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Original article

Nitrogen-15 labelling of the nitrogenous fractions of milk using oral administration of labelled ammonium sulphate in the diet

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Summary — In an attempt to obtain ^{15}N-labelling milk proteins for digestive studies in humans, labelled ammonium sulphate [$(^{15}NH_4)_2SO_4$] was incorporated in the diet of dairy cattle. Cows were fed a total mixed ration including 300 g $(NH_4)_2SO_4$. In trial 1, three cows received a single dose of 50, 150 or 300 g $(^{15}NH_4)_2SO_4$ in substitution for an equal dose of non-labelled product. In trial 2, three cows received 300 g $(^{15}NH_4)_2SO_4$ the first day plus 150 g 24 h and 150 g 48 h after the initial supply. Feed intake, individual milk yields and nitrogen (N) content of the milk were recorded. Kinetic evolution of the ^{15}N enrichment of total (TN) soluble (SN) and casein (CN) nitrogen was followed. In trial 1, ^{15}N enrichment of protein reached maximum levels 36 h after the supply of labelled product. In trial 2, the administration of repeated doses of ^{15}N ammonium sulphate resulted in an enrichment plateau from 36 h to 84 h after beginning labelled product administration. The respective levels in ^{15}N enrichment of TN, SN and CN have been discussed.

^{15}N labelling / nitrogen fraction / milk

Résumé — Marquage au ^{15}N des fractions azotées du lait par apport oral de sulfate d'ammonium dans la ration. En vue d'effectuer des études sur la digestion des protéines laitières chez l'humain, du lait marqué au ^{15}N a été produit en incorporant du sulfate d'ammonium marqué dans la ration de vaches laitières. Les vaches ont reçu une ration complète distribuée à volonté, contenant 300 g de sulfate d'ammonium. Dans l'essai 1, trois vaches ont reçu une dose unique de 50, 150 ou 300 g de sulfate d'ammonium marqué en substitution à une dose égale de produit non marqué. Dans l'essai 2, trois vaches ont reçu 300 g de sulfate d'ammonium le 1er jour, puis 150 g 24 h et 150 g 48 h après le premier apport. Les quantités d'aliment ingérées, la production laitière et la composition azotée du

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lait ont été mesurées individuellement. Les cinétiques d'évolution de l'enrichissement en ^{15}N des fractions azote total, azote soluble et azote caséique du lait ont été suivies. Dans l'essai 1, l'enrichissement en ^{15}N du lait est maximal 36 h après ingestion du produit marqué, respectivement 115, 491 et 980 ‰ pour les doses 50, 150 et 300 g. Dans l'essai 2, l'administration de doses répétées de sulfate d'ammonium marqué au ^{15}N amène à un plateau d'enrichissement observé de 36 à 84 h après la première distribution du produit marqué. Dans les deux essais, l'enrichissement de la fraction azote soluble (SN) du lait est plus rapide que l'enrichissement de l'azote total (TN). La quantité totale de ^{15}N retrouvée dans le lait au cours des 7 à 8 jours suivant le premier apport représente en moyenne 16 % de ^{15}N administré.

enrichissement en ^{15}N / fraction azotée / lait

INTRODUCTION

Milk proteins represent a high part of dietary proteins for humans and numerous researchers are interested in the study of their digestive fate. As stable isotope labelling techniques are suitable to directly distinguish endogenous and exogenous protein fractions in the intestinal lumen (Delange et al, 1989), isotopic labelling of milk proteins is considered one of the most important stages for further nutritional investigations in human. The labelling must be sufficient to meet the requirements of subsequent nutritional studies. Currently, the best way to obtain homogeneously marked products has been via the synthesis of milk proteins by ruminants (Gruhn and Thelemann, 1973; Bequette et al, 1994; Mahé et al, 1994a). Though stable isotopes could be administered intravenously (Oddy et al, 1988; Bequette et al, 1994; Boirie et al, 1995), or directly in the rumen via a fistula (Mahé et al, 1994a), it was easier and more harmless for the animals to incorporate them into the diet. However, the production of labelled milk proteins required the research of food easily enriched in stable isotope. Moreover, as the use of feed by ruminants differs according to their nature, this food must also have a sufficient use yield. As ^{13}C-labelled protein information could be diluted either by the carbon skeleton or by derivatization, and as ^{15}N isotopic information also allowed nitrogenous metabolism to be followed, ^{15}N-labelled products were more convenient for such studies. Thus, the purpose of the present work was: 1) to study the efficiency of labelling milk proteins with ^{15}N from labelled ammonium sulphate incorporated into the diet of dairy cattle; 2) to determine the most efficient treatment for adequate labelling of the milk proteins and production of large amounts of high labelled proteins to carry out digestive studies.

