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Effect of Linoleic Acid Concentration on Conjugated Linoleic Acid Production by *Butyrivibrio fibrisolvens* A38

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Butyrivibrio fibrisolvens **A38 inocula were inhibited by as little as 15 μM linoleic acid (LA), but growing cultures tolerated 10-fold more LA before growth was inhibited. Growing cultures did not produce significant amounts of *cis*-9, *trans*-11 conjugated linoleic acid (CLA) until the LA concentration was high enough to inhibit biohydrogenation, growth was inhibited, and lysis was enhanced. Washed-cell suspensions that were incubated anaerobically with 350 μM LA converted most of the LA to hydrogenated products, and little CLA was detected. When the washed-cell suspensions were incubated aerobically, biohydrogenation was inhibited, CLA production was at least twofold greater, and CLA persisted. The LA isomerase reaction was very rapid, but the LA isomerase did not recycle like a normal enzyme to catalyze more substrate. Cells that were preincubated with CLA lost their ability to produce more CLA from LA, and the CLA accumulation was directly proportional ($r^2 = 0.98$) to the initial cell density. Growing cells were as sensitive to CLA as LA, the LA isomerase and reductases of biohydrogenation were linked, and free CLA was not released. Because growing cultures of *B. fibrisolvens* A38 did not produce significant amounts of CLA until the LA concentration was high, biohydrogenation was arrested, and the cell density had declined, the flow of CLA from the rumen may be due to LA-dependent bacterial inactivation, death, or lysis.**

In the 1930s, Booth et al. (3) noted that summer milk had a greater absorbance at 233 nm than milk produced in the winter, and later work indicated that rats fed summer milk grew better than those fed winter milk even if the fat content was similar (2). In 1963, Riel (28) noted that summer milk fat had more conjugated dienoic acid than winter milk fat. More recently, conjugated linoleic acid (CLA) has been shown to inhibit chemically induced tumors (1, 10, 17), prevent atherosclerosis (24), and improve the protein-to-fat ratio in experimental animals (8).

CLAs can be produced by alkaline isomerization, but there are as many as 16 isomers which are not fully characterized (26, 29). Ruminant nutritionists have attempted to increase the naturally occurring CLA content of cow's milk via diet changes and alterations of ruminal fermentation (9). Recent work indicated that polyunsaturated oil supplements could increase the CLA content of milk, but these diet-dependent increases were often small or transitory (9, 19).

Many ruminal bacteria are inhibited by long-chain fatty acids (25), and gram-positive bacteria are more sensitive than gram-negative species (12). Polyunsaturated fatty acids are particularly toxic (21), but some ruminal bacteria are able to saturate the double bonds via a process known as biohydrogenation (27). In the 1960s, Kepler et al. (22) studied the biohydrogenation of *Butyrivibrio fibrisolvens* and demonstrated that linoleic acid (LA) was first converted to *cis*-9, *trans*-11 CLA. The reductase steps were inhibited by oxygen, but the LA isomerase could continue to produce *cis*-9, *trans*-11 CLA even if oxygen was present (15).

Since *B. fibrisolvens* A38 has a greater CLA-producing capacity than other ruminal bacteria, it has often been used as a model of CLA production (16, 20, 27). Washed-cell suspensions of *B. fibrisolvens* produced CLA, but the CLA production of growing cultures was not examined (15, 20). The following question then arose: is CLA a normal end product or is it simply an artifact of cells that could not biohydrogenate? Recent work indicated that mammalian tissues could also produce *cis*-9, *trans*-11 CLA from *trans*-octadecenoic acid (*trans*-$C_{18:1}$), and the significance of ruminal CLA production has been questioned (9).

The experiments described here sought to define more precisely the effect of LA on the biohydrogenation and CLA production of *B. fibrisolvens*.

