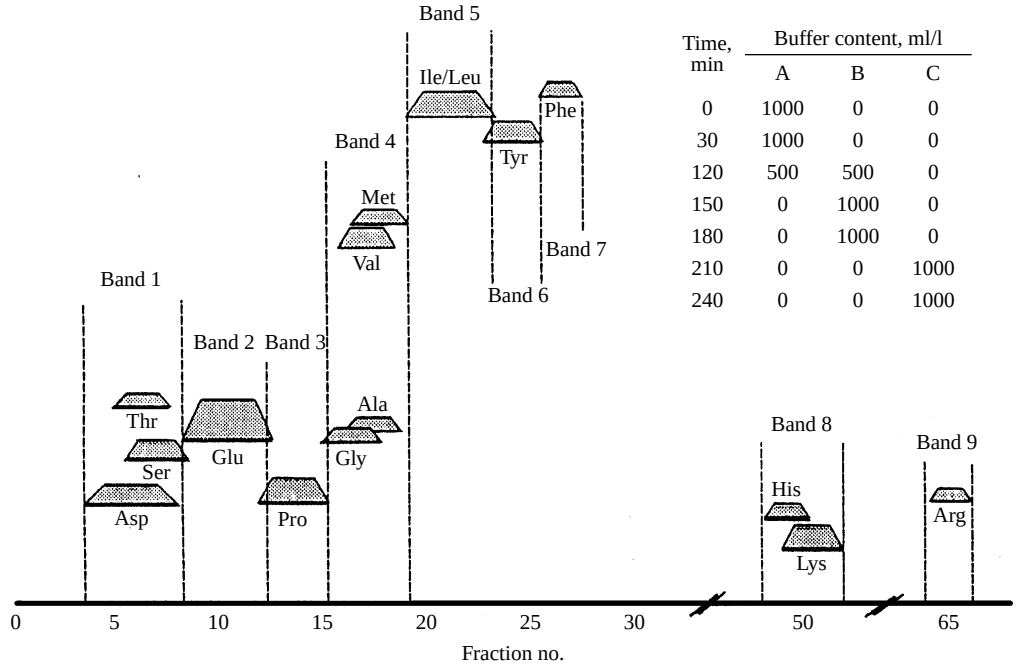


Fig. 1. ^{15}N enrichment rates of ○, milk and ●, plasma samples from four lactating cows receiving three successive daily (at 0, 24 and 48 h) oral doses of 300, 150 and 150 g $(^{15}NH_4)_2SO_4$ (10 atom per cent isotopic enrichment). Values are means with SEM indicated by vertical bars. For the plasma values, SEM are often smaller than the symbols.



Time, min	Buffer content, ml/l		
	A	B	C
0	1000	0	0
30	1000	0	0
120	500	500	0
150	0	1000	0
180	0	1000	0
210	0	0	1000
240	0	0	1000

Fig. 2. Scheme used for the chromatographic fractionation of mixtures of amino acids (400 mg casein hydrolysate) by elution on a column of sulphonated polystyrene resin. Elution was carried out at 60 °C with a series of buffers of progressively increasing pH: A, 2·8; B, 6·4; C, 14·0. Fraction volume was 3 ml.

$(^{15}NH_4)_2SO_4$ to lactating cows (Table 2). The precision of the ^{15}N measurements was $\pm 0{\cdot}0007$ APE (four measurements for each amino acid sample) for individual amino acid samples, but could reach $\pm 0{\cdot}0033$ APE for the groups of amino acids (probably owing to a certain heterogeneity in these samples). These results (Table 2) indicated

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Table 2. *Specific ^{15}N enrichment of amino acids isolated from [^{15}N]milk casein (15NCA) and from [^{15}N]plasma proteins (15NPP) from cows given ^{15}N-labelled ammonium sulphate, determined by isotope ratio mass spectrometry*

(Enrichment values are expressed as APE, means ± SEM for $n = 4$)

Amino acid fraction	15NCA	15NPP	15NPP : 15NCA
Asx, Thr, Ser	0·1980 ± 0·0025	0·0420 ± 0·0002	0·21
Glx	0·2377 ± 0·0003	0·0596 ± 0·0001	0·25
Pro	0·1987 ± 0·0004	0·0326 ± 0·0001	0·16
Gly, Ala, Val, Met	0·2064 ± 0·0013	0·0391 ± 0·0002	0·19
Ileu, Leu	0·2013 ± 0·0009	0·0336 ± 0·0002	0·17
Tyr	0·2092 ± 0·0018	0·0281 ± 0·0003	0·13
Phe	0·1210 ± 0·0018	0·0182 ± 0·0001	0·15
His, Lys	0·2188 ± 0·0001	0·0286 ± 0·0002	0·13
Arg	0·1850 ± 0·0005	0·0525 ± 0·0002	0·28

