Body Fat Deposition Does Not Originate from Carbohydrates in Milk-Fed Calves¹,²

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Abstract

Milk-fed heavy calves utilize dietary protein with a low efficiency and often develop hyperglycemia and insulin resistance. Distributing the daily nutrient intake over an increasing number of meals increases protein deposition and improves glucose homeostasis. Therefore, we examined effects of feeding frequency (FF) and feeding level (FL) on the diurnal pattern of substrate oxidation and on the fate of dietary carbohydrates in milk-fed heavy calves. Eighteen milk-fed calves weighing 136 ± 3 kg were assigned to FF (1, 2, or 4 meals daily) at each of 2 FL (1.5 or 2.5 times maintenance), except for calves at FF1 (only at a low FL). Urea, leucine, and glucose kinetics were assessed for each treatment by use of [¹³C]urea, [¹³C]leucine, [U-¹³C], and [2-¹³C]glucose, respectively. FF altered the diurnal pattern, but not the total, of urea production. Although urea production correlated well with nitrogen retention, oxidation of oral l-[¹³C]leucine did not. Dietary glucose was almost completely oxidized (80% based on [¹³C]glucose and 94% from indirect calorimetry measurements) regardless of FL. Fatty acid synthesis from glucose appeared to be negligible based on similar recoveries of [¹³C]CO₂ from orally supplied [U-¹³C]glucose and [2-¹³C]glucose. The increased fat deposition at the higher FL originated almost exclusively from greater transfer of fatty acids to body lipid stores. These findings contrast with both glucose and lipid metabolism in growing pigs and indicate that alternative adaptive mechanisms operate in heavy milk-fed calves. J. Nutr. 137: 2234–2241, 2007.

Introduction

During the past decades, skimmed milk protein in calf milk replacer has largely been substituted by more rapidly hydrolyzable (i.e. nonclotting) protein sources such as vegetable proteins and whey. This change, coupled with a high feeding level (FL) and low feeding frequency (FF; twice daily is common practice) results in rapid absorption of nutrients, such as amino acids, glucose, and galactose, soon after ingestion (1). The calves need adaptive mechanisms to cope with these high loads of nutrients, with partition toward either nutrient deposition or oxidation. Importantly, milk-fed heavy calves often develop insulin resistance (2), reflected in hyperglycemia (2) and low efficiency of dietary protein utilization (3). The insulin resistance may impact also on the capability to process large amounts of other rapidly absorbed nutrients toward anabolic outcomes.

Under such conditions, more efficient nutrient utilization may occur if the smaller amounts of nutrient are absorbed over short time intervals and this can be achieved by increasing FF at the same FL. Indeed, increased FF improves insulin sensitivity in calves, as indicated by lower postprandial insulin to glucose ratios and lower glucose excretion in urine (4). Furthermore, fat deposition is enhanced (5) by increasing FF, but whether this is due to either increased channelling of glucose into fatty acid synthesis de novo, as occurs in nonruminants (6), and/or better utilization of dietary fatty acids is not known. In other species, including obese and insulin-resistant human subjects (7,8), improved insulin sensitivity increases conversion of glucose to fatty acids and alters metabolic fuel selection, affecting diurnal patterns of glucose and fatty acid oxidation (OXF). Nonetheless, milk-fed heavy calves are ruminants ontologically but are maintained in a preruminant state by a liquid diet that supplies large amounts of digestible glucose and galactose. Fat synthesis from glucose is not a major route in ruminants, because acetate released by rumen fermentation provides ample precursor and there is not the reliance on either citrate lyase or malate dehydrogenase as observed in nonruminants (9). In the milk-fed heavy calf, however, it is unclear whether nutrient source or developmental physiology has the greater impact on the precursor for fat deposition.

Despite the improvements in the insulin-glucose axis with increased FF in these calves (8), the effect on amino acid metabolism
is unclear. For example, increasing the FF did not improve protein utilization when skimmed milk protein was used as the sole protein source for milk-fed calves (10) but did when nonclotting whey protein was provided at a similar daily digestible nutrient intake (5). This suggested that the within-day pattern of amino acid oxidation and, thus, urea production may be linked to the altered diurnal amino acid availability, with this most pronounced when a rapidly hydrolyzable protein source is used.

Therefore, the aims of this study were to: 1) identify the mechanisms of glucose and fatty acid metabolism that operate in milk-fed heavy calves; and 2) quantify the effects of FF at a low and high FL on diurnal variation in amino acid oxidation. We hypothesized that, at equal daily nutrient intakes, an increased FF would lower glucose oxidation (OXCHO) whereas fatty acid synthesis de novo would increase and that there would be decreased urea production and postprandial leucine oxidation. Whole body protein and fat deposition as well as hormone and metabolite responses in this study have been reported elsewhere (4,5).