MATERIALS AND METHODS

Bacterial growth. *B. fibrisolvens* A38 was grown anaerobically at 39°C in basal medium containing (per liter) 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4,000 mg of Na_2CO_3, 600 mg of cysteine hydrochloride, 10 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 2.5 g of yeast extract, and branched-chain volatile fatty acids (1 mmol each of isobutyrate, isovalerate, and 2-methylbutyrate), plus hemin, vitamins, and trace minerals (6). Glucose (2 mg/ml, final concentration) was prepared as a separate solution and was added after autoclaving. Cultures were grown anaerobically under O_2-free CO_2 in 150- by 18-mm tubes that were capped with butyl rubber stoppers and aluminum seals. Growth rate was estimated from the increase in optical density (OD) at 600 nm (1-cm cuvettes). Cultures were sometimes grown in serum bottles (160 ml) that were prepared in a similar fashion. The relationship of OD and bacterial protein was typically 220 mg of protein/liter/OD unit.

Fatty acid preparation. Concentrated LA (Sigma Chemical Co., St. Louis, Mo.) and CLA (75% *cis*-9, *trans*-11 isomer; Matreya, Inc., Pleasant Gap, Pa.) solutions (0.1 g/ml of water with 20% bovine serum albumin) were sterilely filtered (pore size, 0.2 μm). The stability of the emulsion could successfully be maintained with 20% bovine serum albumin throughout the experimental periods. LA and CLA stock solutions were then serially diluted in sterile anaerobic water to decrease the concentration. The LA and CLA solutions were then added to cultures or washed-cell suspensions (10 μl/ml).

Washed-cell suspensions. Cultures (typically 10 ml) were harvested by centrifugation (3,500 × *g*, 5°C, 10 min), and cell pellets were washed twice with anaerobic medium lacking Trypticase, yeast extract, ammonia, and glucose and resuspended in K_2HPO_4 (50 mM, pH 7.5) that was prepared anaerobically or aerobically. Cell ODs were typically 1. All incubations were performed at 39°C. The pH of the potassium phosphate buffer was decreased by adding HCl. When the pH was higher than 7.5, Tris buffer (50 mM) was used, and pH was adjusted with NaOH.

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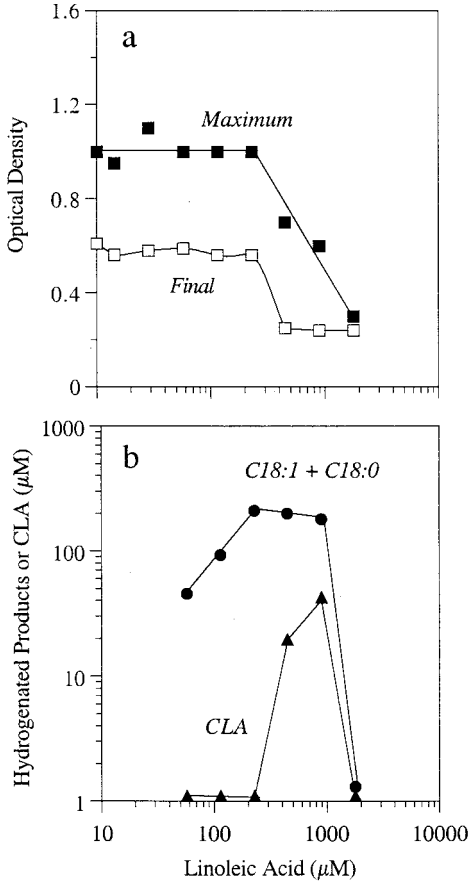

FIG. 1. The effect of LA concentration on the maximum and final ODs of *B. fibrisolvens* cultures (a). LA was added to actively growing cultures at an OD of 0.3, and the final OD was measured at 24 h. LA additions caused an increase in OD, but the OD of inoculated controls could be subtracted to determine the culture OD. (b) Effects of LA hydrogenated end products and CLA.