APE, atoms percent excess; 15NCA, [^{15}N]casein (0·2509 APE) extracted from a ^{15}N-labelled milk mixture of several milkings taken from four lactating cows receiving three successive daily oral doses of 300, 150 and 150 g $(^{15}NH_4)_2SO_4$ (10 atom percent isotopic enrichment); 15NPP, [^{15}N]plasma proteins (0·0577 APE) extracted from pooled venous blood (removed on the fourth day after the start of $(^{15}NH_4)_2SO_4$ administration from the four lactating cows.

high ^{15}N incorporation into all individual amino acids or groups studied. The much lower enrichment in 15NPP compared with 15NCA is noteworthy. The degree of ^{15}N incorporation in the proteins studied, however, appeared to be markedly enhanced in some amino acids but diminished in others. Glx (Glu + Gln), for example, had the highest level of ^{15}N incorporation in 15NCA and in 15NPP. Arg, on the other hand, while one of the least enriched amino acids in 15NCA, was highly enriched in 15NPP. The ^{15}N specific enrichment of Phe was the lowest of all the amino acids in 15NCA and in 15NPP.

DISCUSSION

Procedural considerations

Certain amino acids were lost during acid hydrolysis (Zumwalt *et al.* 1987). Asn and Gln lost their amide groups and contributed to the ^{15}N content of the Asp and Glu groups (designated Asx for Asp + Asn, and Glx for Glu + Gln). Most if not all the Trp and Met residues were destroyed upon hydrolysis. In addition, Cys residues were reduced to cysteic acid, which was eluted with Asx, Thr and Ser. Trp, Met and Cys did not make major nitrogen contributions (Swaisgood, 1973) to the particular amino acid fraction with which they were grouped (Asx, Thr and Ser, or Gly, Ala and Val, Met). Hence, these residues are unlikely to interfere significantly with the ^{15}N enrichment for these fractions. Unstable molecules resulting from Maillard condensations (with Lys) were probably converted into NH_3, CO_2 and other compounds such as brown polymers and volatile products (Cheftel & Cheftel, 1977). Nevertheless, these ^{15}N isotopic fractionation products could be neglected relative to the ^{15}N enrichment level of the labelled proteins.

Fig. 2 presents the schematic profile for amino acid isolation from hydrolysate using the IELC technique. Amino acid separations depend on both the differences in the ionic natures of the amino acids and the structures of their side chains. The order of elution of the amino acids was substantially that of the isoionic pH values. However, solubility effects and short-range adsorption forces, associated in the main with ring structures, strongly retarded certain amino acids more than would be predicted (e.g. Pro and Phe). Tyr crystallized in the fraction tubes after chromatographic isolation because of its low solubility in aqueous solution. The

IRMS method used for the ^{15}N measurement of organic molecules avoided contamination by exogenous nitrogen compounds and provided good precision ($\pm 0{\cdot}0002$ APE). Owing to the low content of nitrogen in amino acids, relatively large samples were needed for complete baseline chromatographic separation in order to collect milligram quantities of nitrogen from amino acids. Therefore, under the chromatographic conditions used, the separation of amino acids was not complete and grouping was necessary, which could have involved a risk of isotopic fractionation. Furthermore, the relatively high quantity of citrate in the effluent tended to interfere with the isolation of pure compounds. Phe and Tyr have lower nitrogen contents than the other amino acids and groupings, so probably the nitrogen content was less than required to provide a satisfactory signal:noise ratio for these two amino acids. In addition, during acid hydrolysis most of the resulting ammonia would be converted into salts (e.g. hydroxylamine hydrochloride, ammonium chloride) that were not completely removed from the purified hydrolysate before IELC analysis. Ammonia eluted from the cation-exchange column as a peak very close to Phe, and the ammonia peak may have interfered with the determination of the ^{15}N enrichment of Phe.

Although the separations of amino acid in the hydrolysate should be capable of improvement, these results are original with regard to the samples analysed. Moreover, this procedure (comparison of the ^{15}N distribution across individual or groups of amino acids and between casein and plasma proteins) may constitute a very powerful tool for studying nitrogen metabolism in the lactating ruminant. The separation of amino acids could probably be improved by using a longer column and a smaller resin bead. A smaller internal diameter column would also have favoured the chromatographic isolation of amino acids, but was not compatible with these preparative methods. In addition, it will be necessary in future to validate the ion-exchange methods for amino acid isolation by analysing the ^{15}N enrichment of a range of ^{15}N-labelled amino acid standards before and after IELC isolation to see whether the LC affected the ^{15}N values.

^{15}N labelling characteristics of milk casein and plasma protein amino acids

The experiment was designed to determine the ^{15}N enrichment levels and distribution in casein and plasma proteins (15NCA and 15NPP). Since these samples were extracted from pooled milk and pooled blood samples from different cows, the ^{15}N distribution in individual or groups of amino acids should contribute to our understanding of nitrogen transport in the lactating cow.