MATERIALS AND METHODS

Animals

Trial 1

Three lactating cows of Prim' Holstein breed were chosen for their milk production (average for the 2 weeks before treatment: 25.9, 32.9 and 26.3 kg/day). One cow was in second lactation, one in third lactation and the third was in fourth lactation. At the beginning of the trial, the cows were 30, 97, and 95 days in milk. They were housed in free stalls and fed a total mixed ration ad libitum once each morning. This ration contained 68.1% (dry matter, DM basis) maize silage, 3.5% chopped straw, 17.7% barley, 8.9% formaldehyde-treated soybean/rapeseed (50:50), plus 300 g ammonium sulphate and two mineral supplements (0.6% $CaCO_3$ and 0.5% of a mineral with 14% Ca and 14% P). The diet was replenished and orts were collected once per day before feeding. Individual feeding took place via electronic feed gates with each cow fitted with and electronic transponder that allowed access to one of the gates. The proportions of different feeds in

the diet were chosen so that there was a good balance between energy and crude protein supply and between rumen degradable and undegradable proteins. For the average DM intake (20.4 kg DM/cow), the total mixed ration provided 1.58 Mcal net energy for lactation and 14.1% crude protein per kg DM, with 40% rumen undegradable protein (estimated from the values of N theoretical degradability in sacco; INRA, 1988).

Trial 2

Three lactating cows of Prim' Holstein breed were used in this trial; two were in second lactation, and one in third lactation. Their milk production for the 2 weeks before treatment was 27.8, 34.0 and 34.5 kg/day. At the beginning of the trial, the cows were 57, 96 and 58 days in milk. They were housed in free stalls and fed a total mixed ration ad libitum once a day in the morning. This ration consisted of 66.5% (DM basis) maize silage, 4.5% chopped straw, 15.6% wheat, 2.2% soyabean meal, 9.4% formaldehyde-treated soybean/rapeseed (50:50) and a mineral supplement (1.8%). For a DM intake of 20 kg/cow/day, 300 g ammonium sulphate were added to the diet, and the quantity was adjusted according to intake. The diet was replenished and orts were collected once per day before feeding. Individual feeding took place as for trial 1. For the average DM intake (19.2), the total mixed ration provided 1.60 Mcal net energy for lactation and 15.0% crude protein per kg DM, with 41% rumen undegradable protein (estimated from the values of N theoretical degradability in sacco; INRA, 1988).

^{15}N-labelling procedures

After 3 weeks of transition and adaptation to the total mixed ration, the cows in trial 1 respectively received a single dose of 50, 150, or 300 g $(^{15}NH_4)_2SO_4$ (10 atoms % isotopic enrichment) in substitution for an equal dose of non-labelled $(NH_4)_2SO_4$ and in trial 2, 300 g $(^{15}NH_4)_2SO_4$ on the first day plus 150 g $(^{15}NH_4)_2SO_4$ 24 h and 150 g $(NH_4)_2SO_4$ 48 h after the initial supply.

With the aim of improving $(^{15}NH_4)_2SO_4$ intake, the ration was then fed twice a day. Three-quarters of the DM intake (average of DM intake during the previous week) were provided in the morning, and if there were no orts the complement plus 10% were supplied in the evening. Labelled and non-labelled ammonium sulphate was integrated into the total mixed ration supplied in the morning.

Sampling and analysis

Feed intakes and orts were measured daily 5 days per week for each cow. Individual milk yields were automatically recorded at each milking. In trial 1, milk samples were taken from each cow during the milking that preceded ^{15}N administration, from the six following milkings and from every second milking until the 14th milking after treatment (milking interval: 12 h on average). In trial 2, milk samples were taken from the milking preceding the first ^{15}N administration, from the eight following milkings, and the 10th, 12th and 16th milking after the initial supply of $(^{15}NH_4)_2SO_4$.

Aliquots from each sample were used to measure total nitrogen (TN) and soluble nitrogen (SN) content after casein precipitation in acetic acid (1 N, pH 4.6) using the method of Rowland (1938) (automatic titration on a Vapodest 6; Gerhardt, Bonn, Germany). Samples were freeze-dried before ^{15}N-enrichment (E_{TN} and E_{SN}) determination by mass spectrometry (Finnigan-Mat Delta E spectrometer; Orsay, France) as described below.