Materials and Methods

Experimental design. The experimental design has been described in detail by Van den Borne et al. (5). Briefly, 18 male Holstein Friesian calves weighing 136 ± 3 kg were used in 9 trials, each with 2 calves of similar age. The trials consisted of 2 consecutive experimental periods each of 6 wk, 4 of which were for adaptation to the treatments. Period 1 was at low FL and period 2 at high FL. Within each trial, 1 particular FF was applied, because the visual and (limited) auditory contact between the individual calves could affect physical activity and cephalic phase reflexes. Animals used in a 2 × 3 factorial arrangement with FL [1.5 × metabolizable energy requirements for maintenance (MEm) and 2.5 × MEm] and FF (1, 2, or 4 meals per day). Six calves were studied per treatment. Animals on the high FL could not consume such an intake within a sensible period if this was offered as 1 meal and so this treatment was not included. Calves at FF 1 served as controls for the effect of experimental period, because FL was confounded with period in this study.

Diets and feeding. Calves were fed according to their metabolic body weight (kg 0.75). Feed supply was adjusted daily for a projected mean daily gain of 0.5 kg at the low FL and 1.5 kg at the high FL. The MEm was assumed to be 460 kJ/kg 0.75·d. Whey was used as the only protein source in the milk replacer; this provides a rapidly hydrolyzable protein source that can be included at high levels in diets for milk-fed calves. The ingredients and analyzed nutrient composition of the experimental milk replacer are presented in Table 1. Nutrient analyses of the milk replacer have been described previously (5). Milk replacer was reconstituted with water (140 g/L) and supplied by bucket at a temperature of ~40°C. Rougshage was not supplied. Feeding times were 0000 (FF 2 and 4), 0600 (FF 4), 1200 (FF 1, 2, and 4), and 1800 (FF 4). In addition, calves at FF 1 served with 3 L of warm water (40°C) at 0600 and 1800 to prevent dehydration. Calves were allowed 15 min to consume the meal. Mean energy and protein intakes were 753 ± 3.4 kJ/kg 0.75·d and 6.9 ± 0.04 g/kg 0.75·d for calves at the low FL and 1228 ± 6.4 kJ/kg 0.75·d and 11.4 ± 0.07 g/kg 0.75·d at the high FL.

Measurements. The calves were housed for 10 d in a metabolic cage within respiration chambers followed by 4 d when the calves were maintained in a metabolic cage within a climate-controlled stable (Fig. 1).

Oxidation of leucine was quantified from 13CO2 excretion after an oral bolus dose of 25.2 μmol·h-1·[1-13C]leucine (99.0 atom%; Cambridge Isotope Laboratories) per gram dietary protein was added to the milk replacer offered at 1200. Glucose oxidation was determined from the amount of 13CO2 exhaled after an oral bolus dose of 2.5 μmol [U-13C]glucose (99.0 atom%; Cambridge Isotope Laboratories) per gram dietary lactose was added to the milk replacer supplied at 1200. The [2-13C]glucose (99.0 atom%; Cambridge Isotope Laboratories) was supplied similarly but at 15 μmol/g dietary lactose. Differences in oxidation of [U-13C]glucose and [2-13C]glucose would reflect, indirectly, fatty acid synthesis from glucose, with a greater reduction in the amount of 13CO2 released from [2-13C]glucose if pyruvate is converted to acetate, by the action of pyruvate dehydrogenase, and this is used for fatty acid synthesis (11). The labeled C-atom from [2-13C]glucose is not released if acetate is formed and used for fatty acid synthesis.

Table 1. Ingredient and analyzed nutrient composition of the experimental diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Nutrient</th>
<th>g/kg</th>
<th>g/kg DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey</td>
<td>Dry matter</td>
<td>372.9</td>
<td>978.0</td>
</tr>
<tr>
<td>Whey protein concentrate</td>
<td>Crude ash</td>
<td>360.0</td>
<td>73.1</td>
</tr>
<tr>
<td>Defactored whey</td>
<td>Crude protein, N × 6.25</td>
<td>70.0</td>
<td>190.5</td>
</tr>
<tr>
<td>Soy oil</td>
<td>Crude fat</td>
<td>144.0</td>
<td>396.6</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>Lactose</td>
<td>36.0</td>
<td>470.3</td>
</tr>
<tr>
<td>ni-Methionine</td>
<td>Gross energy, MJ/kg</td>
<td>1.32</td>
<td>20.6</td>
</tr>
<tr>
<td>Mono potassium phosphate</td>
<td>Lysine</td>
<td>2.32</td>
<td>16.2</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>Methionine</td>
<td>6.60</td>
<td>5.0</td>
</tr>
<tr>
<td>Magnesium oxide</td>
<td>Cystine</td>
<td>0.84</td>
<td>5.0</td>
</tr>
<tr>
<td>Vitamin and mineral premix</td>
<td>Threonine</td>
<td>6.00</td>
<td>13.2</td>
</tr>
<tr>
<td></td>
<td>Tryptophan</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>Isoleucine</td>
<td>3.0</td>
<td>11.4</td>
</tr>
</tbody>
</table>