Fatty acid analyses. Cultures or washed cell suspensions (typically 10 ml) were extracted with a mixture of organic solvents (2 ml; 1 part hexane to 3 parts isopropanol to 1 part acetone; 1 min using a Vortex mixer). The suspensions were then centrifuged (1,000 × *g*, 3 min, 20°C). The solvent layer (top) was removed and flushed with nitrogen until dry. The fatty acids were then dissolved in toluene (1 ml). The fatty acids were methylated as previously described by Kim and Liu (23). Fatty acid methyl esters were separated by a Supelcowax-10 fused silica capillary column (60 m by 0.53 mm, 0.5-μm film thickness; Supelco., Inc, Bellefonte, Pa.) using a Hewlett Packard model HP5890 gas chromatograph equipped with a flame ionization detector and model HP3392 integrator. The conditions were as follows: helium flow, 2.4 ml/min; injector, 200°C; detector, 250°C; column chamber temperature, initially 40°C (5 min) and then increased to 220°C at 20°C/min and held for 30 min. A sample (1 μl) containing 0.5 to 5 μg of LA or CLA was injected into the column in a splitless mode. Heptadecanoic acid ($C_{17:0}$) was used as an internal standard. *cis*-9, *trans*-11 octadecadienoic acid (>98% purity) was used as a CLA standard. The recovery of CLA was 83%, and that of $C_{17:0}$ was 80%. A known standard mixture of fatty acids was used to identify other fatty acids. This protocol was able to separate eight isomers of LA, but it could not differentiate *cis*, *trans* versus *trans*, *cis* configurations in the same position. *B. fibrisolvens* A38 produced only the *cis*-9, *trans*-11 isomer.

Statistical analyses and design. All incubations were performed three times. Mean values and standard deviations of the mean are shown.

RESULTS

When *B. fibrisolvens* A38 was incubated in basal medium lacking fatty acids, the culture grew rapidly (0.46 h^{-1}) and the maximal cell density was approximately 1.0 (Fig. 1a). Stationary-phase cells lysed, and the OD at 24 h was only 0.6. When low concentrations of LA (as little as 35 μM) were added to the growth medium at inoculation, growth was not observed. Similar concentrations of a CLA mixture (75% *cis*-9, *trans*-11 CLA) also inhibited growth.

Actively growing cultures tolerated higher concentrations of LA (Fig. 1a), and virtually all of the LA was converted to hydrogenated products (primarily *trans*-$C_{18:1}$ and small amounts of stearic acid) (Fig. 1b). If the LA concentration was 350 μM or greater, growth was inhibited, hydrogenated products declined, CLA accumulated, and more of the cells lysed. When the LA concentration was 1,800 μM, growth was completely inhibited and LA was not converted to either hydrogenated products or CLA.

When washed stationary-phase cells were incubated anaerobically with 350 μM LA, most of the LA was converted to hydrogenated products (Fig. 2a). The CLA concentration was as high as 13 μM (Fig. 2b). However, if the cells were incubated for more than 2 min, the CLA concentration declined, and after only 30 min CLA could no longer be detected. Washed cells that were incubated aerobically produced little hydrogenated product (Fig. 2a), and the CLA accumulation was at least twofold greater (Fig. 2b). The CLA concentration eventually declined, but the concentration at 30 min was greater than 15 μM. Aerobic-cell suspensions had a pH optimum for CLA production of 7.5, but pH values from 5.5 to 8.5 did not have a marked impact on the CLA production (Fig. 3).

B. fibrisolvens A38 cultures that had been treated with more than 50 μM LA could not be transferred successively, but cultures that were gradually adapted to increasing amounts of

