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Variation of Milk Citrate with Stage of Lactation and De Novo Fatty Acid Synthesis in Dairy Cows

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ABSTRACT

Citrate is a normal constituent of milk that affects milk-processing characteristics. It is an intermediate in the tricarboxylic acid cycle and plays an indirect role in fat synthesis by providing reducing equivalents in the form of NADPH. The objective of this study was to investigate variation in citrate with stage of lactation and de novo fatty acid synthesis, without confounding dietary effects. Twenty-four cows were fed the same diet, and milk citrate and fatty acids were determined over a 10-d period. Eight cows were in early lactation [13 ± 1.8 d in milk (DIM; mean ± standard error], 8 in midlactation (130 ± 4.6 DIM), and 8 in late lactation (283 ± 3.4 DIM). For cows in early, mid, and late lactation, milk yield was 34.4, 34.4, and 21.4 L/d [standard error of difference (SED) 1.78]; milk fat was 50.4, 40.3, and 41.4 g/L (3.68); milk citrate was 11.3, 9.7, and 10.1 mmol/L (0.64); the ratio of 4–14 C:18–20 C fatty acids was 0.9, 1.3, and 1.2 (0.07). Activity of the fatty acid synthase enzyme system (EC 2.3.1.85) was calculated as acetate used for chain elongation (ACE); ACE (mol/d) for cows in early, mid, and late lactation, was 7.3, 11.1, and 8.1 (SED 1.05). For individual cows, citrate (mmol/L) = 14.3 − 0.44 × ACE (r^2 = 0.58). We propose that ACE provides a more accurate indication of synthase activity than do fatty acid ratios or yields. This study confirms the hypothesis that variation in milk citrate with stage of lactation is related to de novo synthesis of fatty acids and that the relationship is independent of diet and milk yield.
Key words: dairy cow, milk citrate, stage of lactation, fatty acid synthesis

INTRODUCTION

Citrate is a normal constituent of milk and forms one of the main buffer systems that regulates the equilibrium between Ca^{2+} and H^+ ions (Faulkner and Peaker, 1982). Citrate affects milk-processing characteristics because it interacts with other milk constituents to influence coagulation of milk protein and its fermentation products yield distinct aromatic flavors characteristic of fermented milk products (Rosenthal, 1991). The biological role of citrate in milk is unknown, but its main role is thought to be maintenance of fluidity through its effects on structure of casein micelles (Faulkner and Peaker, 1982).

Citrate plays a central role in cellular energy metabolism, being an intermediate in the tricarboxylic acid cycle. It has been proposed as an indicator of energy status in the cow, being correlated with ketones in milk (Baticz et al., 2002). However, mammary epithelium is impermeable to citrate in both directions (Linzell et al., 1976), so milk citrate concentration reflects mammary activity rather than general metabolism.

In ruminants, citrate is not a significant intermediate for fatty acid synthesis. Nevertheless, citrate has an indirect role in fat synthesis by providing reducing equivalents in the form of NADPH, which are required for de novo synthesis of fatty acids (Faulkner and Peaker, 1982). In de novo synthesis, each cycle of chain elongation uses 2 molecules of NADPH, similar amounts of which are produced by the pentose phosphate pathway and by the isocitrate cycle (Moore and Christie, 1981). In the isocitrate cycle, NADPH is produced by conversion of isocitrate to α-ketoglutarate. Isocitrate is an isomer of citrate and the 2 are maintained in equilibrium in the cell (Peaker and Faulkner, 1983). Therefore, if de novo synthesis of fatty acids increases, isocitrate concentration decreases, and citrate concentration decreases. These relationships are supported by the studies of Banks et al. (1984, 1990), which used fat supplements to decrease de novo synthesis of fatty acids in the mammary gland and found proportional increases in milk citrate concentration.

In long-term studies, concentrations of citrate in milk have been found to vary according to season and stage of lactation. In general, citrate concentrations are higher during the grazing season (Holt and Muir, 1979; Mitchell, 1979; Keogh et al., 1982) and during early lactation

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(Braunschweig and Puhan, 1999). These effects might be related to de novo synthesis of fatty acids in the mammary gland, which is reduced when cows are fed fresh pasture (Lock and Garnsworthy, 2003) or when cows are rapidly mobilizing body fat (Peaker et al., 1981). However, in all long-term studies of milk citrate, effects of stage of lactation and season have been confounded not only with each other, but also with changes in diet.

The objectives of this study were to determine variation in milk citrate concentration with stage of lactation independently of dietary or seasonal effects, and to see if variation in milk citrate is related to de novo synthesis of fatty acids in the mammary gland. These objectives were achieved using milk samples from cows at different stages of lactation fed the same diet at the same time.