1 Provided per kilogram of the experimental diet: 7.8 g calcium; 6.5 g phosphorous; 1.4 g magnesium; 7.5 g retinol; 0.05 mg cholecalciferol; 80 mg dl-tocopherol; 84 mg zinc; 9 mg copper; 48 mg iron; 15 mg manganese; 0.1 mg selenium; and 1.9 mg cobalt. 2 Analyzed content, unless indicated otherwise. DM, dry matter. 3 Calculated content.

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into a 10-mL Externa tube (Labco) and 50 μL of 12 mol/L HCl was added and mixed. After evaporation to dryness in a water bath at 40°C under a gentle stream of nitrogen, 1 mL of (boiled) water and 300 μL of 1 mol/L HCl were added, and completely evaporated again. Then, 1.5 mL of a cold (4°C) buffer solution (27 g KH₂PO₄ and 53.5 g Na₂HPO₄·12H₂O, pH 7.0) containing urease (50 kU/L; Sigma Chemical) was added on ice. The tubes were covered with rubber lids, flushed with argon for 10 s to replace the air in the headspace, and then incubated at 37°C with gentle shaking in a water bath for 45 min. The reaction was stopped by injection of 0.5 mL 7 mol/L phosphoric acid and the ¹³C enrichment in CO₂ measured in the headspace using a Finnigan Delta C continuous-flow isotope ratio mass spectrometer (Finnigan MAT). The enrichment was expressed as atom% ¹³C.

Calculations. The derivative of a generalized Michaelis-Menten equation, as proposed by López et al. (14) for growth curves, was used to express ¹³C production in time. The model was fitted to the 30-min means of ¹³C excretion in breath (corrected for background enrichment) after ingestion of L-[¹-¹³C]leucine, [¹⁵N]urea, and [¹³C]glucose and after infusion of [¹³C]sodium bicarbonate:

\[ y = \frac{b_0 \cdot t - e^{-c \cdot t}}{1 + (b_1/t)^2}, \]

where \( y = \) ¹³C production (micromoles per minute) at time \( t \) (minutes); \( b_0, b_1, \) and \( c \) (all > 0) are parameters that define the curve.

The nonlinear least squares regression procedure (PROC NLMIN, SAS Institute) was used for curve fitting. Oxidation of the tracer metabolites was calculated by integration of the area under the ¹³C excretion curve over a period of 24 h after administration of the isotope, \( b_0/(1 + b_1 \cdot 1440) \) and expressed relative to the amount of ingested or infused isotope by dividing the area under the curve by the dose of isotope corrected for enrichment and chemical purity. The time to peak was calculated as \( t_{\text{max}} = b_1 / (1 - (c - 1) / (c - 1)) \) (1/²). The maximum ¹³C excretion (mmol/h) was calculated as \( y_{\text{max}} = b_0 \cdot t_{\text{max}} / (1 + b_1 / t_{\text{max}})^2 \) and was expressed as percentage of the dose.

Recoveries and maxima for the ¹³C excretion after the ingestion of leucine and glucose tracers were corrected for bicarbonate sequestration.

Goodness of fit for predicted against experimental data were assessed from the mean squared prediction error (MSPE) (15). The MSPE and goodness of fit for predicted against experimental data were assessed from the mean squared prediction error (MSPE) (15). The MSPE was used to compare individual treatments. Recovery of ¹³C, the maximum ¹³C excretion, and the time of the maximum ¹³C excretion were compared between treatments for each isotope tested. In addition, the ¹³C recoveries of [¹⁵N]urea and [¹³C]glucose were compared. Hourly means and 12-h rates of ¹³C production were used. The daily oxidation rate of amino acids (OXP) was calculated as 6.25 times the urinary N excretion [g/(kg⁰.⁷⁵ h)] after feed intake (AUC12–18). The metabolic values could be affected by FL and FF, the responses in excess of preprandial values (i.e., 2 h before feeding) were also calculated (ΔAUC12–18).