This study has demonstrated high ^{15}N incorporation in all individual amino acids and groups in 15NCA and 15NPP (Table 2). In contrast to ^{13}C-labelled milk proteins (Boirie *et al.* 1995; Rubert-Aleman *et al.* 1997), in which the label is mainly [^{13}C]Leu, oral administration of an ^{15}N-labelled ammonium salt to lactating cows resulted in a uniform distribution of the tracer within plasma and milk proteins (Table 2). This must be because all absorbed microbial amino acids are synthesized *de novo* in the rumen from carbon skeletons and peptides or ammonia (Journet *et al.* 1995), [^{15}N]ammonia in this study.

Comparisons between casein and plasma protein ^{15}N enrichments help to clarify the different amino acid precursor pools that these tissues (liver and mammary gland) use for protein synthesis. 15NCA represented an integrated sample from 36–96 h of the dosing period while 15NPP was extracted at 72 h after the tracer administration. Thus, the absolute values in Table 2 are not directly comparable but they provide relative information on the patterns of amino acids. The much lower

(3·5–7·7-fold) enrichments in 15NPP compared with 15NCA (Table 2) suggested considerable intracellular dilution at the site of liver protein synthesis. In fact, these dilutions may be even larger when one considers that the enrichment of the amino acids presented to the liver would have been much higher than those in the arterial supply to the mammary gland. Furthermore, the different plasma and milk labelling rates and enrichment levels for the time period between 24 and 96 h after the tracer supply probably indicated that plasma protein synthesis and turnover followed different time courses from those of milk proteins (Fig. 1). However, it is important to note that the ratios of the ^{15}N contents for plasma protein and casein (15NPP : 15NCA) were similar (0·13–0·17, Table 2) for the essential amino acids (i.e. His, Lys, Phe, Ile, Leu) while for the most part the ratios for the non-essential amino acids were greater (0·21–0·28, Table 2). This is probably due to the transfer of labelled nitrogen to the non-essential amino acids in the liver. These results demonstrated a certain homogeneity of inorganic ^{15}N metabolism from the rumen to the mammary gland of different lactating cows. This observation was supported by the small SE (Fig. 1), indicating comparable ^{15}N enrichment rates for both plasma and milk for the different lactating cows. For the plasma enrichment rates, the SE were so small that many are not visible in Fig. 1.

Although ^{15}N enrichment and distribution appeared to be uniform (i.e. to the same extent) in the proteins studied, the specific ^{15}N enrichments of some individual amino acids are worthy of comment. In all proteins studied, Glx appeared to be more enriched than all other amino acids (Table 2). Glu and its amine (Gln) are known to be involved in transamination reactions, as nitrogen donors in nitrogen metabolism and the urea cycle, and as ammonia carriers in the blood. These amino acids also play a key role in the metabolism of the other amino acids. Perhaps the rate of absorption of microbial Glu and Gln was greater than their accumulation from dietary protein degradation, relative to other amino acids. In contrast, Phe enrichment was particularly low in both 15NCA and 15NPP. If this is a real effect, it suggests either Phe absorption from intact derived protein (rumen by-pass), which will be unlabelled, or dilution in the body with the Phe from endogenous sources (muscle, skin, etc). That this dilution occurred in both casein and plasma protein suggests that the portal vein Phe enrichment was diluted, pointing towards a dilution effect from dietary by-pass proteins. However, this result could also reflect an experimental artifact. Since the ratio of 15NPP : 15NCA Phe enrichment (Table 2) was similar to those of other essential amino acids, it is likely that there was a systematic dilution of the Phe label (i.e. from the ammonia peak during ion exchange) or that Phe was diluted in the body. Arg was also one of the less enriched amino acids in 15NCA. This result could be explained by the route of Arg synthesis in the urea cycle, resulting in the contribution of unlabelled or less enriched nitrogenous molecules to the Arg pool. Another amino acid group can be distinguished, that containing aliphatic molecules with a single amino function (Asp, Thr, Ser, Gly, Ala, Val, Met, Ile, Leu), whose ^{15}N enrichment was high in both protein groups.

Analysis of the ^{15}N isotopic content of casein or plasma protein amino acids by IRMS could also be used for the ^{15}N-labelled amino acid analysis of microbial proteins, thus providing a more precise technique for studying microbial amino acid metabolism from the rumen to the mammary gland. Further studies are necessary to improve the separation of amino acids and to carry out physiological studies: for example, to determine the actual ^{15}N-labelled amino acid absorption in portal blood and availability for casein synthesis in the mammary gland. Such studies appear possible and a preliminary step has been made with the comparison of ^{15}N

distribution in caseins and plasma proteins. Thus, the isotopic analysis of individual or groups of ^{15}N-labelled amino acids represents a potentially valuable tool for physiological investigations in ruminants. Furthermore, this new research tool should permit new developments in nutrition as well as in dairy studies.

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