Use of naturally enriched mixed food in ^{13}C breath tests applied in young sucking calves

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Utilization of three milk diets including cream, casein or whey, each naturally labelled with ^{13}C (1 mmol ^{13}C excess) from C_4 sources, by six young male calves of the Deutsche Fleckvieh breed was investigated in a Latin-square split-plot design. Each milk diet was examined under resting conditions and during a short period of physical exercise on a treadmill. $\delta^{13}C$ values (‰) in carbon dioxide in expired air were measured at intervals of about 1 h during 6·5 h after food intake. Expired air samples for CO_2 isolation, subsequent isotopic analysis, measurement of CO_2 production and respiratory quotient were taken at about hourly intervals and ^{13}C recovery rates over 6·5 h were calculated. Feeding milk containing enriched milk casein, cream, or whey resulted in maximal significant ^{13}C enrichments over background ($\delta^{13}C$) in CO_2 of +1, +2·4 and +2·2‰, and recovery rates of 3·6, 9·9 and 12·2% respectively. This comparison shows the different kinetic behaviour of the main nutrients during the oxidation in tissues. The short exercise period (5 min at 1 J/s per kg body-weight +5 min at 2 J/s per kg body-weight) did not influence the recovery rates significantly. However, after 10 min of muscular exercise there was a brief decrease in $\delta^{13}C$ value of expired air which disappeared within the first 5 min of rest. These experiments demonstrate for the first time the applicability of the ^{13}C breath test with naturally enriched diets in animal nutrition research and that quantitative results may be obtained.

^{13}C breath test: Naturally-enriched milk diet: Physical activity

Investigations with carbon-labelled material on the metabolism of nutrients or the biosynthesis of body substances in animal nutrition are commonly performed with ^{14}C (Linzell, 1974), while the stable isotope ^{13}C is not used very often. A reason for this may be the higher costs of stable-C-labelled substrates, and the ease of measuring the radioactivity of a tracer without any separation from the matrix. Furthermore, the scintillation technique is quite widespread and based on an extensive literature. To avoid the hazards of radioactivity, stable isotope tracers are widely used with humans, e.g. in the so-called ^{13}C breath test used with medical, pharmacological and nutritional problems (Matthews & Bier, 1983; Schmelz & Schmidt, 1984; Klein & Klein, 1985; Ghoos *et al.* 1986*a*).

Recently studies in human nutrition have been carried out using 'naturally labelled' substrates taking advantage of the higher natural ^{13}C enrichment of C_4 plants (maize, sugar cane, sorghum; $\delta^{13}C$ −15 to −9‰) in contrast to C_3 plants (potatoes, European cereals, vegetables; $\delta^{13}C$ −30 to −22‰) (Hatch & Slack, 1970; O'Leary, 1981). $\delta^{13}C$-value (‰) is the deviation of the $^{13}C:^{12}C$ ratio in a sample relative to the carbonate standard (Pee Dee Belemnite (PDB), SC, USA). The carbon isotope ratio of PDB is 0·0112372. $\delta^{13}C$ values are calculated according to $\delta^{13}C$ (‰) = $[[(^{13}C:^{12}C)_{sample} - (^{13}C:^{12}C)_{PDB}]/(^{13}C:^{12}C)_{PDB}] \times 1000$.

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In most cases, ^{13}C breath tests with these substrates have been performed with the easily available carbohydrates from C_4 plants (Lacroix *et al.* 1973; Mosora *et al.* 1976, 1981; Ravussin *et al.* 1980; Duchesne *et al.* 1982; Krzentowski *et al.* 1983). The application of secondary 'naturally labelled' nutrients, e.g. from milk of cows fed with C_4 plants, in the ^{13}C breath test have been reported recently (Ghoos *et al.* 1986*b*, 1988; Hiele *et al.* 1988; Metges, 1988). However, corresponding research with animals is limited to a few measurements of correlations between ^{13}C abundances of diet and body fluids, tissues, and expired carbon dioxide which have been made on cattle, insects and mice (Minson *et al.* 1975; De Niro & Epstein, 1978; Jones *et al.* 1981; Pelletier *et al.* 1984; Tyrrell *et al.* 1984).

Other investigations have dealt with C kinetics of body product synthesis and ^{13}C content in expired air after feeding naturally labelled compounds to ruminants and poultry (Schroeder & Plavnik, 1984; Schroeder & Ben-Ghedalia, 1986; Boutton *et al.* 1988). In most cases quantitative aspects are lacking, especially with regard to the application of naturally labelled compounds which have been demonstrated very recently, opening up fundamental insights into the potential of this technique (Metges *et al.* 1990).