Statistical analysis. All data were analyzed for the effect of FF, FL, the interaction between FL and FF, and period by ANOVA using PROC MIXED in SAS. The factorial design was analyzed by a mixed model for the fixed effects of FF, FL, and period and the random effect of each calf (Eq. [3]).

\[ Y_{ijkl} = \mu + FF_i + FL_j + (FF\times FL)_{ij} + P_k + e_{ijkl}. \]

where \( Y_{ijkl} \) = dependent variable; \( \mu = \) mean intercept; \( FF_i = \) effect of FF (i = 1, 2, 4); FL_j = effect of FL (j = 1, 2); \( P_k = \) effect of period (k = 1, 2), and \( e_{ijkl} = \) error term, which represents the random effect of calf within FL (i = 1, ..., 6). If main effects were significant, post hoc t tests were used to compare individual treatments. Recovery of ¹³C, the maximum ¹³C excretion, and the time of the maximum ¹³C excretion were compared between treatments for each isotope tested. In addition, the ¹³C recoveries of [¹⁵N]urea and [¹³C]glucose were compared. Hourly means and 12-h rates of ¹³C production were used. The daily recoveries of [¹⁵N]urea and [¹³C]glucose were compared between treatments. Pearson correlation coefficients were calculated for relationships between urinary N excretion and ¹³C excretion for FF 2 and 4, and between mean daily OXP and L-[¹-¹³C]leucine oxidation for all treatments. The SAS software package version 9.1 (SAS Institute) was used for all statistical evaluations.

Results

General. Two calves at FF 1 were excluded from the experiment because of illness and feed refusals. Another calf, at FF 2, was excluded from 1 of the experimental periods (at a low FL). The results were not affected (P > 0.10) by the experimental period. Therefore, the effect of experimental period was not included in the results.

Urea production. FF had limited effect on hourly urea production rates, except that a greater FF increased (P < 0.05) OXCHO and OXF rates were calculated hourly from gas exchange rates with correction for urea-N production rates (protein oxidation). The constants of Brouwer were used to calculate hourly rates of OXCHO and OXF (see 18):

\[ \text{OXCHO} \text{ [g/(kg}^{0.75} \text{ h}]} = -2.968 \cdot O_2 [\text{g/(kg}^{0.75} \text{ h})] + 4.174 \cdot \text{CO}_2 [\text{g/(kg}^{0.75} \text{ h})] - 2.446 \cdot \text{urea-N [g/(kg}^{0.75} \text{ h})].\]

The same formulas were used for calculation of the mean daily OXCHO and OXF, but daily urinary N excretion values, instead of urea-N production rates, and mean daily rates of O₂ consumption and CO₂ production were used. The daily oxidation rate of amino acids (OXP) was calculated as 6.25 times the urinary N excretion [g/(kg⁰.⁷⁵ h)] after feed intake (AUC12–18).
urea production rates at 1130 for both FL (Fig. 2A,B), with a decrease observed at 1630 for the low FL (Fig. 1A). The higher FL increased \( P < 0.05 \) or tended to increase \( P < 0.10 \) hourly urea production rates at various timepoints (compare Fig. 2A,B). The 12-h urea production rate was not affected by FF but was greater \( P < 0.001 \) with the higher FL (Table 2). The AUC\(_{12-18}\) for urea production increased \( P = 0.004 \) with higher FL and tended to decrease \( P = 0.053 \) with greater FF (Table 2), whereas the \( \Delta \)AUC\(_{12-18}\) decreased with FF \( P = 0.016 \) but was not affected by FL.

**OXCHO and OXF: indirect calorimetry.** At higher FF, the hourly OXCHO values were greater \( P < 0.001 \) until 1330 but lower \( P < 0.001 \) thereafter (Fig. 3). All hourly OXCHO values (except at 1330) were greater \( P < 0.001 \) at the higher FL. Hourly OXF values were greater \( P < 0.01 \) at the lower FF until 1330, whereas OXF increased \( P < 0.05 \) with greater FF after 1630. All hourly OXF values (except 0730 and 1230) were lower \( P < 0.05 \) at the higher FL.

Daily OXP, as a percentage of the protein intake, decreased \( P = 0.038 \) with greater FF but was not affected by FL (Fig. 4).

