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Title: The Effects of Alfalfa Hay Fiber and Vitamin B_{12} Levels in Body Fluids on Milk Fat Production in High-Producing Dairy Cows

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The research presented in this thesis was designed to evaluate the effects of feeding alfalfa hays from the Klamath Basin of Oregon in practical dairy rations on milk fat production in high-producing Holstein cows. Thirty-two second lactation or older animals estimated to be capable of producing at least 70 lbs of milk daily were utilized in a 4 X 4 latin square experiment with eight replications. Four rations based upon four different lots of hay, ranging from 15.2 to 22.0% crude protein (CP), from 29.9 to 35.1% acid detergent fiber (ADF) and from 109 to 132 in Relative Feed Value, were fed during four periods, each lasting about 28 days. Rations were formulated to be comparable in caloric and nitrogen content, and all consisted (as a percent of ration dry matter) of 50% concentrate, 30% chopped alfalfa and 20% corn silage, fed as a mixed, complete ration. A digestibility study was also carried out involving four steers fed the four alfalfa hays only.

The digestibility trial showed that there were no significant differences among the four alfalfa hays in feed intake, % digestible dry matter, % digestible ADF and % digestible CP.

In the production trial, no differences were seen in feed intake or persistency, and body weight changes were significantly different only for periods. Both daily 4% fat-corrected milk (FCM) and milk fat percentage were observed to have highly significant differences for all sources of variation except treatments. Treatment means for rations 1 through 4 for 4% FCM were 48.88, 48.02, 48.38 and 48.24 lbs, respectively, and those for percent milk fat were 3.13, 3.12, 3.05 and 3.01, respectively. Differences in other sources of variation for these two measurements were attributed to variation among cows and stage of lactation effects.

Important changes were noted in the relative proportions of volatile fatty acids in the rumen. While there were no significant changes in rumen acetate concentration, highly significant differences in propionate content were seen for all sources of variation. Treatment means for rations 1 to 4 were 18.14, 17.56, 19.44 and 23.03 μmoles /ml of rumen fluid. This response was reflected also in highly significant differences in ruminal acetate:propionate (A:P) ratios. Means here for rations 1 through 4 were 2.82, 3.00, 2.36 and 2.23, respectively. Correlation coefficients were determined for A:P ratios and several hay and ration characteristics, and only that with hay % NDF was significant. It was concluded that changes in rumen fermentation typical of milk fat depression occurred which were not severe enough to actually reduce butterfat levels.

The literature indicates that there may be a relationship between milk fat synthesis and tissue vitamin B_{12} status, i.e., that B_{12}-deficient animals will secrete milk with reduced fat percentage. In this experiment, serum vitamin B_{12} levels did not respond significantly

to treatments, nor did fat percentage, the correlation between them was very low, and there was no indication of vitamin B_{12} deficiency among experimental animals. Our results, therefore, are interpreted as not being in conflict with the hypothesized relationship.

The Effects of Alfalfa Hay Fiber and Vitamin B_{12} Levels in Body Fluids on Milk Fat Production in High-Producing Dairy Cows

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INTRODUCTION

The problem of milk fat depression has long been recognized as one of considerable importance throughout the dairy industry. Among dairymen, of course, the chief concern is an economic one, particularly given the current system of pricing based on milk fat production. Other things remaining equal, a dairyman milking 200 cows could easily lose over $100 daily as a result of a 1% drop in average herd butterfat content. Clearly, with today's trend toward larger, more intensive, operations, the potential impace of such losses cannot be ignored.

It is well known that many factors can lead to variation in the fat percentage of milk produced by dairy cows. Indeed, much of this variability is considered normal, such as that among breeds, or that occuring during the usual course of the lactation of a particular cow. However, circumstances can exist which result in the production of milk having a fat percentage (MF%) which is lower than normal. The most commonly cited causes relate to the fiber or roughage fraction of the diet.

The present study has been part of a larger investigation into the quality of alfalfa hay raised in the Klamath Basin region of southern Oregon. Our investigations have involved two feeding trials: first, a digestibility study in which four steers were used to determine percent digestibilities for crude protein (CP), acid detergent fiber (ADF) and dry matter (DM) of four lots of alfalfa hay; and second, a production study in which 32 Holstein cows from the Oregon State

University dairy herd were fed a practical diet based on the same four hay lots and tested for treatment effects on feed intake, body weight changes, milk yield and fat percentage, persistency, rumen fermentation patterns and serum vitamin B_{12} content. In the production trial, the four rations were formulated to have a DM content of 50% concentrates (90% DM), 30% chopped alfalfa hay (90% DM) and 20% field corn silage (27% DM). They were fed as a thoroughly mixed complete ration.

The objectives of this experiment were as follows:

1. To determine digestibility characteristics of four lots of alfalfa hay.
2. To relate these characteristics, as well as those of the complete ration, to body weight changes, milk protein, persistency, rumen fermentation patterns, serum vitamin B_{12} levels and DM intake.
3. To determine whether these hays, fed as indicated above, would cause MF% depression or differences in butterfat production, or lead to changes in rumen fermentation which are typical of fat depression.
4. To assess the possibility of a relationship between milk fat synthesis and vitamin B_{12} status.