The objectives of the present work were: first, to apply the ^{13}C breath test with naturally labelled substrates to animal nutrition and to check its validity; second, to compare the oxidation of exogenous carbohydrates, lipids and proteins, supplied from a mixed diet; third, to study the influence of a brief period of physical exercise on the change in enrichment of expired CO_2. In order to avoid the problem of ^{13}C enrichment in expired CO_2 of ruminants (enrichment with ^{13}C is due to CO_2 production during rumen fermentation; Metges *et al.* 1990) young milk-fed calves were chosen as the test animals.

MATERIALS AND METHODS

Animals and feeding

Six male, unweaned young calves (age 3 weeks) of the Deutsche Fleckvieh breed with similar weights (mean 73 (SD 3·3) kg) were housed in an air-conditioned stall with a slatted floor (20°; 60% relative humidity). During the pre-experimental and the experimental periods diets were given at 07.30 and 16.00 hours in 5 kg portions in buckets at a temperature of 35°. Milk which was left in the buckets was weighed and the actual amounts consumed by the calves were recorded. Water was supplied *ad lib.*

Diet preparation

In order to supply differentially enriched raw milks for the preparation of diets, cows were fed on either pure C_3 or C_4 plant diets. Milk of each type was pooled for 3 d and was separated into cream, casein, and whey, representing predominantly lipid, protein, and carbohydrate fractions respectively. Hence, the C_3 and C_4 milks were centrifuged and the residual skim milks were turned sour with L-lactic acid to pH of 4·7. In order to preserve typical enrichment for each skim milk, the corresponding L-lactic acid from fermentation of C_3 or C_4 plant carbohydrates respectively ($\delta^{13}C$ −26·5 and −10·6‰) was used. After cooling to room temperature, the casein was isolated from whey by filtration through a cotton cloth. From these C_3 and C_4 milk fractions, three different milk diets, each containing one 'enriched' (C_4) and two 'unenriched' (C_3) milk fractions were prepared (Table 1). Diet P (protein) was obtained by mixing (g/kg) C_4 casein (118) with C_3 cream (236) and C_3 whey (646) to the final weight (5 kg); diet C (carbohydrate) included C_4 whey, C_3 casein and C_3 cream, and diet L (lipid) consisted of C_4 cream, C_3 casein and C_3 whey in the same ratio. In this way milk diets with approximately 32 g protein and 44 g fat/kg were obtained. In order to supply fresh diets during the whole experimental period, five

Table 1. *Composition and mean $\delta^{13}C$* values (‰) of experimental diets*

(Mean values and standard deviations)

	C4-milk fractions†		C3-milk fractions†			Experimental diets		
	Mean‡	SD	Casein	Whey	Cream		Mean§	SD
Casein	−14·0	0·2	−	+	+	P	−24·1	0·8
Whey	−14·5	0·5	+	−	+	C	−23·4	0·5
Cream	−16·2	0·4	+	+	−	L	−21·2	0·4

P, protein; C, carbohydrate; L, lipid; −, omitted; +, included.
* For details, see p. 43.
† Mean $\delta^{13}C$ value of whole C_3 and C_4 milk was −26·8 and −15·2‰ respectively.
‡ Mean of five sampling times each.
§ Mean of five preparations each.

separations of C_3 and C_4 milk, performed as described previously, were necessary (for corresponding mean $\delta^{13}C$ values see Table 1). Before the experiment the calves were equilibrated with a C_3-based sour milk ($\delta^{13}C$ −26·1‰) for 5 d in the morning and in the evening. This period allowed stabilization of the exhaled ^{13}C-enriched CO_2.

On experimental days the animals were given the enriched milk in the morning; in the evenings and between experimental days C_3 sour milk was fed (Table 2).

Experimental design

The experimental design was a 6 × 6 Latin square split-plot design (Gill, 1981), involving a repetition of six treatments (A–F; three diets, with or without locomotion) in six animals and six periods in a randomly chosen sequence (Table 2).

For technical reasons three treatments were performed with three animals on one experimental day. Consequently, to obtain a 6 × 6 Latin square two experimental days were pooled in each period, but each animal was treated on 1 d only of the period. To ensure total disappearance of enrichment from the particular preceding diet a delay of at least 1 d was kept between the experimental days.

Treatments A, C and E correspond to the feeding of diets P, C and L (see Table 1) respectively, with the calves at rest. Treatments B, D and F correspond to the feeding of diets P, C and L, with a brief physical exercise period (locomotion programme) within the same experimental day.

An experimental day was divided into seven intervals of approximately 1 h (starting after feeding in the morning), in which respiratory data and breath samples were collected. If the treatment included a locomotion programme, this was performed always within the third interval of the experimental day.