**FIGURE 2** Influence of FF 1, 2, and 4 at a low [690 kJ metabolizable energy (ME)/kg\(^0.75\) · d]; A] and high [1150 kJ ME/kg\(^0.75\) · d]; B] FL on the diurnal pattern of urea production in heavy milk-fed calves. Values are means ± SEM, \( n = 8 \) (FF 1), \( n = 5 \) (FF 2, in A), \( n = 6 \) (FF 2, in B), or \( n = 6 \) (FF 4). All calves were fed at 1200 (\( \downarrow \)). Contrast comparisons \( P < 0.05 \) for FF 1 vs. FF 2 (a), FF 1 vs. FF 4 (b), and FF 2 vs. FF 4 (c) are indicated.

Daily OXCHO expressed as percentage of the lactose intake was high with 94.2 and 91.5% oxidation at the low and high FL, respectively, but was not affected by either FF or FL. Daily OXF, as a percentage of the fat intake, tended to be affected by FF \( P = 0.058 \) and was greater for the lower FL (77.7 vs. 30.6%; \( P < 0.001 \)).

\( \nu \)-\[\text{L-1}\text{[13C]}\]leucine oxidation. Curve fits for [\(^{13}\)CO\(_2\)] from \( \nu \)-\[\text{L-1}\text{[13C]}\]leucine were good and the MSPE averaged 17.6% \( (r = 0.94) \), with the majority of the prediction error \( (\geq 95\%) \) attributable to random variation. For [\( ^{13}\)C]bicarbonate, curve fits were also good (MSPE 16.3%; \( r = 0.97 \)), with 72% of the injected dose recovered as [\(^{13}\)CO\(_2\)] for both FL. The proportion of the \( \nu \)-\[\text{L-1}\text{[13C]}\]leucine oxidized was greater with increasing FF at the low FL but decreased with increasing FF at the high FL (FF × FL; \( P < 0.001 \); Table 3). The maximum of [\(^{13}\)CO\(_2\)] excretion tended to increase \( P = 0.068 \) with greater FF. The time until maximum [\(^{13}\)CO\(_2\)] excretion was reached decreased \( P < 0.001 \) with greater FF.

**Glucose oxidation: \[\text{U-13C}\]glucose and \[2\text{-13C}\]glucose.** Curve fits were good and gave an MSPE of 17.0% for [\(\text{U-13C}\)]glucose \( (r = 0.95) \) and 17.6% for [\(2\text{-13C}\)]glucose \( (r = 0.92) \), with the majority of the prediction error \( (95\%) \) attributable to random variation. Recoveries of the [\(\text{U-13C}\)]glucose dose as [\(^{13}\)CO\(_2\)] were high \( (79.5\%) \) and were unaffected by either FF or FL (Table 3). The maximum of [\(^{13}\)CO\(_2\)] production was greater \( P < 0.001 \) with increasing FF. The time to maximum decreased \( P < 0.001 \) substantially with greater FF. Recoveries of the [\(2\text{-13C}\)]glucose dose as [\(^{13}\)CO\(_2\)] averaged 78.8%, similar \( P > 0.10 \) to [\(\text{U-13C}\)]glucose, and were unaffected by FF or FL (Table 3). The maximum [\(^{13}\)CO\(_2\)] production was greater \( P < 0.001 \) with increasing FF, whereas the time to maximum decreased \( P < 0.001 \).

**Discussion**

This study showed that urea production increased after milk intake in calves, was less at the lower FL and tended to decrease with greater FF. Extrapolated daily rates of urea production did not significantly decrease with greater FF. This contrasts with an increased protein deposition from N balance measurements and may indicate that longer-term (i.e. 24 h or longer) measurements of urea production are needed to detect differences in diurnal kinetics of amino acid oxidation. Oral glucose was almost completely oxidized and this was unaffected by both FF and FL, indicating that fatty acid synthesis de novo from glucose was negligible in milk-fed calves. The increase in fat deposition with increased feed intake originates almost exclusively from a reduced oxidation of fatty acids.

**Urea production.** Amino acid oxidation was assessed by 2 approaches: ureagenesis based on [\(^{13}\)C]urea kinetics and catabolism of oral \( \nu \)-[\(1\text{[13C]}\]leucine, in addition to the previously reported N balance data (5). Urea production rates showed marked diurnal fluctuations but, in general, increased after meal ingestion at 1200. Postprandial urea production (AUC\(_{12-18}\)) was greater at the higher FL and tended to be greater with decreasing FF (larger meal size). In rats, larger meals also tended to increase amino acid oxidation (and thus urea production) in the postprandial state (19). After the 1200 meal, the hourly urea production rate approximately doubled in calves fed 1 or 2 meals daily, similar to findings in pigs (20) and humans (21). Although
labeled free amino acids to a meal (26–29). From breath $^{13}\text{CO}_2$ indeed, the correlation between recovery as exhaled $^{13}\text{CO}_2$ from that less L-[1-13C]leucine would be oxidized with increasing FF. Urea production data and apparently contradicts our hypothesis FL (FF $^3 \ P^2$) after a bolus dose, and was independent of FL. Values similar to recoveries in human (31,32) and animal studies calculated, with this corrected to allow for bicarbonate sequestration. Nonetheless, the daily urea-N production showed a strong positive correlation ($r = 0.98; \ P < 0.001$) with the 10-d urinary N excretion across FF.