The overall ($^{15}N/^{14}N$) isotope ratios for organic matter were determined by isotope ratio mass spectrometry. An aliquot of dry sample was burnt in the combustion unit of an elementary analyzer (NA 1500; Fisons, Manchester, UK). In the presence of pure oxygen, the organic compounds were converted into CO_2, N_2, NO_x and H_2O during passage through an oxidation oven. While passing through a reduction reactor NO_x was reduced into N_2 and any oxygen released from the oxidation reactor was removed. The combustion water was trapped by anhydrone $[Mg(ClO_4)_2]$ on a column, and for the ^{15}N measurements all the CO_2 was removed by passage through sodium hydroxide on support. The analyzer was coupled via a splitter valve to an isotope ratio mass spectrometer (Delta E; Finnigan Mat, Orsay, France; or Optima; Fisons, Manchester, UK) and the N_2 content of the helium stream analyzed for the relative contributions at masses 28, 29 and 30.

The N content and the ^{15}N-enrichment of casein (CN, E_{CN}) were calculated as follows:

$$CN = TN - SN$$

$$E_{CN} = \frac{(E_{TN} \times TN) - (E_{SN} \times SN)}{CN}$$

RESULTS

Feed intake and milk yield

For the sampling period, the cows had an average intake of 20.4 (± 4.6) kg of DM/day in trial 1 and 19.2 (± 2.5) kg DM/day in trial 2. In trial 1, they produced 23.1 (± 1.9), 25.0 (± 2.0) and 30.5 (± 1.5) kg milk per day respectively for the doses 50, 150 and 300 g of $(^{15}NH_4)_2SO_4$ and in trial 2, 30.4 (± 2.2), 32.7 (± 1.5) and 34.3 (± 6.0) kg milk/day.

Nitrogen content of milk

The TN, SN and CN contents of the milk collected are presented for each trial and each cow in table I. The N contents were higher in trial 2 than in trial 1 for each fraction, but the proportion of casein in TN was similar (80.8% in trial 1 and 79.6% in trial 2).

Nitrogen enrichment of milk

Trial 1

The evolution of the isotopic labelling of TN over the period and for the different doses is shown in figure 1a. The enrichments increased to a maximum reached at 36 h after treatment of 115, 491 and 980‰ of the air labelling, respectively. The higher enrichment corresponded to a labelled N content of 7.25 g ^{15}N/kg N; 120 h after treatment (10th milking), all enrichment values were lower than 150% whatever the dose.

After the supply of $(^{15}NH_4)_2SO_4$, the enrichment of SN (E_{SN}) increased to a maximum reached at 36 h after treatment for doses 50 and 150 g, and at 48 h after treatment for dose 300 g (fig 2a). However, whatever the dose, at 12 and 24 h after treatment E_{SN} was higher than E_{TN}: on average, E_{SN} represented respectively 214 and 120% of E_{TN} (table II). Thirty-six h after treatment, E_{SN} decreased to 95 and 76% of E_{TN} respectively for 150 and 300 g of $(^{15}NH_4)_2SO_4$ supplied, but was still higher than E_{TN} (114%) for the dose of 50 g. After 48 h, the values of E_{Nsol} and E_{TN} were close

Table I. Nitrogen content of milk.
Teneurs en azote du lait.

	Trial 1 [1]			*Trial 2* [2]		
	50 g	*150 g*	*300 g*	*A*	*B*	*C*
TN content (g N/L)	4.42 (± 0.14)	4.86 (± 0.25)	5.21 (± 0.23)	4.95 (± 0.32)	5.83 (± 0.30)	4.88 (± 0.29)
SN content (g N/L)	0.79 (± 0.10)	0.90 (± 0.07)	0.89 (± 0.08)	1.01 (± 0.05)	1.14 (± 0.08)	1.04 (± 0.05)
CN content (g N/L)	3.57 (± 0.15)	3.93 (± 0.26)	3.36 (± 0.21)	3.94 (± 0.28)	4.69 (± 0.24)	3.84 (± 0.28)
CN/TN (%)	81.9 (± 2.4)	81.3 (± 1.6)	79.1 (± 1.2)	79.5 (± 0.9)	80.5 (± 0.7)	78.7 (± 10.3)

[1] Dose of labelled ammonium sulphate.
[2] Cow.