^{13}C breath tests

Expired air was collected in every interval after the test diet intake in the morning. For breath collection and breath data recording, a mobile Metabolic Measurement Cart (MMC) (Beckman, Anaheim, CA, USA) was used. The calves stood in the MMC and wore a light face mask connected by a flexible pipe to a one-way valve and a breath-mixing chamber inside the MMC. In the last 5 min of each interval, after flushing the mask and apparatus with breath appropriately, three 15 ml Vacutainers® (Becton Dickinson, Heidelberg, Germany) were filled with expired air by means of an adapter connected to the entrance to the mixing chamber. The remaining expired air entered the MMC, and

Table 2. *Experimental design*

Animal no.	Period ...	Total experimental period											
		1		2		3		4		5		6	
	Day no. ...	1	3	4	5	8	9	11	12	15	16	18	19
1		A	—	C	—	B	—	E	—	F	—	D	—
2		C	—	E	—	D	—	A	—	B	—	F	—
3		E	—	A	—	F	—	C	—	D	—	B	—
4		—	B	—	D	—	C	—	F	—	A	—	E
5		—	D	—	F	—	E	—	B	—	C	—	A
6		—	F	—	B	—	A	—	D	—	E	—	C

A, C, E, feeding diets P, C and L during rest; B, D, F, feeding diets P, C and L as well as brief physical exercise. For details of composition of diets P, C and L, see p. 44 and Table 1.

subsequently the CO_2 production ($\dot{V}_{CO_2}$), the oxygen consumption ($\dot{V}_{O_2}$), and the respiratory quotient (RQ) in each interval were calculated. The same collection procedure was applied during the locomotion programme.

Locomotion programme

In order to study the influence of a brief period of exercise upon the $^{13}C:^{12}C$ isotope ratio in expired air while ingesting a naturally enriched milk diet, a programme of locomotion on a treadmill, always at the third interval of the experimental days, was combined with each of the three diets (see p. 44). This programme consisted of two work periods between two rest periods.

After a rest of 5 min, level walking for 5 min at 1 m/s (1 J/s per kg body-weight) was followed by upward slope walking on a 6° gradient for 5 min (2 J/s per kg body-weight). A final rest period of 5 min finished the programme. While the calves were walking on the treadmill, $\dot{V}_{CO_2}$, $\dot{V}_{O_2}$, RQ and heart rate were recorded (Fig. 2).

During this programme expired air was collected just before exercise began (pre-exercise; corresponding to the regular third interval breath sample), in the last minute of the slope walking period (end-exercise), and at the end of the second rest period (post-exercise) (Table 3). Breath collection was done by means of the face mask as described previously (^{13}C breath tests).

Isotopic analysis

C isotopic analysis of the CO_2 in breath samples was performed by a fully-automated isotope-ratio mass spectrometer (SIRA 24; VG Isogas Ltd, Middlewich, UK) after cryogenic isolation of the CO_2 in a vacuum line (Auto sampler BAS 50; VG Isogas Ltd). Solid samples were combusted to CO_2 according to established procedures (Winkler & Schmidt, 1980). All sample measurements were duplicated. The isotope ratios were measured against a laboratory CO_2 standard. Results were automatically corrected for ^{17}O (Craig, 1957) and expressed in the international $\delta^{13}C$ (‰) notation *v.* Pee Dee Belemnite carbonate (PDB).

Calculations and statistics

From the course of $\delta^{13}C$ values in expired air over seven intervals (6·5 h), the total dose of ^{13}C administered (mmol ^{13}C excess) and the CO_2 production rates, the recovery of labelled C was calculated. Total dose of ^{13}C administered was computed from the actual amount of milk consumed and the ^{13}C excess in the particular diet.

Table 3. *Mean changes in $\delta^{13}C$* values (‰) in expired carbon dioxide in sucking calves after intake of C_3-based milk or experimental diets (protein (P), carbohydrate (C) and lipid (L))† during the locomotion programme‡*

(Mean values and standard deviations)

Exercise ... / Diets	No. of replicates§	δ^{13}C (‰) Pre Mean	Pre SD	End Mean	End SD	Post Mean	Post SD
C_3 milk	6	−25·91	0·66	−26·31	0·55	−25·93	0·80
Diet P	6	−26·08	0·52	−26·52	0·36	−25·98	0·27
Diet C	5	−24·47	0·78	−25·12	0·42	−24·68	0·52
Diet L	6	−25·21	0·60	−25·75	0·38	−25·13	0·66
Mean	23	−25·46[a]	0·86	−25·96[b]	0·67	−25·46[a]	0·78

[a, b] Means with the same superscript letter were not significantly different ($P = 0·05$).
* For details, see p. 43.
† For details, see p. 44 and Table 1.
‡ For details, see p. 46 and Table 2.
§ Replicates are missing if animals refused drinking.