As expected, urea production increased with increasing FL, as commonly observed across species (21–23). The urea-N production exceeded the urinary N excretion by 39%. Urea-N production is still considerable better at the high FL ($r = 0.77; \ P = 0.003$) than at the low FL ($r = -0.34; \ P = 0.149$). Several explanations may account for the apparent discrepancy between leucine oxidation and urea production. First, to obtain a quantitative value for leucine oxidation requires measurement of the flux through the free leucine pool, but this was not determined in the current study as it would not extend over a meal interval (12 h) with the dietary bolus dose method employed. Changes in leucine flux can influence markedly the net oxidation. For instance, Motil et al. (35) reported fractional leucine oxidation rates in response to protein intake in meal-fed humans of 16.3, 18.2, and 29.3% of the dose, whereas absolute oxidation rates were 11.8, 21.6, and 46.3 $\mu$mol/(kg · h), respectively. Second, ingestion of labeled leucine creates an isotopic gradient with the higher leucine enrichment in the gastrointestinal tract and this may overemphasize the contribution of enterocytes to whole body amino acid oxidation. Leucine can be extensively oxidized by enterocytes (36,37), but the proportion oxidized decreases with increasing FL (38). This would make a larger fraction of the dose available for peripheral tissue metabolism and may explain the better correlation with N balance at the higher FL. Third, a time lag between the absorption of free amino acids, such as the added t-[1-13C]leucine, and those originating from protein digestion may have resulted in a temporal amino acid imbalance. Leucine-rich fed calves, hydrolysis of urea might be low due to the lack of fermentable substrates in the gastrointestinal tract, but even assuming that 75% of the urinary N originated from urea and a urea entry into the gastrointestinal lumen of 20% in exclusively milk-fed calves, hydrolysis of urea and a urea entry into the gastrointestinal tract and this may overemphasize the contribution of enterocytes to whole body amino acid oxidation. Leucine can be extensively oxidized by enterocytes (36,37), but the proportion oxidized decreases with increasing FL (38). This would make a larger fraction of the dose available for peripheral tissue metabolism and may explain the better correlation with N balance at the higher FL. Third, a time lag between the absorption of free amino acids, such as the added t-[1-13C]leucine, and those originating from protein digestion may have resulted in a temporal amino acid imbalance and catabolism of t-[1-13C]leucine. Differences in oxidation patterns between free amino acids and intrinsically labeled proteins have been reported in rats (39). In summary, although the 13C-leucine breath test may be useful for clinical diagnosis (40) or studying the fate of dietary free amino acids (39), it is not a suitable method to measure postprandial amino acid oxidation in milk-fed calves.

**Dietary amino acid oxidation.** The metabolic fate of dietary amino acids has often been studied by adding $^{13}$C- or $^{14}$C-labeled free amino acids to a meal (26–29). From breath $^{13}$CO$_2$ or $^{14}$CO$_2$ excretion, the proportion of the dose oxidized can be calculated, with this corrected to allow for bicarbonate sequestration (30). The recovery of $^{13}$Cbicarbonate averaged 72%, values similar to recoveries in human (31,32) and animal studies (33,34) after a bolus dose, and was independent of FL.