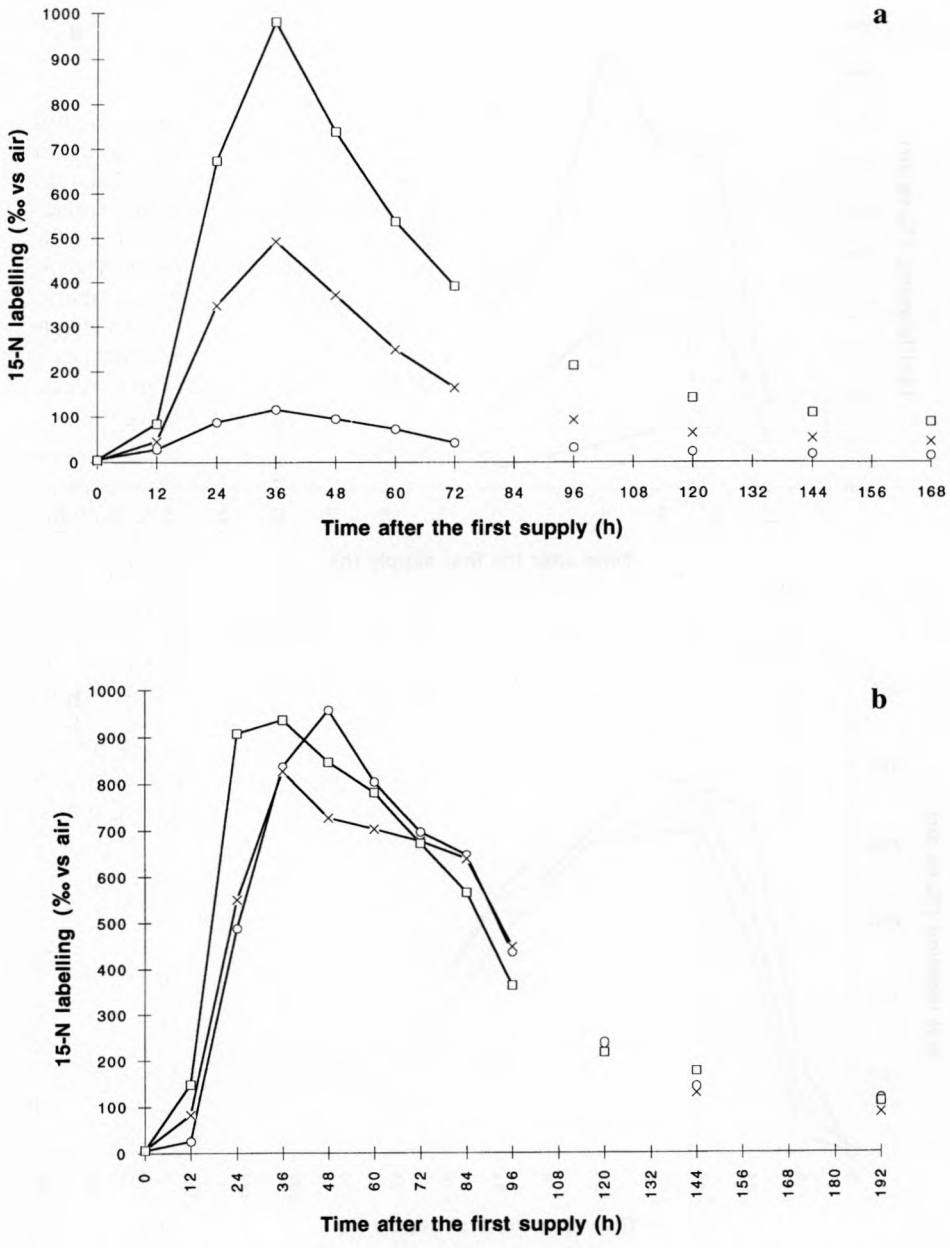

Fig 1. ^{15}N labelling of the total nitrogen in milk. **a.** A single dose of labelled ammonium sulphate; —○—: 50 g; —×—: 150 g; —□—: 300 g ^{15}N ammonium sulphate. **b.** Supply of 300 + 2 × 150 g of labelled ammonium sulphate; —○—: cow A; —×—: cow B; —□—: cow C.

