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Lipoprotein Patterns in Normal Lactating Holstein Cows Bled at Various Times: Effects of Milking (40944)

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Abstract. Six lactating Holstein cows (60–90 days) fed normal rations were bled via jugular catheters at 1130 (pre-milking), 1300 (post-milking), 1900, 2330 (pre-milking), 0100 (post-milking), and 0700 hr. Serum lipoproteins were isolated and fractionated by gel chromatography into three major fractions: very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). VLDL protein content was high at both pre-milking times but decreased following milking. HDL protein showed an inverse relationship to that of VLDL, with peak HDL protein concentrations occurring after both milking times. Although no other dependent variables exhibited statistically significant time effects, mean VLDL triglyceride concentrations did decline after each milking time. The results suggested an increased rate of VLDL catabolism following milking.

Studies have previously characterized bovine serum lipoproteins with respect to chemical and physical properties (1, 2). However, little attention has been focused on factors which induce variations in serum lipoprotein concentration, distribution, or composition in the dairy cow. One exception has been reports of alterations in bovine lipoprotein concentrations with stage of lactation (3). Numerous sources of variation have been reported in other species, e.g., diet, age, sex, and disease (4, 5). Recently, Puppione (2) suggested the possibility of a diurnal variation in bovine lipoprotein concentrations, citing an early paper by Gage and Fish (6) which demonstrated a rise in chylomicrons during the night in dairy cows fed butter fat. In a preliminary study we tested for diurnal variations in lipoprotein concentrations in normal lactating dairy cows fed standard rations (7) and found that one animal did exhibit a significant alteration in the protein content of very low density lipoprotein. The present investigation employed a larger number of animals than our pilot study and tested for the effect of milking as well as for possible diurnal variation in serum lipoproteins.

Methods. Six Holstein cows, lactating 60 to 90 days, were fitted with jugular catheters and bled (200 ml) at: 1130 (pre-milking), 1300 (post-milking), 1900, 2330 (pre-milking), 0100 (post-milking), and 0700 hr. These were normal production dairy cows and milking was performed at the routine times for these animals. Milking at different times, in order to separate the milking effect from any other possible time-dependent effects, might have induced further variables, e.g., stress. The animals were kept in stanchions except when moved for milking. Feed was provided twice daily. The ration consisted of 75% corn silage (30–33% dry matter) and 25% a concentrate mixture principally of corn and soybean meal fortified with adequate minerals and fat-soluble vitamins to meet or exceed NRC recommendations for lactating cows (8). To minimize stress, all cows were placed in the stanchions 3 days prior to bleeding, and catheters were inserted 1 day before bleeding. Serum was isolated by centrifugation (4000*g*), and EDTA (0.02%) was added to prevent lipid oxidation and microbial contamination. Because of large sample numbers it was necessary to freeze serum (−20°) to prevent degradation. Foreman *et al.* (9) have observed that a

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single freezing at −20° does not significantly affect the subsequent fractionation of serum lipoproteins. Samples were thawed at room temperature, total lipoproteins were isolated by ultracentrifugation (100,000*g*, 40 hr), and lipoproteins were subsequently fractionated by agarose gel column chromatography (Biorad A5M, 2.5 × 100-cm column). All procedures were performed using the techniques of Rudel *et al.* (10) which we have previously used in fractionating and characterizing bovine serum lipoproteins (11). The lipoprotein fractions eluted from the agarose columns were: I, very low density lipoprotein (VLDL); II, low density lipoprotein (LDL); and III, high density lipoprotein (HDL) (11). Protein was determined by the method of Lowry (12) and total cholesterol by the BMC manual enzymatic assay (Biodynamics, Indianapolis, Ind.) (13). Peak I triglycerides were also measured using a BMC manual enzymatic kit (14). Peak II and III triglycerides were estimated using Phillip's manual fluorometric procedure (15).

Analyses of variance (ANOV) (16) were used to determine the effects of time and animal on the dependent variables: protein, cholesterol, and triglyceride concentrations in VLDL, LDL, and HDL. Differences between individual levels of significant ($P < 0.05$) factors were analyzed using Duncan's multiple-range test (16). The specific effect of milking was also tested by comparing the mean of all pre-milking values with the mean of all post-milking values using a single degree of freedom contrast (16).