MATERIALS AND METHODS

Animals, Diets, and Sampling

Twenty-four Holstein cows were selected from the University of Nottingham dairy herd according to DIM, with 8 cows in early lactation (4 to 29 DIM), 8 in midlactation (103 to 156 DIM), and 8 in late lactation (265 to 306 DIM). All cows were fed ad libitum the same TMR, which consisted of corn silage (34% of DM), grass silage (14%), soybean meal (17%), wheat (15%), brewers' grains (9%), palm kernel meal (8%), ruminally inert fat (Megalac, Volac International, Royston, UK; 2%) and a mineral and vitamin supplement (Hi-Phos, Bibby Agriculture, Peterborough, UK; 1%). The composition of the TMR was 459 g of DM/kg, 12.1 MJ of ME/kg of DM, 180 g of CP/kg of DM, 207 g of starch/kg of DM, 49 g of oil/kg of DM, and 347 g of NDF/kg of DM. All cows were fed as one group, so individual feed consumption could not be measured. The TMR had been fed to the herd for more than 1 mo before this study commenced. Cows were milked twice daily between 0500 and 0700 h, and between 1500 and 1700 h. Milk samples were collected daily at both milkings for 10 consecutive days. One 20-mL aliquot of each milk sample was stored at 4°C with preservative (30 mg of potassium dichromate; Lactab MkIII tablet, Thomson and Capper Ltd., Runcorn, Cheshire, UK) until analyzed for fat, protein, lactose, and SCC by infrared analysis at the National Milk Records Laboratory (Harrogate, Yorkshire, UK) using reference method (AOAC, 1990; method no. 972.16). Two additional aliquots were stored without preservative at –20°C for determination of milk citrate and fatty acids. Milk samples from a.m. and p.m. milkings were analyzed separately.

Milk Analysis

Milk citrate was determined by HPLC. Samples were thawed at room temperature and defatted by centrifugation at $1,970 \times g$ for 10 min to separate the fat layer. Deproteinization was carried out by treatment of a 0.4-mL aliquot of skimmed milk with 3.6 mL of cold 3% TCA to precipitate proteins, followed by centrifugation at $17,700 \times g$ for 10 min. A 1.5-mL aliquot of supernatant was placed in an HPLC vial, and stored at –20°C until analysis.

The HPLC apparatus (model Gynkotek, Jaytee Biosciences Ltd, Whitstable, Kent, UK) consisted of a gradient pump (m480G), a Gina 50 autosampler, a UVD340 diode array detector, and an Inertsil C8, 5-μm column (150 × 4.6 mm i.d.). The mobile phase was 98% 0.1 *M* KH_2PO_4 (pH 3.0 with H_3PO_4 + 2% acetonitrile). The flow rate was 1.0 mL/min and the UV detector was set at 218 nm. The column temperature was 40°C. Data were analyzed by Chromeleon software (Dionex, Camberley, UK).

Milk fat was extracted by centrifugation and proportions of individual fatty acids were determined as fatty acid methyl esters (**FAME**) by gas chromatography following the procedures described by Feng et al. (2004). A butter-oil reference standard (CRM 164; Commission of the European Community Bureau of References, Brussels, Belgium) was used as a routine check for recoveries and correction factors for individual fatty acids.

Calculations and Statistical Analysis

Individual yields of milk constituents at morning and afternoon milkings were summed each day and divided by daily milk yield to produce daily mean concentrations. All daily data were averaged for each cow before statistical analysis.

Proportions of individual milk fatty acids determined by gas chromatography were converted to molar proportions and daily yields following the calculations of Schauff et al. (1992). Area percentages for FAME were corrected using the butter-oil correction factors to account for recoveries of individual FAME. Corrected areas (g/100 g of FAME) were then adjusted by removing the mass of the methyl group to produce proportions of fatty acids (g/100 g of fatty acids). Molecular weights of fatty acids were then used to calculate molar proportions (mmol/mol of fatty acids). Unidentified fatty acids were assigned a dummy molecular weight equivalent to 18:1 after examination of their contributions to the total peak area and retention times relative to identified fatty acids. It was assumed that milk fat was 100% triglycerides containing 3 mol of fatty acids/mol of glycerol (Schauff et al., 1992). Therefore, molar proportions

of fatty acids, plus 333 mmol of glycerol/mol of fatty acids, were used to calculate the relative weight proportions of fatty acids in milk fat (g/100 g of fat) after allowing for the mass of hydrogen and oxygen used in esterification. The relative weight proportions of fatty acids in milk fat were then used to calculate daily yields of fatty acids (mol/d) in milk.

De novo synthesis of fatty acids was examined using 2 indicators. For the first indicator, the sum of fatty acids with chain lengths of 4 to 14 C, which are all synthesized de novo in the mammary gland, was divided by the sum of fatty acids with chain lengths of 18 or 20 C, which are all derived from plasma fatty acids. This ratio is commonly used to indicate mammary synthase activity; it ignores fatty acids with 16 C because they are partly synthesized de novo and partly derived from plasma. For the second indicator, acetate used for chain elongation during de novo synthesis of fatty acids (**ACE**) was calculated because this is related to use of NADPH. It was assumed that 50% of the initial C_4 moiety comes from acetate and 50% from BHBA, and that chain elongation using acetate accounts for all of the milk fatty acids from 6:0 to 14:0 and 60% of 16:0 (Moore and Christie, 1981). Thus, if L = chain length, synthesis of 1 mole of a fatty acid requires (L/2 – 1.5) moles of ACE. Total ACE was calculated as the sum of ACE in 4:0 to 16:0. As discussed later, for cows in early lactation with milk fat >40 g/kg, ACE was adjusted to (L/2 – 1.75), and the proportion of 16:0 synthesized de novo was reduced from 60% to 10%, to allow for mobilized body fat.