Marquage au ^{15}N de l'azote total du lait. ***a.*** *Apport d'une dose unique de sulfate d'ammonium marqué ;* ***b.*** *Apport de 300 + 2 × 150 g de sulfate d'ammonium marqué.*

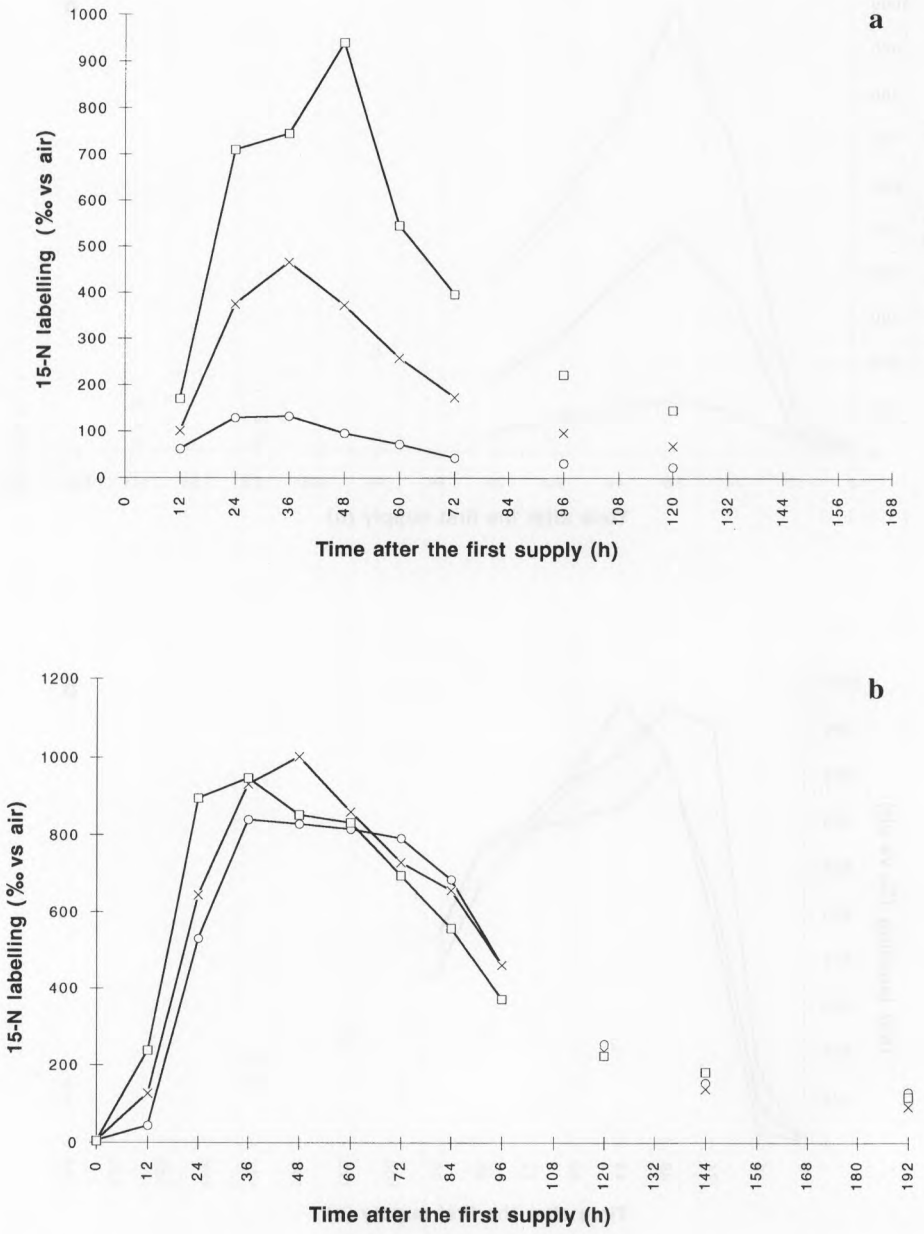

Fig 2. ^{15}N labelling of the soluble nitrogen of milk. **a.** A single dose of labelled ammonium sulphate; —○—: 50 g; —×—: 150 g; —□—: 300 g ^{15}N ammonium sulphate. **b.** Supply of 300 + 2 × 150 g of labelled ammonium sulphate; —○—: cow A; —×—: cow B; —□—: cow C.

Marquage au ^{15}N de l'azote soluble du lait. ***a.*** *Apport d'une dose unique de sulfate d'ammonium marqué ;* ***b.*** *Apport de 300 + 2 × 150 g de sulfate d'ammonium marqué.*

(between 98 and 105%), except for the dose of 300 g $(^{15}NH_4)_2SO_4$ (128%) (table II).