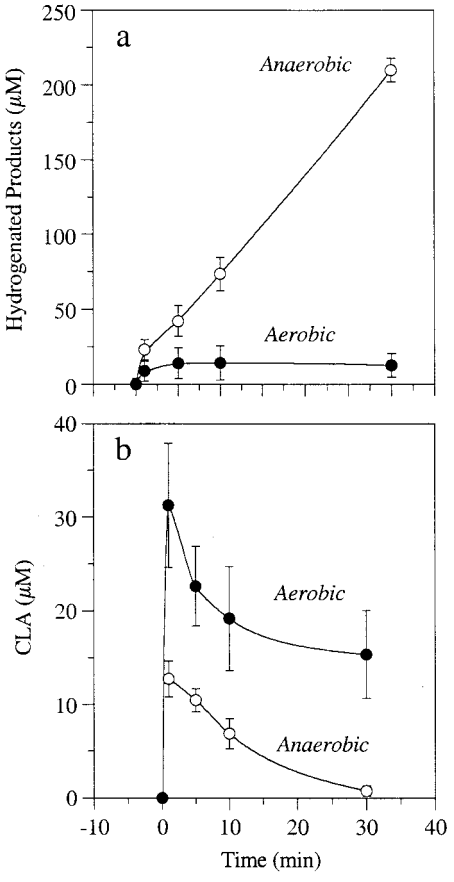

FIG. 2. Conversion of LA to hydrogenated products (*trans*-$C_{18:1}$ and $C_{18:0}$) (a) or CLA (b) by washed *B. fibrisolvens* cells. The initial LA concentration was 350 μM, and the cell OD was 1. The incubations were performed in triplicate, and the values are the means ± the standard deviations.

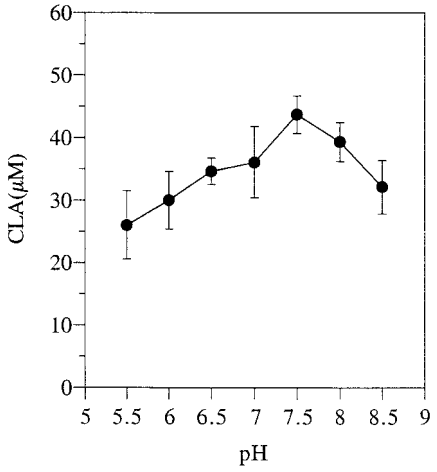

FIG. 3. Effect of pH on CLA production of washed *B. fibrisolvens* cells that were incubated aerobically. The initial LA concentration was 350 μM, and the cell OD was 1. The incubations were performed in triplicate, and the values are the means ± the standard deviations.

LA (0 to 50 μM) grew repeatedly with 35 μM LA. LA-adapted cells that were washed and incubated aerobically produced less CLA and more hydrogenated products than unadapted cells (Fig. 4). When unadapted cells were given a 350 μM dose of LA, CLA increased rapidly, but a second dose of LA did not cause a further increase in CLA (Fig. 5a). Washed-cell suspensions that were given a larger dose of LA (700 μM) produced approximately the same amount of CLA as those given a single dose of 350 μM or two doses of 350 μM. When washed-cell suspensions were provided with LA at concentrations ranging from 0 to 350 μM, CLA concentrations increased but only at LA concentrations lower than 350 μM. Cells that were preincubated with 35 μM CLA produced less additional CLA than those that were not preincubated with CLA (Fig. 6). The CLA production was greater if more cells were added, and the relationship between CLA and cell density was linear (Fig. 7).

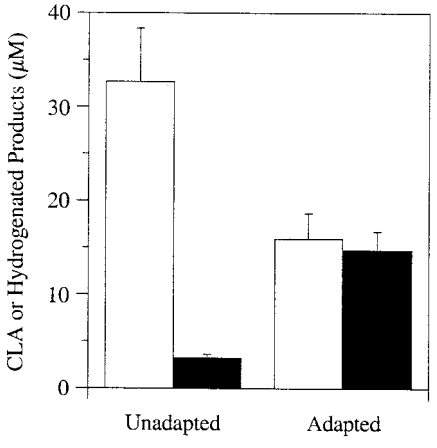

FIG. 4. Conversion of LA to CLA (open bars) or hydrogenated products (dark bars) by washed *B. fibrisolvens* cells that were incubated aerobically. The initial LA concentration was 350 μM, and the cell OD was 1. The unadapted cells had not been grown with LA, but the adapted cells had been repeatedly transferred with 35 μM LA. The incubations were performed in triplicate, and the values are the means ± the standard deviations.