Activity of the Δ^9-desaturase (stearoyl-CoA desaturase) enzyme system (EC 1.14.19.1), which adds a double bond between C 9 and 10, was calculated using the 14:0 and *cis*-9 14:1 content of milk fat: [14:1c9]/[14:1c9 + 14:0] × 100.

Statistical analyses were conducted using Genstat 6.0 (Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Effects of stage of lactation on milk yield and composition were examined by simple one-way ANOVA, using the model $y_{ij} = \mu + e_i + \varepsilon_{ij}$, where y_{ij} is the value measured for individual j in lactation-stage group i, μ is the overall mean, e_i is the fixed effect of lactation-stage group i (i = 1, 2, 3), and ε_{ij} is the residual error. Where stage of lactation had a significant effect, group means were compared by LSD test. Relationships between milk citrate concentration and de novo fatty acid synthesis were examined by simple linear regression.

RESULTS

Milk Yield and Composition

Average milk yield was the same for cows in early and midlactation, but was lower for cows in late lactation (Table 1). Milk fat concentration was similar in mid and late lactation, but was higher in early lactation; milk protein concentration was similar in early and late lactation, but was lower in midlactation; milk lactose concentration was not affected by stage of lactation (Table 1). Daily yield of fat decreased with stage of lactation; yields of lactose and protein were similar for cows in early and midlactation but lower for cows in late lactation (Table 1).

Table 1. Days in milk, milk yield, and milk solids in groups of cows at 3 stages of lactation (n = 8); all cows were fed the same diet

	Stage of lactation				
	Early	Mid	Late	SED	*P*
DIM	13	130	283	4.9	
Milk yield, L/d	34.4[a]	34.4[a]	21.4[b]	1.78	***
Fat					
g/L	50.4[a]	40.3[b]	41.4[b]	3.68	*
g/d	1,812[a]	1,394[b]	887[c]	146.5	***
Protein					
g/L	34.8[a]	32.2[b]	35.2[a]	0.89	**
g/d	1,194[a]	1,107[a]	752[b]	59.3	***
Lactose					
g/L	46.5	45.7	45.2	0.72	NS
g/d	1,598[a]	1,573[a]	968[b]	82.0	***

[a–c]Means within row with different superscripts differ ($P < 0.05$).
***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; [NS]$P > 0.05$.

Milk Fatty Acids

Molar proportions of 4:0, 16:1, 18:1c9, 18:1c11, and 18:2c9c12 were greater for cows in early lactation, and molar proportions of 10:0, 12:0, 14:0, 14:1, 16:0, 18:2c9t11, and unidentified fatty acids were lower for cows in early lactation, but molar proportions of these fatty acids were similar for cows in mid and late lactation (Table 2).

Yields of 10:0, 12:0, 14:0, 14:1, 15:0, 15:0iso, 15:0anteiso, 16:0, 17:0, 18:1t16, 18:2c9t11, 18:3, and 20:0 were lower for cows in late lactation, but were similar for cows in early and midlactation; yield of 18:0 was higher in early lactation, but was similar in mid and late lactation; yields of all other identified fatty acids were greatest in early lactation, lower in midlactation and lowest in late lactation; yield of unidentified fatty acids was higher in midlactation than in early or late lactation (Table 3).

Milk Citrate and Fatty Acid Synthesis

Milk citrate concentration was significantly higher for cows in early lactation than for cows in midlactation, but milk citrate concentration for cows in late lactation was intermediate and not significantly different from

Table 2. Molar proportions of fatty acids (mmol/mol of total fatty acids) in groups of cows at 3 stages of lactation (n = 8); all cows were fed the same diet

	Stage of lactation[1]				
Fatty acid	Early	Mid	Late	SED	*P*
4:0	112[a]	96[b]	97[b]	3.0	***
6:0	43	42	42	1.8	NS
8:0	19	20	20	1.1	NS
10:0	31[a]	38[b]	37[b]	2.1	**
12:0	32[a]	39[b]	37[b]	1.7	***
14:0	89[a]	118[b]	116[b]	3.1	***
14:1	8[a]	11[b]	10[b]	0.9	*
15:0	9	11	10	1.0	NS
15:0iso	2	3	3	0.3	NS
15:0anteiso	3	4	5	0.4	NS
16:0	269[a]	301[b]	290[b]	9.8	*
16:1	20[a]	16[b]	15[b]	1.5	**
17:0	5	6	6	0.4	NS
18:0	86	76	82	6.3	NS
18:1c9	197[a]	138[b]	146[b]	8.9	***
18:1c11	7[a]	4[b]	4[b]	0.5	***
18:1t6-8	2	2	2	0.2	NS
18:1t9	2	2	2	0.1	NS
18:1t10	4	4	3	0.4	NS
18:1t11	9	10	11	0.7	NS
18:1t12	4	4	4	0.2	NS
18:1t16	3	3	4	0.2	NS
18:2c9c12	19[a]	16[b]	17[ab]	1.2	*
18:2c9t11	3[a]	5[b]	5[b]	0.3	***
18:3	3	3	4	0.3	NS
20:0	1	1	1	0.1	NS
Unidentified	17[a]	29[b]	30[b]	3.0	*

[a,b]Means within row with different superscripts differ ($P < 0.05$).
[1]Mean DIM: Early = 13; Mid = 130; Late = 283.
***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; [NS]$P > 0.05$.