Analysis of variance was performed using SAS program version 5 (SAS 1985*a*, *b*), run on the IBM-3090 system of the Bayerisches Staatsministerium für Ernährung, Landwirtschaft und Forsten. The data were pooled per treatment and interval, and computed with a mixed model including four main variables (animal, period, treatment and interval) as well as three two-way (animal × interval; period × interval; treatment × interval) interactions and one three-way (animal × period × treatment) interaction. Means per treatment, period and animal were tested against the three-way interaction as an error term (17 df). Means per interval were tested against the animal × interval two-way interaction (30 df). Resulting values from the locomotion programme (experimental and pre-experimental) were analysed separately with the previously described mixed model. For variables which occurred only once per animal and treatment (rates of recovery; administered ^{13}C excess in the diets), a two-factorial model (animal, treatment) was used. To investigate whether there were differences between the intervals within the treatment, an analysis of variance was conducted separately with a two-factorial model (animal, interval) for every treatment. Similarity of baseline δ^{13}C values between the treatments were tested by means of a single-factorial model (treatment). The effects of the main factors of all models described were tested by the *F* test. If the effect was significant ($P \leqslant 0·01$), comparison of means were made by the Student-Newman-Keuls test with a significance level of 5%.

RESULTS

Variations of the $\delta^{13}C$ value in expired CO_2 after feeding the C_3-based milk

In order to control and stabilize the natural ^{13}C background of the expired CO_2 during the experiments, an unenriched milk diet (δ^{13}C −26·1‰) based on C_3 sources was devised and offered for 5 d before starting with the test diets. After equilibration of the calves with this milk, its influence on the ^{13}C background of expired CO_2 during rest and during the programme of locomotion was investigated. During rest the maximal δ^{13}C shift observed was +0·3‰ compared with the baseline value, and the δ^{13}C values between intervals nos.

Table 4. *Mean* $\delta^{13}C$* (‰) *in expired carbon dioxide and respiratory quotient (RQ) values in sucking calves after intake of experimental diets (protein (P), carbohydrate (C) and lipid (L))† with (treatments B, D, F) and without (treatments A, C, E) the locomotion programme‡*

(Mean values for no. of replicates shown in parentheses; replicates are missing if animals refused drinking)

Interval no.	Treatment											
	A (*n* 5)		B (*n* 6)		C (*n* 4)		D (*n* 5)		E (*n* 6)		F (*n* 6)	
	$\delta^{13}C$	RQ	$\delta^{13}C$	RQ	$\delta^{13}C$	RQ	$\delta^{13}C$	RQ	$\delta^{13}C$	RQ	$\delta^{13}C$	RQ
1	$-26{\cdot}45^{c}$	$0{\cdot}80^{a}$	$-26{\cdot}40^{c}$	$0{\cdot}85^{a}$	$-26{\cdot}27^{c}$	$0{\cdot}85^{a}$	$-26{\cdot}28^{c}$	$0{\cdot}87^{a}$	$-26{\cdot}76^{e}$	$0{\cdot}78^{a}$	$-26{\cdot}55^{d}$	$0{\cdot}83^{a}$
2	$-26{\cdot}15^{b,c}$	$0{\cdot}79^{a}$	$-26{\cdot}13^{b,c}$	$0{\cdot}80^{b,a}$	$-25{\cdot}45^{b}$	$0{\cdot}79^{a,b}$	$-25{\cdot}03^{b}$	$0{\cdot}81^{b,a}$	$-26{\cdot}04^{d}$	$0{\cdot}78^{a}$	$-26{\cdot}10^{d}$	$0{\cdot}80^{a,b}$
3	$-25{\cdot}95^{b,a}$	$0{\cdot}78^{b,a}$	$-26{\cdot}08^{b,c}$	$0{\cdot}82^{a}$	$-25{\cdot}00^{b,a}$	$0{\cdot}79^{a,b}$	$-24{\cdot}47^{b,a}$	$0{\cdot}82^{b,a}$	$-25{\cdot}55^{c}$	$0{\cdot}79^{a}$	$-25{\cdot}21^{c}$	$0{\cdot}83^{a}$
4	$-25{\cdot}63^{a}$	$0{\cdot}80^{a}$	$-25{\cdot}87^{b,a}$	$0{\cdot}76^{b}$	$-24{\cdot}75^{a}$	$0{\cdot}78^{b}$	$-24{\cdot}47^{b,a}$	$0{\cdot}74^{b}$	$-24{\cdot}99^{b}$	$0{\cdot}78^{a}$	$-24{\cdot}70^{b}$	$0{\cdot}74^{b}$
5	$-25{\cdot}53^{a}$	$0{\cdot}77^{b,a}$	$-25{\cdot}82^{b,a}$	$0{\cdot}75^{b}$	$-24{\cdot}56^{a}$	$0{\cdot}77^{b}$	$-24{\cdot}04^{a}$	$0{\cdot}74^{b}$	$-24{\cdot}66^{b,a}$	$0{\cdot}77^{a}$	$-24{\cdot}36^{b}$	$0{\cdot}75^{b}$
6	$-25{\cdot}56^{a}$	$0{\cdot}73^{b,c}$	$-25{\cdot}61^{a}$	$0{\cdot}74^{b}$	$-24{\cdot}46^{a}$	$0{\cdot}74^{b}$	$-24{\cdot}09^{a}$	$0{\cdot}72^{b}$	$-24{\cdot}30^{a}$	$0{\cdot}74^{a}$	$-24{\cdot}16^{b}$	$0{\cdot}74^{b}$
7	$-25{\cdot}54^{a}$	$0{\cdot}71^{c}$	$-25{\cdot}56^{a}$	$0{\cdot}74^{b}$	$-24{\cdot}52^{a}$	$0{\cdot}74^{b}$	$-24{\cdot}46^{b,a}$	$0{\cdot}73^{b}$	$-24{\cdot}35^{a}$	$0{\cdot}74^{a}$	$-24{\cdot}10^{a}$	$0{\cdot}74^{b}$

^{a,b,c,d} Means with the same superscript letter were not significantly different ($P = 0{\cdot}05$).