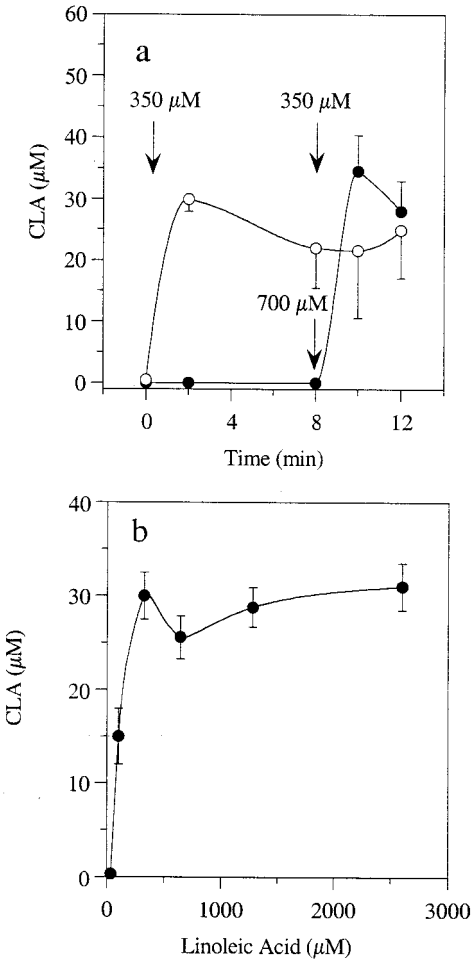

FIG. 5. CLA production by washed *B. fibrisolvens* cells that were incubated aerobically. (a) Cells received two doses of LA (350 μM) or a single dose of LA (700 μM) as indicated by the arrows. (b) The LA concentration was increased from 0 to 2,800 μM. The cell OD was 1. The incubations were performed in triplicate, and the values are the means ± the standard deviations.

DISCUSSION

Henderson (12) noted that *B. fibrisolvens* was very sensitive to long-chain fatty acids, but he did not examine the effect of LA or CLA. Our results indicated that unadapted *B. fibrisolvens* A38 inocula could not initiate growth if the LA or CLA was included at a concentration of 15 μM. Other strains of *B. fibrisolvens* did not produce as much CLA as A38 did. When the LA concentration was increased in a stepwise fashion, *B. fibrisolvens* A38 tolerated more LA, but the maximal LA concentration that allowed growth was only 35 μM. Growing cultures (OD, 0.3) were, however, 10-fold more LA resistant, but these cultures could not be transferred successively.

Dawson and Kemp (7) noted that saturated fatty acids were less toxic to ruminal bacteria than polyunsaturated fatty acids and suggested that biohydrogenation was a detoxification mechanism. Growing *B. fibrisolvens* A38 cultures biohydrogenated LA, but this capacity could be overcome by a high LA concentration. If the LA concentration was greater than 350 μM, biohydrogenation was incomplete, CLA could be detected, and the cultures were no longer viable. These results indicated that CLA was not a normal end product of growing cultures, and CLA accumulated only if biohydrogenation was inhibited.

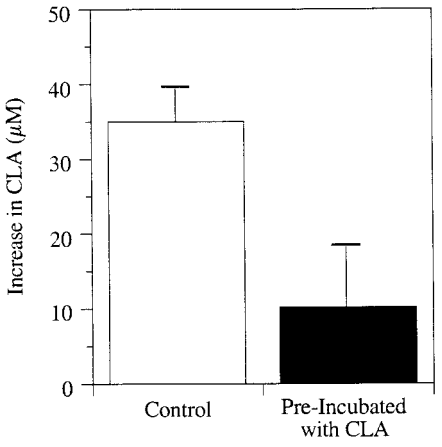

FIG. 6. CLA production by washed *B. fibrisolvens* cells that were incubated aerobically with 350 μM LA. Control cells were not preincubated with CLA; preincubation was done with 35 μM CLA. The incubations were performed in triplicate, and the values are the means ± the standard deviations.