Table 3. Daily yield of fatty acids (mmol/d) in groups of cows at 3 stages of lactation (n = 8); all cows were fed the same diet

	Stage of lactation[1]				
Fatty acid	Early	Mid	Late	SED	*P*
4:0	649[a]	441[b]	287[c]	45.1	***
6:0	270[a]	209[b]	132[c]	18.0	***
8:0	120[a]	101[b]	64[c]	9.3	***
10:0	198[a]	199[a]	124[b]	18.2	***
12:0	207[a]	203[a]	127[b]	18.9	***
14:0	588[a]	622[a]	390[b]	50.2	***
14:1	54[a]	59[a]	35[b]	6.0	***
15:0	55[a]	58[a]	34[b]	5.0	***
15:0iso	15[a]	15[a]	10[b]	1.1	***
15:0anteiso	22[a]	24[a]	15[b]	1.6	***
16:0	1,801[a]	1,613[a]	997[b]	144.2	***
16:1	138[a]	87[b]	51[c]	14.8	***
17:0	33[a]	30[a]	19[b]	3.4	***
18:0	593[a]	408[b]	277[b]	64.8	***
18:1c9	1,325[a]	742[b]	490[c]	88.0	***
18:1c11	47[a]	22[b]	12[c]	4.6	***
18:1t6-8	16[a]	11[b]	7[c]	0.8	***
18:1t9	13[a]	10[b]	7[c]	0.7	***
18:1t10	25[a]	19[b]	10[c]	2.4	***
18:1t11	62[a]	51[b]	36[c]	4.6	***
18:1t12	26[a]	21[b]	14[c]	1.5	***
18:1t16	21[a]	18[a]	12[b]	1.4	***
18:2c9c12	128[a]	87[b]	56[c]	9.2	***
18:2c9t11	21[a]	24[a]	17[b]	2.0	**
18:3	18[a]	19[a]	12[b]	1.6	**
20:0	4[a]	4[a]	3[b]	0.4	**
Unidentified	115[a]	158[b]	103[a]	13.1	***

[a–c]Means within row with different superscripts differ ($P < 0.05$).
[1]Mean DIM: Early = 13; Mid = 130; Late = 283.
***$P < 0.001$; **$P < 0.01$.

either of the other groups (Table 4). Daily yield of citrate decreased with stage of lactation (Table 4).

The sum of fatty acids 4:0 to 14:1 (synthesized de novo) was lower for cows in early lactation; the sum of fatty acids 18:0 to 20:0 (preformed fatty acids) was higher for cows in early lactation; consequently, the ratio of de novo to preformed fatty acids was lower for cows in early lactation. These sums and ratio were similar for cows in mid and late lactation (Table 4).

Using means for individual cows, a significant relationship was found between milk citrate concentration and the ratio of synthesized to preformed fatty acids. Milk citrate concentration decreased by 3.08 mmol/L for every unit increase in this ratio (Figure 1). A similar relationship was found between milk citrate concentration and ACE (mol/mol of fatty acids). Milk citrate concentration decreased by 3.9 mmol/L for every 1-mol increase in ACE (Figure 2).

The sum of yields for fatty acids 4:0 to 14:1 was similar for cows in early and midlactation, but was lower for cows in late lactation; the sum of yields for fatty acids 18:0 to 20:0 was highest in early lactation, lower in midlactation, and lowest in late lactation; the ratio of these sums was lower for cows in early lactation, but was similar for cows in mid and late lactation (Table 4).

Daily total ACE (mol/d) was highest in early lactation, lower in midlactation, and lowest in late lactation when calculated using the assumption that 50% of C_4 moieties and 60% of 16:0 came from acetate. For individual cows in mid and late lactation, a significant relationship was found between milk citrate concentration and ACE. Milk citrate concentration decreased by 0.26 mmol/L for every 1 mol/d increase in ACE for these cows (Figure 3a). Six cows in early lactation, however, did not fit this relationship; these cows were characterized by milk fat concentrations >40 g/L.

When the calculation of ACE was adjusted for cows in early lactation with milk fat >40 g/L, to assume that 25% of C_4 moieties came from acetate and 75% from BHBA, and that 10% of 16:0 and 16:1c9 was synthesized de novo, ACE for cows in early lactation was similar to ACE for cows in late lactation (Table 4). Furthermore, all cows now fitted one highly significant relationship; milk citrate concentration decreased by 0.44 mmol/L for every 1 mol/d increase in ACE (Figure 3b).

Activity of the Δ^9-desaturase enzyme system, measured as the percentage of 14-C fatty acids present as 14:1, did not differ with stage of lactation (Table 4).