* For details, see p. 43.

† For details, see p. 44 and Table 1.

‡ For details, see p. 46 and Table 2.

Table 5. *Administered ^{13}C excess, maximal ^{13}C enrichment in expired carbon dioxide, CO_2 production and calculated rates of recovery in sucking calves after intake of experimental diets (protein (P), carbohydrate (C) and lipid (L))* with (treatments B, D, F) or without (treatments A, C, E) the locomotion programme†*

(Mean values and standard deviations)

Treatment			No. of replicates§	^{13}C excess (mmol)		Maximal $\delta^{13}C$‡ over Baseline (‰)	CO_2 production (ml/min)	Recovery rate¶ (%)	
	Diet	Locomotion		Mean‖	SD			Mean	SD
A	Diet P	−	5	1·110[a]	0·08	0·96	320[a]	3·62[a]	2·09
B	Diet P	+	6	0·998[a]	0·15	1·03	343[a]	3·17[a]	1·82
C	Diet C	−	4	1·058[a]	0·37	2·12	404[a]	9·89[b]	4·93
D	Diet C	+	5	1·242[a]	0·23	2·20	401[a]	9·69[b]	4·14
E	Diet L	−	6	0·978[a]	0·31	2·44	368[a]	12·17[b]	3·91
F	Diet L	+	6	1·018[a]	0·26	2·39	343[a]	10·14[b]	6·02

[a, b] Means with the same superscript letter were not significantly different ($P = 0·05$).
* For details, see p. 44 and Table 1.
† For details, see p. 46 and Table 2.
‡ For details, see p. 43.
§ Replicates are missing if animals refused drinking.
‖ Means are calculated from ^{13}C excess actually administered and amounts drunk.
¶ Recovery rates for 6·5 h.

2–6 did not differ significantly from each other ($P > 0·05$). The mean $\delta^{13}C$ values for all animals ranged from −26·2 (interval no. 1) to −25·9‰ (interval no. 6). Therefore, the morning intake of the control diet caused no change in exhaled ^{13}C enrichment, and no correction for diurnal or feed-induced background drift was necessary.

However, during locomotion a shift in the $\delta^{13}C$ values became evident (Table 3). In the CO_2 expired at the end of the walking periods (end-exercise) a small but perceptible ^{13}C depletion was found compared with the pre-exercise value. After 5 min of rest (post-exercise) the enrichment of breath $^{13}CO_2$ increased again to the pre-exercise level.

Influence of treatments and time on ^{13}C breath tests and RQ values

When examined by analysis of variance, the main factors (treatment (A–F) and time of day (interval)) were found to have a significant effect on the $\delta^{13}C$ values in exhaled air ($P = 0·0005$; $P = 0·0001$). Rates of ^{13}C recovery were affected significantly by the treatment ($P = 0·0009$). Also, analysis of variance within treatments revealed the significance of interval on RQ values ($P = 0·0005$) and $\delta^{13}C$ values ($P = 0·0002$) (Tables 4 and 5). It should be pointed out that in all animals for every treatment the $\delta^{13}C$ values in the first interval were equal ($P = 0·66$), indicating similar starting conditions for each animal.

The following changes in ^{13}C content in expired CO_2 after intake of naturally labelled milk were found (Fig. 1): (a) milk with C_4 casein (diet P, treatments A and B) caused a small but significant ^{13}C enrichment of maximal +1‰ in the 7th interval compared with the baseline value (Table 5). The course of the $\delta^{13}C$ values in breath CO_2 during treatment B was somewhat lower. A pseudo-plateau of $\delta^{13}C$ values was reached beginning in the 4th interval with treatment A (Table 4, Fig. 1). The mean rates of ^{13}C recovery from C_4 casein were below 4% within the observed period (6·5 h) (Table 5). (b) The exhalation curves after the administration of milk diet with the C_4 whey (diet C) indicated a maximum ^{13}C enrichment of +2·12 and +2·20‰ for treatments C and D respectively (Table 5). The corresponding rates of recovery were 9·9% for treatment C and 9·7% for treatment D.