Regarding E_{TN}, E_{CN} reached maximal levels at 36 h after treatment; these were 113, 497 and 1039‰ respectively for the doses 50, 150 and 300 g $(^{15}NH_4)_2SO_4$ (fig 3a). At 12 and 24 h respectively, E_{CN} represented on average 71 and 95% of E_{TN}. These results were consistent with the higher levels of E_{SN} than of E_{TN} at the first two milkings. Afterwards, E_{CN} were similar (E_{CN}/E_{TN} varied between 98 and 106%) except for the dose of 300 g at 48 h (table II).

Trial 2

E_{TN} increased after the first supply, and the maximum was obtained 36 or 48 h after the initial supply of $(^{15}NH_4)_2SO_4$ (826 to 955‰ for the different cows; fig 1b). The repeated supply of $(^{15}NH_4)_2SO_4$ allowed the ^{15}N enrichment to be maintained at over 500‰ 36 h after the last supply, ie, 84 h after the first supply. Eighty-four h after the last supply, the average level of ^{15}N enrichment for the three cows was still 150‰.

The kinetics of ^{15}N enrichment of SN were close to those of TN; the more significant differences were observed just after the initial supply (fig 2b). Twelve h after the first supply, the ^{15}N enrichment of SN was, on average, 1.6 times higher than the ^{15}N enrichment of TN. Then the ^{15}N enrichment levels became closer, but E_{SN} was always higher than E_{TN} (table II). The ratio of ^{15}N enrichment of SN and TN varied between 103 and 110% on average for the three cows.

Regarding E_{TN}, E_{CN} attained maximal levels between 36 and 48 h after the initial supply of $(^{15}NH_4)_2SO_4$ (800 to 989‰ for the different cows) (fig 3b). Twelve h after the first supply of $(^{15}NH_4)_2SO_4$, E_{CN} constituted only 85% of E_{TN}, consistent with the higher level of E_{SN} compared to E_{TN} at the same time. Afterwards, the kinetics of the ^{15}N enrichment of CN were very close to

Table II. Ratios of soluble and casein nitrogen ^{15}N-enrichment in TN enrichment.
Rapports entre les enrichissements en ^{15}N de l'azote soluble et de l'azote caséique par rapport à l'enrichissement de l'azote total.

Time[1] *(h)*	*Enrichment ratios*							
	E_{SN}/E_{TN} (%)				E_{CN}/E_{TN} (%)			
	Trial 1, dose (g)			*Trial 2*	*Trial 1, dose (g)*			*Trial 2*
	50	*150*	*300*	*Average*	*50*	*150*	*300*	*Average*
12	220	222	201	161	73	66	75	85
24	147	108	106	108	89	98	98	98
36	114	95	76	105	98	101	106	99
48	101	100	127	108	100	100	92	98
60	100	104	102	110	100	99	99	97
72	101	105	101	108	100	99	100	98
84	–	–	–	103	–	–	–	99
96	98	104	104	104	100	99	99	99
120	103	106	102	105	99	98	99	99
144	–	–	–	106	–	–	–	98
192	–	–	–	108	–	–	–	98

[1] Time after initial supply.