Table 4. Milk citrate, sums and ratios of fatty acids (FA) synthesized de novo (DN) or preformed (PF), and acetate used for chain elongation (ACE) during de novo synthesis in groups of cows at 3 stages of lactation (n = 8); all cows were fed the same diet

	Stage of lactation[1]				
	Early	Mid	Late	SED	*P*
Citrate					
mmol/L	11.3[a]	9.7[b]	10.1[ab]	0.64	*
mmol/d	387[a]	330[a]	216[b]	32.8	***
Sum 4:0–14:1 (DN; mmol/mol of FA)	335[a]	363[b]	358[b]	9.1	*
Sum 18:0–20:0 (PF; mmol/mol of FA)	340[a]	268[b]	284[b]	13.3	***
Ratio DN:PF	1.0[a]	1.4[b]	1.3[b]	0.07	***
Sum 4:0–14:1 (DN; mmol/d)	2,087[a]	1,820[a]	1,158[b]	156	***
Sum 18:0–20:0 (PF; mmol/d)	2,299[a]	1,435[b]	953[c]	161	***
Ratio DN:PF	0.9[a]	1.3[b]	1.2[b]	0.07	***
ACE,[2] mol/mol of FA	2.1[a]	2.4[b]	2.3[b]	0.06	***
ACE, mol/d	13.9[a]	11.1[b]	8.1[c]	0.95	***
ACE Adjusted,[3] mol/d	7.3[a]	11.1[b]	8.1[ab]	1.05	**
Δ^9-Desaturase activity[4]	8.5	8.7	8.1	0.71	NS

[a–c]Means within row with different superscripts differ ($P < 0.05$).

[1]Mean DIM: Early = 13; Mid = 130; Late = 283.

[2]ACE in de novo synthesis of fatty acids calculated as (chain length/2 – 1.5) mol/mol for fatty acids 4:0 to 14:1 and (chain length/2 – 1.5) × 0.6 mol/mol for 16:0 and 16:1.

[3]For cows in early lactation with milk fat content >40 g/kg, ACE calculated as (chain length/2 – 1.75) mol/mol for fatty acids 4:0 to 14:1 and (chain length/2 – 1.75) × 0.1 mol/mol for 16:0 and 16:1.

[4]Calculated as 14:1/(14:0 + 14:1) × 100.

***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; [NS]$P > 0.05$.

DISCUSSION

Milk Yield, Fat, and Protein

Cows in early lactation might be expected to yield more milk than cows in midlactation. In this study, however, cows in the early lactation group averaged just 13 DIM, so they had not yet reached peak milk yield. Because milk yield was the same, differences between early and mid lactation cows in milk composition can be attributed to differences in secretion of milk solids rather than to dilution. The higher concentrations of milk fat and protein are typical of cows in early

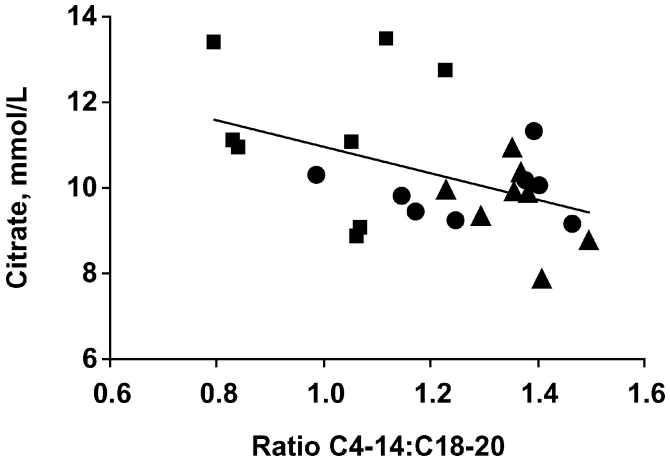

Figure 1. Relationship between milk citrate concentration and ratio of concentrations of fatty acids synthesized de novo (C4–14) to preformed fatty acids (C18–20) for cows in early (■), mid (▲), and late (●) lactation. Mean DIM: Early = 13; Mid = 130; Late = 283. Line shows regression for combined data (citrate = 14.02 – 3.08 × ratio; $r^2 = 0.21$; $P < 0.05$).

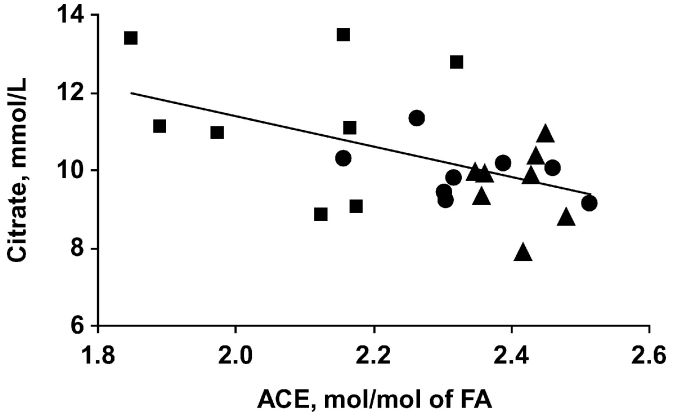

Figure 2. Relationship between milk citrate concentration and estimated quantity of acetate used for chain elongation in de novo synthesis of fatty acids (ACE; mol/mol of fatty acids) for cows in early (■), mid (▲), and late (●) lactation; ACE was calculated as (chain length/2 – 1.5) mol/mol for fatty acids 4:0 to 14:1 and (chain length/2 – 1.5) × 0.6 mol/mol for 16:0 and 16:1. Mean DIM: Early = 13; Mid = 130; Late = 283. Line shows regression for combined data (citrate = 19.2 – 3.9 × ACE; $r^2 = 0.26$; $P < 0.05$).