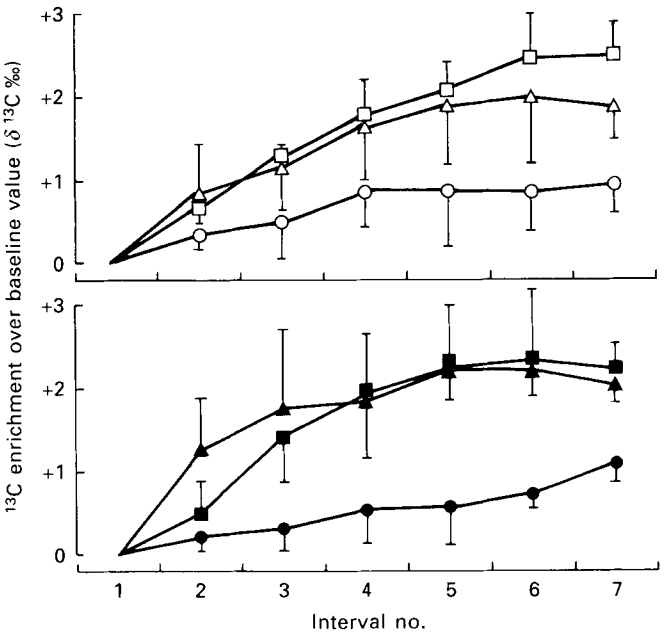

Fig. 1. Mean ^{13}C enrichment in breath carbon dioxide over baseline values (δ^{13}C‰) of sucking calves after intake of milk diets naturally enriched with naturally ^{13}C-labelled casein (treatments A, (○); B, (●)), whey (treatments C, (△); D, (▲)) or cream (treatments E, (□); F (■)) combined either with (●, ▲, ■) or without (○, △, □) locomotion programme. δ^{13}C (‰) = $[[(^{13}C:^{12}C)_{sample}-(^{13}C:^{12}C)_{standard}]/(^{13}C:^{12}C)_{standard}]\times 1000$, where the standard is Pee Dee Belemnite carbonate (Craig, 1957). For details of diets, see p. 44 and Table 1, and for details of treatments, see p. 45 and Table 2. Values are means and standard deviations represented by vertical bars.

Also, a pseudo-plateau was observed starting with the third interval (Table 4). The initial rise in the exhalation curves with diet C seemed to be the highest (Fig. 1). (c) The milk labelled with C_4 cream (diet L) caused the highest δ^{13}C values in breath CO_2 (+2·44‰; Table 5). Recovery rates were 12·2% for treatment E and 10·1% for the treatment F including the locomotion programme.

RQ values were influenced significantly by the interval but not by the treatment. Comparison of means within every treatment revealed decreasing RQ values with increasing number of intervals in all treatments (Table 4). A significant drop of RQ values between the 3rd and the 4th interval occurred which was only found as a consequence of locomotion programme (treatments B, D, F; Table 4).

As the total dose of administered ^{13}C excess did not differ significantly between the treatments (Table 5), the different recovery rates observed must be definitely an effect of the type of treatment. There is no indication from the course of the exhalation curves that the locomotion programme caused any difference in the rates of recovery within the same diet (Fig. 1), but a trend for slightly lower values could be observed with the treatments B, D, and F (Table 5).

Variations of δ^{13}C and RQ values during the locomotion programme

The δ^{13}C values changed during the locomotion programme similar to the changes observed with the unenriched milk (Table 3): therefore, with all diets the physical activity seemed to induce a small decrease of the δ^{13}C value ($P < 0{\cdot}05$). After 5 min of rest, the values reverted to those pre-exercise.