In response to greater FF, recovery of the t-[1-13C]leucine dose as $^{13}$CO$_2$ increased at the low FL but decreased at the high FL (FF × FL; $P < 0.001$). The increased fractional oxidation at the low FL does not correspond with improved N balance (5) or urea production data and apparently contradicts our hypothesis that less t-[1-11C]leucine would be oxidized with increasing FF. Indeed, the correlation between recovery as exhaled $^{13}$CO$_2$ from t-[1-13C]leucine and OXP calculated from N balance was considerably better at the high FL ($r = 0.77; \ P = 0.003$) than at the low FL ($r = -0.34; \ P = 0.149$). Several explanations may account for the apparent discrepancy between leucine oxidation and urea production. First, to obtain a quantitative value for leucine oxidation requires measurement of the flux through the free leucine pool, but this was not determined in the current study as it would not extend over a meal interval (12 h) with the dietary bolus dose method employed. Changes in leucine flux can influence markedly the net oxidation. For instance, Motil et al. (35) reported fractional leucine oxidation rates in response to protein intake in meal-fed humans of 16.3, 18.2, and 29.3% of the dose, whereas absolute oxidation rates were 11.8, 21.6, and 46.3 $\mu$mol/(kg · h), respectively. Second, ingestion of labeled leucine creates an isotopic gradient with the higher leucine enrichment in the gastrointestinal tract and this may overemphasize the contribution of enterocytes to whole body amino acid oxidation. Leucine can be extensively oxidized by enterocytes (36,37), but the proportion oxidized decreases with increasing FL (38). This would make a larger fraction of the dose available for peripheral tissue metabolism and may explain the better correlation with N balance at the higher FL. Third, a time lag between the absorption of free amino acids, such as the added t-[1-13C]leucine, and those originating from protein digestion may have resulted in a temporal amino acid imbalance and catabolism of t-[1-13C]leucine. Differences in oxidation patterns between free amino acids and intrinsically labeled proteins have been reported in rats (39). In summary, although the 13C-leucine breath test may be useful for clinical diagnosis (40) or studying the fate of dietary free amino acids (39), it is not a suitable method to measure postprandial amino acid oxidation in milk-fed calves.

**Glucose and fatty acid oxidation.** Calculation of substrate oxidation from indirect calorimetry in fast-growing animals is often complicated by a high protein-free respiratory quotient (RQ), indicating de novo fatty acid synthesis from glucose (41,42). In our study, however, this RQ did not exceed unity. On the other hand, gluconeogenesis from noncarbohydrate precursors (e.g. alanine) results in a low RQ (42) and would lead to an overestimation of OXF and underestimation of OXCHO. Therefore, the estimates of OXCHO in this study should be considered as minimal values.
A milk-fed calf of 150 kg usually ingests >500 g of lactose in a single meal and portal glucose and galactose appearance is virtually complete within a few hours after feeding (1). Temporary storage of glucose (as additional glycogen) in well-fed animals is very small and therefore glucose is either oxidized or used as a precursor for de novo fatty acid synthesis. From the high 13C recovery of [U-13C]glucose as 13CO2 (mean 80%) and the high OXCHO as a percentage of the lactose intake (mean 94%), it appears that ingested carbohydrates are predominantly oxidized independent of either FF or FL. The lower glucose oxidation observed from the tracer compared with the gas exchange method may be a consequence of the label sequestration through glucose-glycogen interchange, thus reducing the number of 13C glucose molecules that enter the glycolytic pathway. The gas exchange method does not discriminate between oxidation of exogenous and endogenous glucose, so this more closely represents net catabolism of glucose. The net result of an increase in FL was 53–59% more OXCHO. At the same time, OXF decreased markedly at the higher FL. In energetic terms, this could be advantageous, because the efficiency of triglyceride synthesis from fatty acids is considerably greater than from glucose [90 vs. 74%, respectively; (43)]. Nonetheless, this is not simply a direct substitution, because regression analysis revealed that every 1 kJ increase in heat production from glucose resulted in only 0.5 kJ decrease in heat production from fatty acids (r = −0.79; P < 0.001). Partly, this is due to the additional energy utilized by the animal at the higher FL, possibly related to additional demands to support absorption or to drive elevated protein synthesis and urea production (44,45).

**Fatty acid synthesis from glucose.** Other species generally tend to have increased rates of de novo fatty acid synthesis from glucose in the immediate postprandial period with decreasing FF, especially when carbohydrate-rich diets are offered (6). This is accompanied by increased enzyme activities associated with glucose and lipid metabolism and improved sensitivity of adipose tissue to insulin [reviewed in (6)]. This scenario is not true for milk-fed heavy calves, however, because OXCHO is virtually complete, regardless of the FF. Indeed, fatty acid synthesis de novo from glucose can be calculated from the difference between OXCHO plus urinary glucose excretion and digestible lactose intake plus gluconeogenesis from amino acids [as described in (41)]. This produced low values, 5.6 kJ/(kg0.75·d), independent of both FF and FL and was <2% of total fat deposition at a FL of 2.5 × MEm (from 5), contrary to our hypothesis that more glucose is used for de novo fatty acid synthesis with increasing FF in milk-fed calves. This finding does not correspond with the higher activities of ATP citrate lyase observed in liver and adipose tissue from milk-fed calves compared with ruminant calves (46) and is also contrary to findings in pigs, where increasing the FL resulted in almost 700 g of the ingested starch retained as fat (41). Even at close to energy maintenance, pigs use substantial amounts (~150 g) of carbohydrates for fatty acid synthesis while also oxidizing body fat (41).