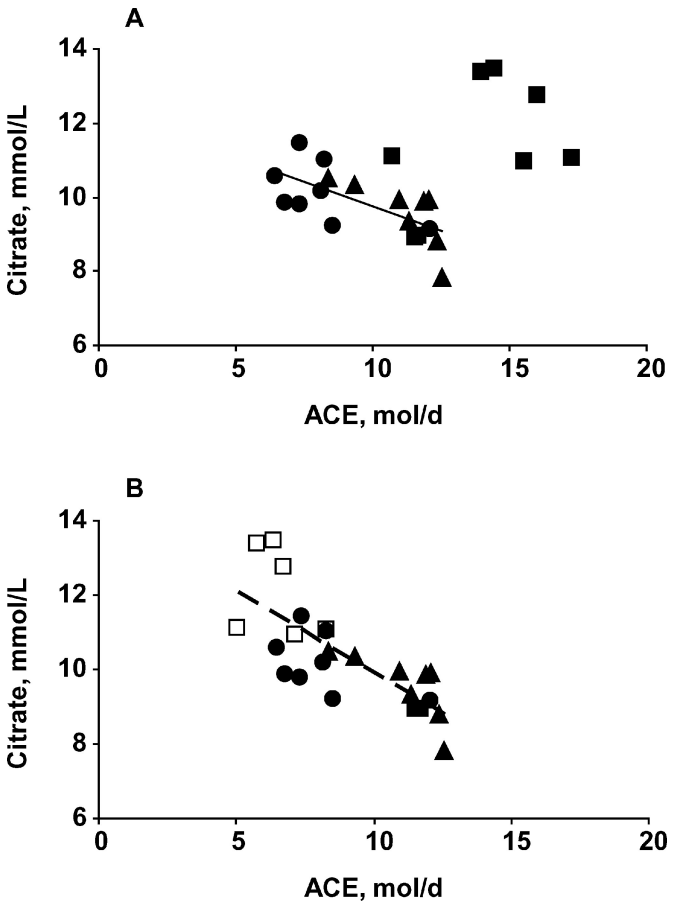

Figure 3. Relationship between milk citrate concentration and estimated daily quantity of acetate used for chain elongation in de novo synthesis of fatty acids (ACE; mol/d) for cows in early (■), mid (▲), and late (●) lactation; mean DIM: Early = 13; Mid = 130; Late = 283. A) ACE was calculated as (chain length/2 – 1.5) mol/mol for fatty acids 4:0 to 14:1 and (chain length/2 – 1.5) × 0.6 mol/mol for 16:0 and 16:1. Solid line shows regression for cows in mid and late lactation (citrate = 12.4 – 0.26 × ACE; $r^2 = 0.43$; $P < 0.01$). B) For cows in early lactation with fat >40 g/kg, ACE was calculated as (chain length/2 – 1.75) mol/mol for fatty acids 4:0 to 14:1 and (chain length/2 – 1.75) × 0.1 mol/mol for 16:0 and 16:1; for other cows, ACE was calculated as in panel A. Dashed line shows regression for all cows (citrate = 14.3 – 0.44 × ACE; $r^2 = 0.58$; $P < 0.001$).

lactation. The decline in milk yield for cows in late lactation, accompanied by an increase in protein concentration, is also typical.

Milk Fatty Acids

The fatty acid profile of milk is a result of complex interactions among diet composition, DMI, rumen fermentation, liver metabolism, body fat mobilization, and mammary absorption and synthesis of fatty acids. In the current study diet composition was constant, thus removing one of the major influences on fatty acid profile. Milk yield and DMI vary with stage of lactation, and therefore influence yield of fatty acids; they might also influence the relative proportions of individual fatty acids indirectly by influencing the balance between body fat mobilization and de novo synthesis of fatty acids in the mammary gland.

Many studies have shown that dairy cows are usually in negative energy balance in early lactation and mobilize considerable amounts of body fat because feed intake is inhibited by body fat (Garnsworthy and Topps, 1982). In adipose tissue, 18:1c9, 16:0, and 18:0 account for nearly 90% of fatty acids in roughly equal molar proportions (Christie, 1981); body fat mobilization would be expected to increase direct incorporation of these fatty acids into milk fat. In terms of molar proportions, only 18:1c9 was higher in early lactation but, more importantly, daily yields of both 18:1c9 and 18:0 were higher in early lactation. In fact, yield of 18:1c9 for early-lactation cows was 180% of the value for midlactation cows, with no difference in milk yield and no change in diet composition. Palmquist et al. (1993) reported that concentration of 18:1 in milk fat was 50% higher in wk 1 of lactation than in wk 16; in that study, however, concentration of 18:0 was also 50% higher. Some 18:1c9 is produced from 18:0 in the mammary gland by the Δ^9-desaturase enzyme system, but desaturase activity was similar for all groups in the current study, so this potential source of variation in 18:1c9 and 18:0 can be discounted. It can be concluded, therefore, that differences in 18:1c9 and 18:0 were due to increased mobilization of body fat in early lactation.

Mobilization of body fat did not increase the molar proportion of 16:0 in early lactation, probably because de novo synthesis of 16:0 was reduced or 16:0 was catabolized for other purposes. Yield of 18:1c9 was 80% greater in early lactation than in midlactation, but yield of 16:0 was only 11% greater. Normally 60% of 16:0 originates from de novo synthesis (Moore and Christie, 1981), corresponding to 970 mmol/d for cows in midlactation. The difference between groups in 18:1c9 yield suggests that 580 mmol/d was derived from mobilized body fat. If the same quantity of 16:0 were derived from mobilized body fat, apparent de novo synthesis of 16:0 in early lactation cows would be reduced to 570 mmol/d, which is an average of 30% instead of 60% of 16:0 yield. The reduction would have varied among individual cows, as discussed later.