Washed-cell suspensions that were incubated anaerobically biohydrogenated LA, with little CLA accumulation, and CLA eventually declined to undetectable levels. When the washed cells were incubated aerobically, biohydrogenation was strongly inhibited, the CLA production was at least twofold greater, and CLA persisted. These results supported the idea that CLA accumulation was due to an inhibition of biohydrogenation.

Our washed-cell suspensions were typically prepared from cultures that had not been exposed to LA. However, experiments with adapted cultures indicated that the CLA production was even lower and even more of the LA was converted to hydrogenated products. These results indicated that CLA production was not being constrained by the absence of an "inducer." Kepler and Tove (20) noted that the LA isomerase had a broad pH range, and we also observed CLA production at pH values ranging 5.5 to 8.5.

Kepler and Tove (20) incubated their *B. fibrisolvens* extracts for less than 2 min, and we also noted a very rapid increase in CLA production. However, the prolonged incubation did not cause a further increase in CLA. The LA isomerase did not recycle like a normal enzyme to catalyze more substrate, and the CLA production was highly cell density dependent. The CLA production increased if more LA was added, but only if the LA concentration was low. Because CLA was as toxic as LA, there was no advantage in releasing large amounts of free CLA.

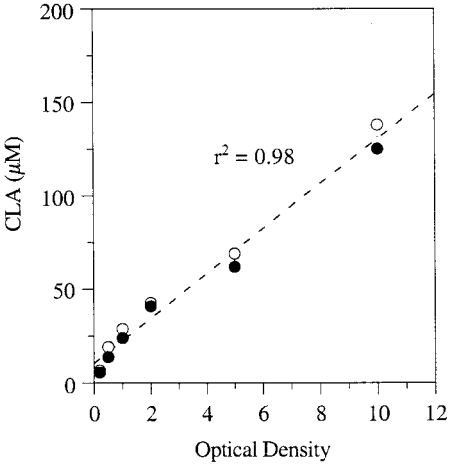

FIG. 7. Effect of cell OD on the conversion of LA to CLA by washed cells of *B. fibrisolvens* that were incubated aerobically with 350 μM LA. Open symbols show a 2-min incubation, and closed symbols show a 5-min incubation. Dotted line represents the regression line of 2-min and 5-min incubations.

When *B. fibrisolvens* cells were sonicated, the membrane fraction (pelleted by centrifugation at 150,000 × *g*) produced at least 10-fold more CLA than the cytoplasmic extract, and the result indicated that the LA isomerase was a membrane-bound enzyme (data not shown). Hughes and Tove (13, 14) extracted *B. fibrisolvens* cells with solvent, and they were able to purify the reductase of biohydrogenation. The LA isomerase activity was also found in the membrane fraction, but the preparations always had large amounts of contaminating carbohydrate (20).

Metabolically active *B. fibrisolvens* A38 cells produced hydrogenated end products rather than CLA, but *B. fibrisolvens* is a bacterium that lyses once it reaches stationary phase. Because membrane fractions retained their ability to convert LA to CLA, it is conceivable that dead or lysed cells could produce CLA in the rumen. When Harfoot et al. (11) incubated particle-associated ruminal bacteria with large amounts of sucrose and LA, biohydrogenation was favored and CLA was not detected. However, if the sucrose was omitted, some of the LA was converted to CLA.

When cattle are fed diets rich in fiber, the CLA content of milk often increases, but the ratio of CLA to total milk fat is usually less than 20 mg/g (28). CLA production in cattle fed hay and grain can be enhanced by LA supplements (oils), and in these cases the ratio of CLA to total milk fat can be greater than 20 mg/g (19). Both of these effects are consistent with the properties of *B. fibrisolvens. B. fibrisolvens* is a hemicellulose-digesting bacterium that is found in high numbers when cattle are fed hay or grass (4), and high concentrations of LA stimulated CLA production by *B. fibrisolvens* A38.