Results. Lipoprotein cholesterol, triglyceride, and protein concentrations are presented in Table I. ANOV indicated a significant effect of bleeding time on VLDL and HDL protein concentrations ($P < 0.05$). Subsequent testing of individual times indicated a significant decrease in VLDL protein at 1300 hr, approximately 1 hr after milking ($P < 0.05$), whereas a significant increase in HDL protein occurred. Following the night milking, a decrease in VLDL protein concurrent with a rise in HDL protein was again observed. However, statistical significance for the midnight milking effect could not be demonstrated using Duncan's procedure. A single degree of freedom contrast was employed to compare specifically the mean of all pre-milking with the mean of all post-milking values (Table II). The mean post-milking VLDL protein value was significantly lower than the mean pre-milking value ($P < 0.003$). Conversely, post-milking HDL protein was higher than the pre-

TABLE I. MEAN TRIGLYCERIDE, CHOLESTEROL, AND PROTEIN CONCENTRATIONS (mg/dl SERUM) OF THE LIPOPROTEIN CLASSES ISOLATED FROM SERUM OBTAINED AT DIFFERENT TIMES

	VLDL			LDL			HDL		
Bleeding times	TG[a]	CHOL[b]	PROT[c]	TG	CHOL	PROT	TG	CHOL	PROT
1130 hr	1.9	1.9	2.7	0.2	6.5	3.8	0.7	56.0	94.9
1200 hr (milk)									
1300 hr	1.3	1.1	0.5[d,e]	0.3	9.2	5.3	0.6	70.3	141.4[f,g]
1900 hr	1.9	2.6	1.9	0.5	8.7	5.7	0.9	70.4	124.4
2330 hr	2.0	2.3	2.6	0.3	9.0	4.5	0.9	57.4	99.0
2400 hr (milk)									
0100 hr	1.7	2.4	1.3[e]	0.3	6.5	3.2	0.4	82.9	131.8[g]
0700 hr	2.1	2.3	1.4	0.3	6.9	3.7	0.6	82.5	138.1
± S $\bar{x}$	0.1	0.2	0.2	0.03	0.5	0.3	0.1	4.7	5.8

[a] Triglyceride.
[b] Cholesterol.
[c] Protein.
[d] Significantly lower than the pre-milking value (Duncan's test, $P < 0.05$).
[e] Mean of both post-milking values is significantly lower than the mean of both pre-milking values ($P < 0.003$).
[f] Significantly higher than the pre-milking value (Duncan's test, $P < 0.05$).
[g] Mean for both post-milking values is significantly lower than the mean of both pre-milking values ($P < 0.007$).

TABLE II. COMPARISON OF PRE-MILKING VERSUS POST-MILKING EFFECTS[a]

Dependent variable	Contrast, pre-milking versus post-milking	Level of significance
VLDL protein	+ 3.4 mg/dl	$P < 0.0031$
HDL protein	− 79.2 mg/dl	$P < 0.0070$

[a] Statistical analysis by single degree of freedom contrast.

milking mean ($P < 0.007$). It was also noted that the decreases in mean VLDL protein following milking were mirrored by similar declines in VLDL triglyceride concentrations (Fig. 1), although ANOV did not indicate these triglyceride changes to be statistically significant.

Discussion. Figure 1 shows that VLDL protein reached a maximum at both pre-milking times, but at both post-milking times, VLDL protein fell to a minimum. HDL protein exhibited an inverse relationship to that of VLDL protein. A possible explanation for these regular lipoprotein fluctuations in the lactating cow may be provided by recent studies on the catabolism of triglyceride-rich lipoproteins in animals and man (5, 17, 18). Briefly, VLDL catabolism is initiated in the blood via the transfer of C apoproteins from circulating HDL to the VLDL molecule. Acquisition of the apo C makes VLDL molecules more susceptible to enzymatic degradation. Lipoprotein lipase on the capillary endothelium then acts on VLDL to liberate fatty acids for uptake by the surrounding tissue. The final lipoprotein product of VLDL catabolism is thought to be intermediate and/or low density lipoprotein. Each time VLDL is acted upon by lipoprotein lipase, not only are triglycerides hydrolyzed, but protein (mainly apo C), phospholipids, and cholesterol also are released. These catabolic products are then transferred either to mature HDL or nascent particles (immature HDL (5, 17, 18).

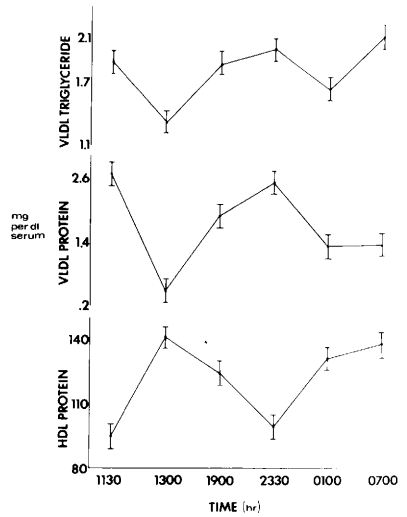

FIG. 1. Comparison of VLDL and HDL protein concentrations with VLDL triglyceride concentrations. Note the simultaneous changes occurring following the 1200 hr milking and again after the 2400 hr milking.