Molar proportions of fatty acids in the range 10:0 to 14:0 were significantly lower in early lactation than in midlactation. Palmquist et al. (1993) and Auldist et al. (1998) reported lower proportions of fatty acids in the range 6:0 to 14:0 by cows in early lactation compared with cows in midlactation, and concluded that de novo synthesis of these fatty acids was inhibited by long-chain fatty acids from body fat. However, in the current

study, yields of 6:0 to 14:0 fatty acids were not different. Therefore, on a daily basis, de novo synthesis of fatty acids was not inhibited in early lactation and reductions in molar proportions and ratios were due to increases in long-chain fatty acids supplied by body fat mobilization.

Molar proportion and yield of 4:0 were higher in early lactation than in midlactation. This could be related to body fat mobilization for ketogenesis and consequently, plasma BHBA concentration, which is known to be elevated immediately after calving. In a survey of 10,199 cows, Ward et al. (1995) found that BHBA rose rapidly after calving to peak at 25 DIM. Although BHBA was not measured in the current experiment, values (mmol/L) for cows with equivalent DIM from the study of Ward et al. (1995) were 0.93 for early lactation, 0.6 for midlactation, and 0.5 for late lactation. Moore and Christie (1981) reviewed tracer studies of milk fatty acid synthesis and concluded that 50% of the initial C_4 moiety comes from acetate and 50% from BHBA; they also concluded that chain elongation to produce 6:0 to 16:0 uses only acetate, although a small proportion of acetate can be derived from cleaved BHBA. Thus, it is possible that the elevated 4:0 was a result of substrate supply and termination of synthesis at 4:0 would be energetically more efficient.

Palmquist et al. (1993) reviewed several studies that found reduced concentrations of short chain fatty acids in early lactation with unchanged or elevated concentrations of 4:0. They suggested that 4:0 synthesis was not reduced because, in addition to direct formation from BHBA, 4:0 can also be formed by the β-reduction pathway, which is independent of malonyl-CoA.

Another alternative is that 4:0 was elevated in milk from cows in early lactation as an obligation for triacylglycerol formation. Jensen (2002) reported that 98.1% of 4:0 is located at the *sn*-3 position, and that 36% of triacylglycerols contain 4:0 or 6:0 plus 2 long-chain fatty acids, the most common being 18:1-16:0-4:0, 16:0-16:0-4:0 and 16:0-14:0-4:0. Thus, elevated 4:0 might have been required to balance increased incorporation of 18:1c9 and 16:0 into triacylglycerols.

Unidentified fatty acids were those for which standards were not available and are likely to be branched-chain, geometric, and positional isomers arising from microbial synthesis or modification of fatty acids. Individual unidentified fatty acids formed less than 1 mmol/mol of total fatty acids; although collectively they formed 17 to 30 mmol/mol. Unidentified fatty acids represent the balance between the sum of identified fatty acids and 100%. Therefore, the significantly lower proportion of unidentified fatty acids in early lactation probably indicates a change in the contribution of body fat mobilization, which increased the proportion of identified fatty acids (especially 18:1c9), relative to the contribution of fatty acids produced in the rumen.

Cows in late lactation produced milk with a fatty acid profile similar to that of cows in midlactation. Differences between mid and late lactation in yield of fatty acids were due to differences in milk fat yield, so it can be concluded that stage of lactation does not affect the relative incorporation of fatty acids from de novo synthesis vs. preformed sources when diet composition is constant.

Milk Citrate Concentration

Some workers have found that milk citrate concentration is affected by mastitis, which alters permeability of epithelial cells in the udder (Hamann and Krömker, 1997). Erhardt and Senft (1982) found that milk citrate decreased rapidly after experimental infection of the mammary gland, but Sloth et al. (2003) found no difference in milk citrate concentration between cows with healthy or infected udders. No cow in the current study showed clinical signs of mastitis. Somatic cell counts averaged 165,000 cells/mL and were below 250,000 cells/mL for all but 3 cows in midlactation. Two of these cows had SCC averaging 358,000 and 380,000 cells/mL, which are considered slightly high; the third cow had a history of high SCC and averaged 1,273,000 cells/mL. There was no relationship between milk citrate concentration and SCC in this study, and milk citrate for the 3 cows with higher SCC was not significantly different from other cows within the midlactation group. Thus, it can be concluded that variations in milk citrate were not a result of (or related to) mastitis.

Milk citrate concentration has been found to vary widely throughout lactation (Banks et al., 1984). Therefore, cows sampled in early, mid, and late lactation were selected to be within a narrow range of DIM to reduce this source of variation within each stage. Citrate concentrations were lowest in midlactation, although milk yields were similar in early and midlactation. The difference in citrate concentration between early and midlactation provides evidence for an effect of lactation stage on citrate that is independent of milk yield and diet. There was no difference in citrate concentration, however, between cows in mid and late lactation. This suggests that stage of lactation only affects citrate concentration in early lactation and that the effect is probably mediated through differences in fatty acid synthesis.