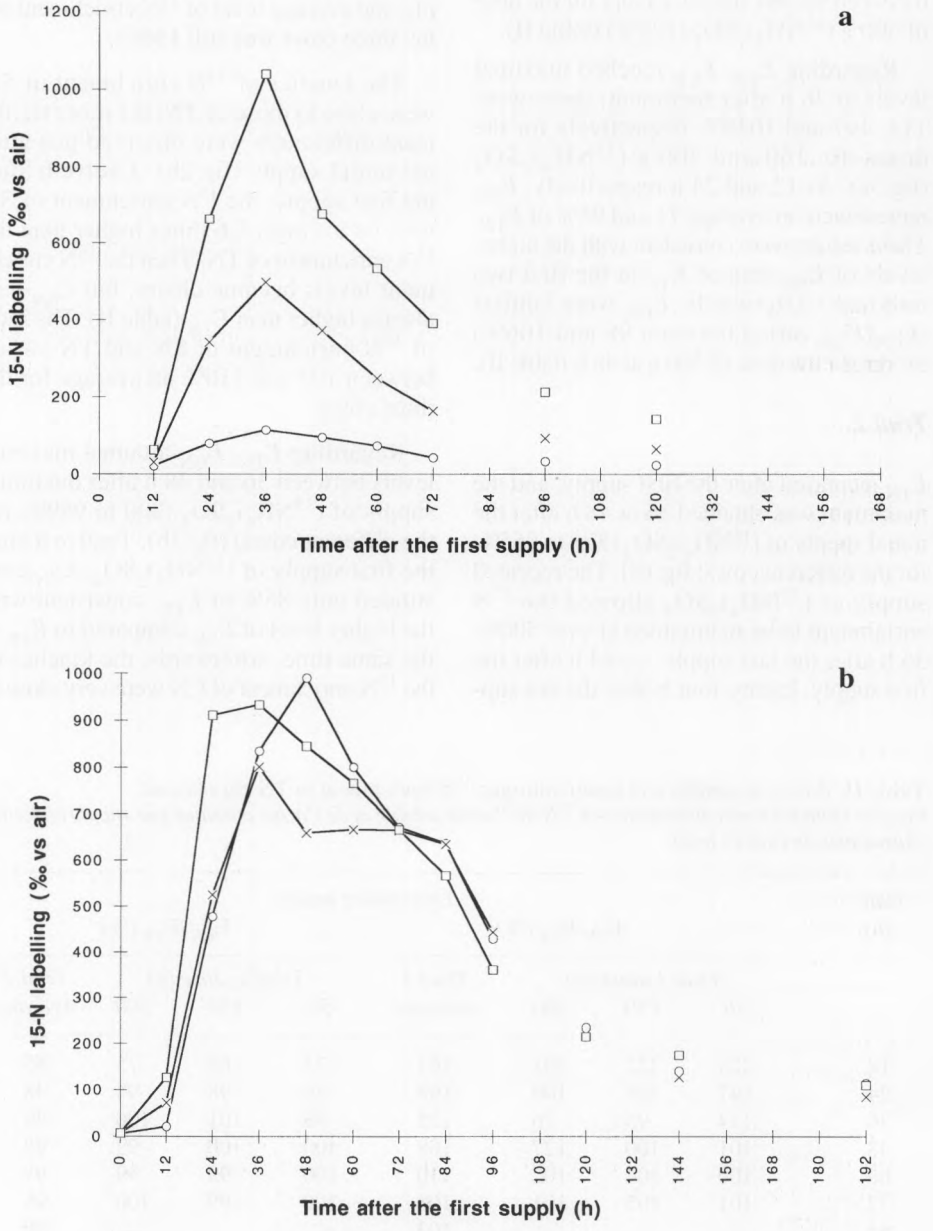

Fig 3. ^{15}N labelling of the casein nitrogen of milk. **a.** A single dose of labelled ammonium sulphate; —○—: 50 g; —×—: 150 g; —□—: 300 g ^{15}N ammonium sulphate. **b.** Supply of 300 + 2 × 150 g of labelled ammonium sulphate; —○—: cow A; —×—: cow B; —□—: cow C.

Marquage au ^{15}N de l'azote caséique du lait. ***a.*** *Apport d'une dose unique de sulfate d'ammonium marqué ;* ***b.*** *Apport de 300 + 2 × 150 g de sulfate d'ammonium marqué.*

those of TN: the ratio of ^{15}N enrichment of CN and TN varied between 97 and 99% on average for the three cows.

Amounts of labelled protein

The amount of total ^{15}N labelled protein produced per cow, with an enrichment of ≥ 140‰, was in trial 1, 1.7 kg for the 150 g dose and 3.2 kg for the 300 g dose and in trial 2, 5.3, 5.7 and 5.9 kg respectively for the three cows.

In trial 1, the amount of ^{15}N recovered in the milk collected during the 7 days following the supply represented 7% of the total administered ^{15}N for the 50 g dose and 15 and 17% for the 150 and 300 g doses. In trial 2, the amount of ^{15}N recovered in the milk collected during the 8 days following the initial supply constituted between 14 and 17% of the total administered ^{15}N for the three cows.

DISCUSSION AND CONCLUSION

As ^{15}N labelled milk proteins were produced from absorbed microbial ^{15}N amino acids which were synthesized from inorganic ^{15}N administered in the diet, we demonstrated in both trials a high ^{15}N incorporation in all studied nitrogenous milk fractions, ie, total nitrogen, casein and whey protein. In fact, we did not measure the ^{15}N labelling directly from the casein. However, the assessment of ^{15}N labelling from the casein in an average milk, made up from a milk mixture (third to the eight's milking after the first supply of ^{15}N ammonium sulphate) in trial 2, provided a similar value to that obtained by calculation from whey protein (respectively 680 and 710‰).