The study of CLA production in ruminants is complicated by the fact that ruminal fermentation is not the only source of CLA. If biohydrogenation is incomplete, some *trans*-$C_{18:1}$ can pass from the rumen (18), and *trans*-$C_{18:1}$ can be converted to CLA by the Δ-9 desaturase of the mammary and adipose tissues (30). When the mammalian Δ-9 desaturase was inhibited by a postruminal infusion of sterculate, endogenous CLA synthesis decreased 40%, but postruminal *trans*-$C_{18:1}$ addition caused only a small increase in the level of CLA in milk (5 to 7 mg/g) (9).

Our results indicate that *B. fibrisolvens* A38 can produce significant amounts of CLA if the LA concentration is high enough to inhibit biohydrogenation, but further work will be needed to define the role of *B. fibrisolvens* and other ruminal bacteria in ruminant CLA production. Because the LA isomerase does not seem to release free CLA and appears to be feedback inhibited, traditional schemes of cloning and overexpression to increase CLA production could be ineffective.

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REFERENCES

1. **Belury, M. A., K. P. Nickel, C. E. Bird, and Y. M. Wu.** 1996. Dietary conjugated linoleic acid modulation of phorbol ester skin tumor promotion. Nutr. Cancer **26:**149–157.
2. **Boer, J., B. C. P. Jansen, A. Kentie, and H. K. Oosterhuis.** 1944. Proeven over de mogelijkheld van adsorptie wan den groeifactor aanwezig in de vetzuren van Zomerboter. Arch. Neerl. Physiol. Homme Anim. **28:**57–72.