Our data suggest that upon removal of milk from the mammary gland, new milk synthesis proceeds immediately. The demand on serum VLDL for its fatty acid contribution to milk fat increases. As VLDL is catabolized by mammary gland lipoprotein lipase, apoproteins are released and transferred to mature and/or immature HDL particles. The result would then be an increase in the serum HDL protein gained from the loss of VLDL protein.

Although the reciprocal relationship between VLDL and HDL protein is evident qualitatively, Table II indicates a 23-fold greater absolute change in HDL protein versus VLDL protein following milking. This most likely reflects differences in the turnover rates of the two lipoprotein fractions. In humans and rats the turnover of VLDL is significantly more rapid than that of HDL (17–19). Assuming the same exists in the cow, a rapid synthesis of new VLDL followed by rapid transfer of apoproteins to HDL should result in a marked increase in HDL protein. The actual VLDL protein concentration in the blood at the time of sampling merely reflects this complex metabolic interaction, but without accurate

turnover data cannot provide a true quantitative profile.

Although these events can only be hypothesized at this time, they receive further support from investigations of milk fat synthesis. Barry *et al.* (20) found significant arteriovenous differences in VLDL across the mammary glands of goats. Using radioactive tracers, Glascock and Welch (21) calculated 70.6% of milk mixed fatty acids were derived from serum VLDL in the bovine. Such findings support the concept of significant VLDL catabolism as blood circulates through the active mammary gland.

One aspect of our data needs further consideration. At night, mean values for VLDL protein and HDL protein were altered in the post-milking serum but the differences were not statistically significant ($P < 0.05$) using Duncan's procedure. Nevertheless, following the noon milking, a statistically significant change in VLDL and HDL protein was demonstrated. If the metabolic scheme we described is valid, protein changes at both milking times should have been statistically significant. Indeed, the statistical contrast between pre-milking and post-milking effects (VLDL and HDL proteins) yielded high levels of significance and thus provided some confidence that the milking effect was real at midday and at midnight. One possible explanation for this discrepancy may lie in the inherent conservativeness of the Duncan's procedure, compounded by the fact that for most parameters involved there was a high degree of within-animal variability. Other investigators reported similar high levels of variation among cows with respect to serum lipid concentrations (21). The decrease in VLDL triglyceride associated with both milking times also would be expected if significant catabolism of VLDL were occurring. Lack of statistical validation was likewise attributed to the large degree of within-animal variability.

Another possible reason for the lack of significant triglyceride differences may be due to instability of the triglyceride moiety prior to analysis. Rudel *et al.* (10) have demonstrated recoveries of whole lipoproteins from 80 to more than 90% using their method of centrifugation and gel filtration. We are confident that our recoveries of whole lipoproteins were achieved with a similar degree of success. However, based on the levels of cholesterol and protein present in the VLDL subfractions the triglyceride values are much lower than they should have been. Recent studies in this laboratory (unpublished) have shown bovine VLDL to contain approximately 75% of its total mass as triglycerides. We have also noticed that large triglyceride-rich lipoproteins (>7500 Å) are unstable in storage, exhibiting significant losses of triglyceride. The protein and cholesterol moities appeared stable.

Gage and Fish (6), using dark-field microscopy, observed that starved dairy cows fed butterfat showed a significant increase in chylomicron absorption after midnight. This absorption was intermittent, with three distinct peaks of chylomicrons occurring at 2400, 300, and 700 hr. They believed that they had observed a similar but less dramatic night-time rise in chylomicrons in dairy cows fed bran and hay, although the significance of this difference is somewhat questionable based on their published absorption curves. There are two possible reasons for our lack of data to support their findings. First, we did not measure just chylomicron particles but also VLDL. Second, they sampled every hour versus our less ambitious bleeding schedule.

Decreased mean VLDL protein, increased mean HDL protein, and decreased mean VLDL triglyceride concentrations all occurred after both milking times. We believe that the simultaneous occurrence of these events argues strongly that we have observed a significant biological event: The catabolism of VLDL by an active lactating mammary gland. Apart from the effects of milking there was little evidence for the existence of diurnal variations (i.e., a daytime versus nighttime difference) in lipoprotein patterns of normal dairy cows on standard rations.

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