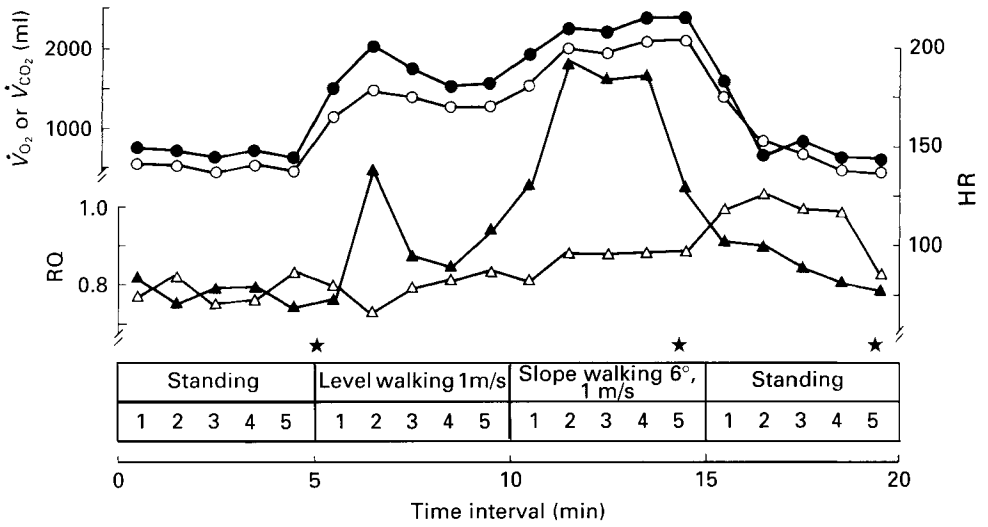

Fig. 2. Variation in respiration variables (oxygen consumption, $\dot{V}_{O_2}$ (●); carbon dioxide production, $\dot{V}_{CO_2}$ (○); respiratory quotient, RQ (△); heart rate, HR (▲)) in sucking calves during intake of milk diets naturally enriched with ^{13}C combined with the locomotion programme (for details of diets, see p. 44 and Table 1, and for details of treatments, see p. 45 and Table 2). ★ Breath-sampling times.

While the calves were walking on the treadmill several respiration variables changed. For example, in one animal (Fig. 2), $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$, as well as heart rate (HR) increased in the first 2 min of exercise.

The incline walking period that followed provoked a further increase in these variables by up to four times the values found during the initial rest period: HR was twice as high as during rest, while RQ was just below 0·9.

The subsequent recovery period while the animal was standing still was characterized by a decrease in $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$. In contrast to the reduced HR, the RQ rose above 1·0 as a result of increased CO_2 excretion. These variations were affected by the physical activity and gave no evidence of an influence of the type of diet.

DISCUSSION

We have shown recently that adult ruminants produce a markedly ^{13}C-enriched expired CO_2 (+4–5‰) relative to the given diet when C_3 plants are consumed. We also showed that the $\delta^{13}C$ value of expired CO_2 is partially determined by the CO_2 produced in the rumen (Metges *et al.* 1990). In contrast to this observation, it can be assumed that the digestion of young sucking calves is similar to that of simple-stomached animals because the rumen in calves is still small and its function is not yet developed (Giesecke & Henderickx, 1973). Because of the reticular groove the milk is immediately transported into the abomasum and digested in the small intestine. Hence, with those calves, the sources of expired CO_2 should be the food and the endogenous metabolism but not the rumen CO_2.

The finding that expired CO_2 was slightly depleted by about 1‰ relative to the diet in insects and human volunteers (De Niro & Epstein, 1978; Metzler *et al.* 1983) cannot be confirmed. The given C_3-based diet (−26·1‰) is equivalent to the measured ^{13}C enrichment in CO_2 (−26‰). Probably, the cited finding is only valid if the organism is in a totally balanced metabolic and isotopic state and these conditions are not fulfilled in a growing organism. Other reasons for the different results are a possible inaccuracy caused

by the estimation of food carbon isotope composition (Metzler *et al.* 1983) and differences between species (De Niro & Epstein, 1978). Moreover, there is a considerable natural variation in C isotope composition of foodstuffs and in breath which make an experimental determination difficult (Schoeller *et al.* 1977; De Niro & Epstein, 1978; Nakamura *et al.* 1982); but we conclude that rumen digestion by the calves plays at most only a minor role and it is justifiable to discuss these results as if they were from simple-stomached animals.

Although the brief exercise period seemed not to affect the subsequent course of $\delta^{13}C$ values in expired air, the considerable drop in RQ values from the 3rd to the 4th interval (Table 4) could be interpreted as a consequence of the locomotion programme which was conducted in the third interval. In contrast, Schoeller *et al.* (1984) reported a 1‰ increase in the $^{13}C:^{12}C$ ratio of breath in men as a consequence of 30 min hard exercise (75% $\dot{V}_{O_2max}$) and interpreted this as an increase in endogenous carbohydrate utilization. However, after 30 min of easy exercise no shift in $\delta^{13}C$ values over the baseline was seen, whereas a decrease of RQ was already visible 10 min after exercise began (Schoeller *et al.* 1984). On the other hand, even a slight physical exercise (35% $\dot{V}_{O_2max}$) without any nutrient administration induced an enrichment of about 3‰ accompanied by a shift in RQ (from 0·74 to 0·97; Wolfe *et al.* 1984).

Why was no systematic change in $\delta^{13}C$ values due to exercise perceptible in our results, except a brief one at the end of the exercise period (Table 3)? Exercise probably caused a partial change to endogenous substrates of lower enrichment compared with the given diet. This hypothesis may be supported by the considerable fall in RQ values in the interval following exercise (Table 4), which indicates a more preferential oxidation of fat and a faster liberation of CO_2 of lower ^{13}C enrichment. This may be supported by the results that Schoeller *et al.* (1984) found with easy exercise. Another explanation for our observations could be that the period of physical exercise was too short and the new isotopic equilibrium would not have been reached to induce a change in the course of ^{13}C enrichment in expired CO_2 in the subsequent interval and later.