3. **Booth, R. G., S. K. Kon, W. J. Dann, and T. Moor.** 1935. A study of seasonal variation in butter fat. II. A seasonal spectroscopic variation in the fatty acid fraction. Biochem. J. **29:**133–137.
4. **Bryant, M. P., and N. Small.** 1956. The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen. J. Bacteriol. **72:** 16–21.
5. **Corl, B. A., S. H. Lacy, L. H. Baumgard, D. A. Dwyer, J. M. Griinari, B. S. Phillips, and D. E. Bauman.** 1999. Examination of the importance of Δ-9 desaturase and endogenous synthesis of CLA in lactating cows. J. Anim. Sci. **77**(Suppl. 1)**:**118. (Abstract.)
6. **Cotta, M. A., and J. B. Russell.** 1982. Effect of peptides and amino acids on efficiency of rumen bacterial protein synthesis in continuous culture. J. Dairy Sci. **65:**226–234.
7. **Dawson, R. M. C., and P. Kemp.** 1970. Biohydrogenation of dietary fats in ruminants, p. 504–518. *In* A. T. Phillipson (ed.), Physiology of digestion and metabolism in the ruminant. Oriel Press, Newcastle-upon-Tyne, United Kingdom.
8. **Dougan, M. E. R., J. L. Aalhus, A. L. Schaefer, and J. K. G. Kramer.** 1997. The effect of conjugated linoleic acid on fat to lean repartitioning and feed conversion in pigs. Can. J. Anim. Sci. **77:**723–725.
9. **Griinari, J. M., and D. E. Bauman.** 1999. Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants, p. 180–200. *In* P. Yurawez, M. M. Mossoba, J. K. G. Kramer, G. Nelson, and M. W. Pariza (ed.), Advances in conjugated linoleic acid research, vol 1. American Oil Chemists Society, AOCS Press, Champaign, Ill.
10. **Ha, Y. L., N. K. Grimm, and M. W. Pariza.** 1987. Anticarcinogens from fried ground beef: heat-altered derivatives of linoleic acid. Carcinogenesis **8:**1881–1888.
11. **Harfoot, C. G., R. C. Noble, and J. H. Moore.** 1973. Factors influencing the extent of biohydrogenation of linoleic acid by rumen micro-organisms in vitro. J. Sci. Food Agric. **24:**961–970.
12. **Henderson, C.** 1973. The effects of fatty acids on pure cultures of rumen bacteria. J. Agric. Sci. **81:**107–112.
13. **Hughes, P. E., and S. B. Tove.** 1980. Identification of an endogenous electron donor for biohydrogenation. J. Biol. Chem. **255:**4447–4452.
14. **Hughes, P. E., and S. B. Tove.** 1980. Identification of deoxy-α-tocopherol as another endogenous electron donor for biohydrogenation. J. Biol. Chem. **255:**11802–11806.
15. **Hughes, P. E., W. J. Hunter, and S. B. Tove.** 1982. Biohydrogenation of unsaturated fatty acids. J. Biol. Chem. **257:**3643–3649.
16. **Hunter, W. J., F. C. Baker, S. Rosenfeld, J. B. Keyser, and S. B. Tove.** 1976. Biohydrogenation of unsaturated fatty acids. J. Biol. Chem. **251:**2241–2247.
17. **Ip, C., M. Singh, H. J. Thompson, and J. A. Scimeca.** 1994. Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. Cancer Res. **54:**1212–1215.
18. **Keeney, M.** 1970. Lipid metabolism in the rumen, p. 489–503. *In* A. T. Phillipson, (ed.), Physiology of digestion and metabolism in the ruminant. Oriel Press, Newcastle-upon-Tyne, United Kingdom.
19. **Kelly, M. L., J. R. Berry, D. A. Dwyer, J. M. Griinari, P. Y. Chouinard, M. E. Van Amburgh, and D. E. Bauman.** 1998. Dietary fatty acid sources affect conjugated linoleic acid concentrations in milk from lactating dairy cows. J. Nutr. **128:**881–885.
20. **Kepler, C. R., and S. B. Tove.** 1967. Biohydrogenation of unsaturated fatty acids. J. Biol. Chem. **242:**5686–5692.
21. **Kepler, C. R., and S. B. Tove.** 1970. Biohydrogenation of unsaturated fatty acids. J. Biol. Chem. **245:**3612–3620.
22. **Kepler, C. R., K. P. Hirons, J. J. McNeill, and S. B. Tove.** 1966. Intermediates and products of the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*. J. Biol. Chem. **241:**1350–1354.
23. **Kim, Y.-J., and R. H. Liu.** 1999. Selective increase in conjugated linoleic acid in milk fat by crystallization. J. Food Sci. **64:**792–795.
24. **Lee, K. N., D. Kritchevsky, and M. W. Pariza.** 1994. Conjugated linoleic acid and atherosclerosis in rabbits. Atherosclerosis **108:**19–25.
25. **Maczulak, A. E., B. A. Dehority, and D. L. Palmquist.** 1981. Effects of long-chain fatty acids on growth of rumen bacteria. Appl. Environ. Microbiol. **42:**856–862.
26. **Nichols, P. L., Jr., S. F. Herb, and R. W. Riemenschneider.** 1951. Isomers of conjugated linoleic acid. I. Alkali isomerized linoleic acid. J. Am. Chem. Soc. **73:**247–252.
27. **Polan, C. E., J. J. McNeill, and S. B. Tove.** 1964. Biohydrogenation of unsaturated fatty acids by rumen bacteria. J. Bacteriol. **88:**1056–1064.
28. **Riel, R. R.** 1963. Physico-chemical characteristics of Canadian milk fat. Unsaturated fatty acids. J. Dairy Sci. **46:**102–106.
29. **Sehat, N., M. P. Yurawecz, J. A. G. Roach, M. M. Mossoba, J. K. G. Kramer, and Y. Ku.** 1998. Silver-ion high-performance liquid chromatographic separation and identification of conjugated linoleic acid isomers. Lipids **33:**217–222.
30. **Tocher, D. R., M. J. Leaver, and P. A. Hodgson.** 1998. Recent advances in the biochemistry and molecular biology of fatty acyl desaturases. Prog. Lipid Res. **37:**73–117.