Results of the first trial in particular indicated the minimal dose required of ^{15}N ammonium sulphate to obtain sufficient enriched milk proteins for metabolic investigations in humans. As an enrichment of 140‰ is needed to perform digestibility experiments with ^{15}N labelled exogenous proteins (Mahé et al, 1994b), we noted that for a single dose of 150 g, ^{15}N enrichment of milk proteins was > 140‰ for milkings at 24 h, 36 h, 48 h, 60 h and 72 h (figs 1a, 3a). This means that as early as 24 h after a single oral administration of 150 g ^{15}N ammonium sulphate, ^{15}N milk can be used for nutrition studies in humans. This result can be linked to the observations of Mahé et al (1994a) who found a ^{15}N milk protein enrichment of 145‰ in a cow which over 5 days received a dose of 25 g ^{15}N amonium sulphate, ie, a total dose of 125 g. However, a single dose of 50 g resulted in a maximum ^{15}N enrichment of 115‰ at 36 h after beginning administration (fig 1a), and thus was < 140‰. On the contrary, administration of a single dose of 300 g resulted 36 h later in ^{15}N milk enrichment, a level that was twice as high as the natural ^{15}N enrichment of the air. We therefore demonstrated for the first time a high enrichment level in milk proteins. Moreover, this labelling was performed under normal feeding conditions in lactating cows.

The administration of repeated doses of ^{15}N ammonium sulphate (trial 2) resulted in an enrichment plateau of ~ 750‰ and 740% in total milk N (fig 1b) and in milk casein (fig 3b) respectively. This enrichment plateau could be observed from 36 to 84 h after beginning ^{15}N ammonium sulphate administration. Enrichment plateaus were previously observed by Boirie et al (1995), who obtained a plateau from 8 to 32 h after a continuous (1-^{13}C)-leucine intravenous infusion, as well as by Mahé et al (1994a) who detected an enrichment plateau after five successive daily doses (25 or 50 g) of ^{15}N ammonium sulphate in lactating cows. In agreement with our observations, these authors showed that milk labelling rapidly dropped once the administration had stopped. We also noticed that the ^{15}N enrichment kinetics of the different milk nitrogenous fractions studied were quite similar for the

three cows (figs 1b, 2b and 3b), thus indicating a strong homogeneity of inorganic ^{15}N metabolism from the rumen to the mammary gland in lactating cows.

The overall tracer recoveries which averaged 15–16% in both experiments were rather high, since Boirie et al (1995) found ~ 24% recovery when the tracer was infused directly in the jugular vein. In other respects, these yields were good considering the transformation yield of the SN of feed into milk protein by the cow (INRA, 1988). They were also consistent with the transformation yields reported by Mertens et al (1994). Furthermore, these results were higher than those obtained in preceding studies on dairy goats (Brun-Bellut and Blanchard, 1994). The latter authors found with a single supply of 20 g of labelled urea (5 atoms %) that 11% of the total administered ^{15}N was recovered in the TN fraction of the milk collected from ten milkings after treatment.

^{15}N incorporation in proteins and in caseins was slower than the enrichment of SN. Non-protein N fraction mainly explained the high enrichment of SN just after ^{15}N ammonium sulphate administration. In fact, E_{NPN}, which was measured in trial 1, was 4.9 times higher at 12 h and twice higher at 24 h than E_{TN}, and NPN constituted 24% of SN. ^{15}N labelling of the non-protein fraction was certainly due to the amount of ^{15}N ammonium sulphate included in the diet and in excess as regards microbial protein synthesis. This excess could partially be eliminated in urea, which represents at least 50% of NPN in milk.

Finally, in contrast to Mahé et al (1994a) and Boirie et al (1995), who used surgically prepared cows to produce isotope-enriched milk, we present here adequate means of obtaining large amounts of ^{15}N enriched milk proteins with cows reared under normal conditions and without surgical intervention. Indeed, in both experiments we decided to provide the diets with proportion of SN supplied by $(NH_4)_2SO_4$ that was not too important and similar to that used on farms. ^{15}N-labelled milk protein production now appears to have been well investigated, and can easily be forecast, as ^{15}N enrichment of milk depends on the administered ^{15}N ammonium sulphate dose. ^{15}N casein or ^{15}N individual caseins as well as ^{15}N whey proteins could therefore be considered as new research tools which will certainly enable new developments both in nutrition and in dairy processes.

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