However, a trend towards lower rates of ^{13}C recovery was found with treatments B, D, and F, although CO_2 production was similar in all treatments (Table 5). This result probably indicates a similarly directed slight influence of the physical activity on absorption of nutrients or on a partial use of fuels with lower enrichment, as stated previously.

The short period of physical activity was always followed by a transient decrease in $\delta^{13}C$ values in expired air (Table 3). Decreased breath $\delta^{13}C$ values are commonly attributed to catabolism of lipids which are isotopically less enriched, as was shown in experiments with fasting rats which oxidize body fat stores (Schoeller *et al.* 1984). On the other hand, it is also known that an immediate energy requirement is met by oxidation of carbohydrates, namely glucose and glycogen. Hence, the increasing RQ value within the two walking periods and the rise above 1·0 in the post-exercise rest period (Fig. 2) may be interpreted as due to hyperventilation or lipogenesis according to Elia & Livesey (1988).

Indeed, the discrepancy between the data of the ^{13}C breath test and indirect calorimetry may reflect a transient change in bicarbonate kinetics. The preferred substrate for resting skeletal muscle is lipid (Wahren, 1977), thus bicarbonate derived from this metabolite will be less enriched. During the change from rest to exercise an increased blood flow to skeletal muscle plus hyperventilation will promote the production of CO_2 from this source. Similar results were obtained in humans by Schoeller *et al.* (1984) in the initial phase of easy and hard exercise. This means that neither the RQ nor the $\delta^{13}C$ values of breath reflect accurately the actual substrate oxidation during short-term moderate physical activity. However, this is not specific for stable tracer work in the range of natural abundances but probably is of general importance.

The kinetics of the $\delta^{13}C$ values for breath CO_2 reflect unequivocally the nature of the administered labelled diet and do not depend on the ^{13}C dose (Tables 4 and 5). While the recovery rates of carbohydrates and lipids from C_4-milk diets were not significantly different, they differed markedly from the low oxidation rate found for casein over 6·5 h. Although baseline values were not reached at the end of the breath sampling period (Fig. 1), this relative comparison made of recovery rates within 6·5 h may be justified: the observed trend is completely in line with the knowledge about the different contribution of nutrients to the energy metabolism of mammals. The two major metabolic fuels are carbohydrates and lipids. Previous results with volunteers showed that ingestion of proteins induces only a very small increase in the $^{13}C:^{12}C$ ratio in breath CO_2 (Schmidt & Metges, 1986).

Nevertheless, in all three diets the calculated total ^{13}C recovery is very low. Particularly when comparing oxidation rates found with pure naturally ^{13}C-labelled carbohydrates and with diet C does this become evident. For example, Mosora *et al.* (1976) observed ^{13}C recovery rates in breath of 30% over 7 h after intake of 100 g maize-glucose by human volunteers. Also, oxidation rates of naturally labelled butter ($\delta^{13}C$ −16·3‰) and milk powder ($\delta^{13}C$ −14·4‰) attained 33·7 and 23% in 8 h respectively, when given as main nutrients with a minimum of additional matrix (Metges, 1988). Thus, in the present experiments there is no doubt that the true oxidation rates are underestimated.

The rate of absorption per unit time and probably the time taken to reach oxidation sites from a mixed diet is different from those of pure nutrients. This will have consequences for the exhalation period of enriched CO_2 because $^{13}CO_2$ exhalation from the enriched fraction is disguised by the CO_2 from the unenriched diet fractions. For example, while giving a 100 g bolus of pure naturally enriched [^{13}C]glucose, the exhalation of enriched CO_2 is complete after approximately 8 h. When giving a similar bolus dose of [^{13}C]glucose together with unenriched fat and protein (carbohydrate, fat and protein providing 58, 18 and 23% of total C respectively), 8 h after administration the expired CO_2 was still 2% enriched relative to the baseline value (Metges & Wolfram, 1990).

In the present experiment the course of ^{13}C enrichment in expired CO_2 was observed only over 6·5 h. As shown in Fig. 1, the CO_2 was still enriched at the end of the measurement period with all three diets.

As a general consequence of these experiences, to prevent underestimation of ^{13}C recovery rates of a single labelled bolus, particularly in those cases when an enriched nutrient is given in a mixed diet, the collection period of exhaled air must be extended until the ^{13}C enrichment has returned to the baseline value. However, it was demonstrated principally that ^{13}C breath tests with naturally enriched diets can be applied in animal nutrition, a quantification of recovery rates can be made, and immediate changes in C isotope composition in breath caused by physical activity can be identified.

A potent method for nutritional investigations is available by combining the ^{13}C breath test with the highly sensitive isotope-ratio-monitoring gas–liquid chromatography–mass spectrometry technique (Barrie *et al.* 1984). While the former gives information about whole-body oxidation, the latter gives the turnover of metabolites (e.g. fatty acids). This allows measurements in the range of natural abundance which opens the possibility of using the inexpensive naturally ^{13}C-enriched compounds and avoiding the hazards of radiotracers.

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