We speculate that the low level of de novo fatty acid synthesis from glucose in milk-fed calves relates to: 1) the (onto)genetic background of the calves belonging to a herbivorous species and may lack enough of the necessary enzyme systems for converting dietary glucose to fat; or 2) the high fat intake and the impact this exerts on the metabolic fate of dietary glucose. Potential mechanisms for the high OXCHO and low utilization
TABLE 3 Influence of FF (1, 2 or 4 meals/d) and FL (high vs. low) on oxidation of orally provided \([1-^{13}C]\)leucine, \([I-^{13}C]\)glucose, and \([2-^{13}C]\)glucose in heavy milk-fed calves\(^1\,\,2\)

<table>
<thead>
<tr>
<th>Item</th>
<th>FF 1</th>
<th>FF 2</th>
<th>FF 4</th>
<th>SEM</th>
<th>FL low</th>
<th>FL high</th>
<th>FL low</th>
<th>FL high</th>
<th>FL × FL</th>
</tr>
</thead>
<tbody>
<tr>
<td>([1-^{13}C])leucine oxidation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery as (^{13}CO_2), % of dose</td>
<td>39.3</td>
<td>41.8</td>
<td>49.7</td>
<td>58.6</td>
<td>38.2</td>
<td>0.52</td>
<td>0.059</td>
<td>0.190</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Maximum (^{13}CO_2) production, % of dose/h</td>
<td>4.2</td>
<td>6.5</td>
<td>7.3</td>
<td>9.3</td>
<td>7.2</td>
<td>0.20</td>
<td>0.068</td>
<td>0.971</td>
<td>0.883</td>
</tr>
<tr>
<td>Time to peak, min</td>
<td>281</td>
<td>169</td>
<td>204</td>
<td>129</td>
<td>163</td>
<td>3.5</td>
<td>&lt;0.001</td>
<td>0.442</td>
<td>0.838</td>
</tr>
<tr>
<td>([I-^{13}C])glucose oxidation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery as (^{13}CO_2), % of dose</td>
<td>75.1</td>
<td>84.6</td>
<td>84.7</td>
<td>79.3</td>
<td>73.8</td>
<td>1.08</td>
<td>0.065</td>
<td>0.093</td>
<td>0.502</td>
</tr>
<tr>
<td>Maximum (^{13}CO_2) production, % of dose/h</td>
<td>6.8</td>
<td>9.6</td>
<td>9.4</td>
<td>11.6</td>
<td>11.5</td>
<td>0.13</td>
<td>&lt;0.001</td>
<td>0.286</td>
<td>0.948</td>
</tr>
<tr>
<td>Time to peak, min</td>
<td>403</td>
<td>290</td>
<td>301</td>
<td>205</td>
<td>217</td>
<td>3.0</td>
<td>&lt;0.001</td>
<td>0.190</td>
<td>0.858</td>
</tr>
<tr>
<td>([2-^{13}C])glucose oxidation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery as (^{13}CO_2), % of dose</td>
<td>73.3</td>
<td>88.2</td>
<td>86.4</td>
<td>74.2</td>
<td>72.0</td>
<td>1.21</td>
<td>0.144</td>
<td>0.782</td>
<td>0.953</td>
</tr>
<tr>
<td>Maximum (^{13}CO_2) production, % of dose/h</td>
<td>6.9</td>
<td>10.3</td>
<td>9.1</td>
<td>10.9</td>
<td>11.7</td>
<td>0.21</td>
<td>0.001</td>
<td>0.858</td>
<td>0.177</td>
</tr>
<tr>
<td>Time to peak, min</td>
<td>393</td>
<td>297</td>
<td>300</td>
<td>243</td>
<td>235</td>
<td>4.4</td>
<td>&lt;0.001</td>
<td>0.904</td>
<td>0.604</td>
</tr>
</tbody>
</table>

1 Values are means, \(n = 8\) (FF 1), \(n = 5\) (FF 2, FL low), or \(n = 6\).

2 Recoveries and maxima are corrected for bicarbonate sequestration.

of glucose for fatty acid synthesis remain to be studied in milk-fed calves.

**Literature Cited**


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33. Moehn S, Bertolo RFP, Pencharz PB, Ball RO. Pattern of carbon dioxide production and retention is similar in adult pigs when fed hourly, but not when fed a single meal. BMC Physiol. 2004;4:1–8.