The majority of studies have reported milk citrate to be high in early lactation and to gradually decrease as lactation progresses (Konar et al., 1971; Illek et al., 1997). Braunschweig and Puhan (1999) found that citrate was significantly higher from 30 to 80 DIM (9.94

mmol/L) than from 170 to 215 DIM (9.16 mmol/L). It is difficult to determine what proportion of variation is related to stage of lactation in other studies because dietary changes are common over the lactation cycle. Seasonal variations have also been reported (Holt and Muir, 1979) but these also might be attributable to changes in diet, such as grazing vs. indoor TMR feeding. Dietary effects can be discounted in the current study, so variations with stage lactation are probably related to changes in intramammary energy metabolism and fatty acid synthesis.

Relationship Between Citrate and Fatty Acid Synthesis

The relationship between citrate and milk fatty acid profile was investigated to determine whether citrate varied with de novo fatty acid synthesis, as suggested in the literature (Faulkner and Peaker, 1982; Banks et al., 1984). Previous studies of this relationship have used dietary changes to manipulate de novo synthesis, but the current study used a single diet. A relationship was found between citrate and the ratio of synthesized to preformed chain fatty acids, suggesting that citrate concentration does reflect de novo fatty acid synthesis in the mammary gland, even in the absence of dietary manipulation. The r^2 value of this relationship was low, however, because of the relatively high citrate concentrations for some cows in early lactation.

Banks et al. (1990) found citrate (mol/L) = 13.38 – 0.216 short-chain fatty acids (**SCFA**, g/L; $r^2 = 0.43$, $P < 0.01$), where SCFA is the sum of 6:0 to 14:1. In the current study, the same variables produced a remarkably similar equation: citrate = 15.74 – 0.217 SCFA ($r^2 = 0.17$, $P < 0.05$). The r^2 value for this equation was relatively low because citrate concentrations for cows in early lactation were generally higher than predicted.

Stoichiometric calculation of acetate required for chain elongation in de novo synthesis of fatty acids (ACE) gives a truer reflection of synthase enzyme activity than either concentration ratios or yields of fatty acids. Ratio of SCFA to long-chain fatty acid concentrations is often used as an indicator of de novo fatty acid synthesis, but this can lead to false conclusions. Fatty acid proportions must sum to 100%, so increased incorporation of long-chain fatty acids into milk will suggest that de novo fatty acid synthesis is inhibited even if, as for early- vs. midlactation cows in the current study, there is no difference in yield of synthesized fatty acids. Yields of individual fatty acids give a better indication of synthase enzyme activity than concentrations. However, total yields (in g/d) bias toward medium-chain fatty acids because, for example, 1 mol of 12:0 has less mass than 2 mol of 6:0. Total yields in moles per day also bias toward medium-chain fatty acids because, for example, 12:0 requires 5 elongation steps during synthesis, whereas 6:0 requires only 2. As far as we are aware, ACE has not been reported in previous studies. We propose that this calculation provides a useful contribution to understanding the relative contributions of chain initiation and elongation when examining de novo fatty acid synthesis.

Adjusting the calculation of ACE for 6 cows in early lactation improved the fit of the relationship between ACE and milk citrate concentration. Adjustment was justified because these cows had higher concentrations of milk fat (53 ± 5.4 g/L) than the other cows in early and midlactation (40 ± 4.5 g/L), even though all cows were fed on the same diet and milk yield was similar for adjusted (34.7 ± 3.0 L/d) and unadjusted (34.2 ± 2.3 L/d) cows. As discussed earlier, group means show that 4:0 yield increased by 50% and suggest that de novo synthesis of 16:0 was reduced to an average of 30% in early lactation. To achieve these group means, a 50% increase in BHBA use and an 83% reduction in 16:0 synthesis were assumed for cows in which ACE was adjusted. These assumptions require testing in further experiments. In practice, the parameters would vary between individual cows, but optimization was not considered appropriate with current data. Although these assumptions are speculative, they are based on observed differences in milk fatty acids and established metabolic responses by dairy cows in early lactation. The effect of adjustment appears to contradict the suggestion that ACE provides a better indication of synthase activity than fatty acid ratios because there is uncertainty over the adjustment factors and when they should be applied. However, this uncertainty applies equally when considering fatty acid ratios or yields; ignoring 16:0 and 4:0, which is the usual practice, introduces greater errors than making assumptions about adjustment factors.

The relationship between milk citrate and ACE provides a unified explanation for variation in milk citrate concentration throughout lactation. Without adjustment, the stoichiometry breaks down in early lactation and previous researchers have speculated that high citrate concentrations immediately after calving might be related to increased permeability of epithelial membranes (Linzell et al. 1976), for which there is no evidence in healthy cows. The adjustment, therefore, allows a more robust explanation for variation in milk citrate with stage of lactation and de novo fatty acid synthesis.

CONCLUSIONS

This study confirms the hypothesis that variation in milk citrate with stage of lactation is related to de novo

synthesis of fatty acids. The relationship is independent of diet and milk yield. For considering the extent of de novo fatty acid synthesis from milk fatty acid profiles, we propose that stoichiometric calculation of acetate used for chain elongation (ACE) provides a better indication of synthase activity than fatty acid ratios.

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