LITERATURE REVIEW

Since the early work of Powell (1939), a large proportion of the nutrition research directed at the problem of MF depression has focused on some aspect of the fiber component of rations. In a 3 year study with the Ralston Purina Company, he found that butterfat levels could be varied "at will" by as much as 60% through manipulations of dietary roughage. While this conclusion may initially have seemed both apparent and direct, much information has since been developed pointing to a complexity of interactions among a variety of factors. For example, the numerous sources of dietary fiber available for use in dairy rations, methods of preparation and feeding, other components of feedstuffs (especially energy), intake and digestibility, and the possible inclusion of various kinds of dietary supplements all need to be considered. All of these factors may be approached from two perspectives: that of the practical dairyman concerned with how to feed his cows, and that of the digestive physiologist, whose interest is to determine why particular feeding programs produce certain effects. Both perspectives will be pursued here in the hope of drawing their respective insights together into an understanding of the milk fat depression problem.

Practical Considerations of Fiber Feeding for Milk Fat Production

Concentrate:Roughage Ratios

Roughages are usually considered to be those feedstuffs having in excess of 16-18% crude fiber (CF) on a DM basis. This classification would thus include forage grasses and legumes, silages, haylages,

straws and hays, as well as such materials as seed screenings, bagasse and nut hulls or shells. In the literature, roughages (high-fiber feeds) are contrasted with concentrates (high-energy feeds), although materials such as corn silage or beet pulp may well have the properties of both. Rations may thus be described as having specific concentrate:roughage ratios.

So far as milk fat production is concerned, concentrate:roughage (C:R) ratios can be of great importance. Bishop et al. (1963) observed significant MF depression when animals were fed diets with high C:R ratios, but not in the case of low ratios. Ingalls and McKirdy (1974) found that a 70:30 ratio depressed MF% while a 50:50 did not, and Bauman et al. (1971) reported MF% values of 1.9 and 4.0 in feeding ratios of 85:15 and 45:55, respectively. Ibrahim and Ingalls (1971) fed concentrates with a variety of roughages in a 60:40 ratio and found no differences in fat percentage. When Whiting et al. (1976) added 4% barley straw to two concentrate mixtures, which were then characterized as being 39.6% and 59.2% roughage, milk fat was reduced for the low roughage diets only, regardless of whether the straw had been included. From feeding three C:R ratios (80:20, 70:30 and 60:40) involving hay and corn silage, Marshall and Voigt (1975) reported average MF% values of 3.4, 3.5 and 4.0, respectively, the first two being significantly lower than the third.

A set of designations, similar to the C:R ratio but less quantitative, which is also commonly used in the literature to describe rations in fat production studies is that of low roughage (LR), restricted roughage (RR), or high grain (HG) rations, as contrasted

with normal or control diets. HG rations are often used to induce milk fat depression for experimental purposes. For example, Beitz and Davis (1964) reported MF percentages of 3.21 and 1.77 using control and HG rations, respectively, and Davis and Sachan (1966), in a similar study, reported similar percentages of 3.3 and 1.4. Kinsella and Houghton (1975) noted that fat production was consistently reduced in RR-fed animals. This type of information, while confirming Powell's (1939) statement about the possiblity of varying MF% "at will," is not able either to establish a sufficiently direct cause for this depression or to describe relationships with other aspects of production.

Roughage and Energy Relationships

The interactions between the fibrous and the more available energy fractions of diets are very complex and often confusing to the study of fat production in milk. In the paper by Marshall and Voigt (1975) already mentioned, "stepwise" increases in milk yield were seen with increases in the proportion of concentrate in the ration. This might suggest that a dairyman seeking to maximize both milk yield and fat percentage with a single ration would always be disappointed. It could also be true that when relatively high proportions of roughage are fed, energy intake or availability could effectively limit milk yield. Putman and Loosli (1959) reported increased TDN and DM intakes with increasing proportions of concentrates when they were included as 20, 30 or 60% of the total ration DM, although there were no differences as to milk yield or fat percentage in their study.

Evans et al. (1975) reported no significant differences for yield or MF% when HR and LR diets isocaloric for digestible energy (DE) were compared. However, Walker and Elliot (1973) observed that roughage restriction reduced both milk production (23.2 vs. 20.9 kg) and MF% (3.36 vs. 2.71) compared to controls. In an effort to separate effects due to fiber levels from those due to energy, Gordon and Forbes (1971) fed low, medium and high fiber in low and high energy rations in a factorial design experiment. They determined that neither milk nor energy yields were significantly different due to fiber levels, but that both responded greatly ($p<.001$) to energy levels. In their experiment, MF% was unaffected by treatment, and the efficiency of conversion of metabolizable energy (ME) to milk energy was 66, 65 and 56% for low, medium and high-fiber diets, respectively.

Given the above evidence that cows can achieve excellent conversion efficiency for energy on a diet containing a "medium" level of fiber, there is also evidence that they may prefer a ration consisting of concentrate and roughage in approximately equal proportion. This was shown by Wiktorrson (1973), who fed grass hay and concentrates ad libitum or a 61:39 C:R control ration. In this study, experimental cows selected a ration which approximated a ratio of 50:50 C:R, but production of actual milk and 4% FCM were higher on the control diet. Since the total DM and ME intakes were not significantly different between groups, the wider ratio was supporting the most efficient production. In earlier work (Wiktorrson, 1971), the same author also found the 60:40 C:R ME ratio superior, from the standpoint of efficiency, to wider ratios in which either hay or concentrate was

included as a still larger proportion of the diet. Research by Satter et al. (1973) showed that cows may be able to adjust feed consumption so as to maintain at least a minimum level of DE intake, or to optimize such intake for a particular ration. They fed graded amounts of aspen sawdust (0, 10, 20 and 30%) in pellets which otherwise contained high-energy ingredients, and found that DM intakes increased with the proportions of sawdust. Interestingly, this result would appear to be in contrast to that of Putnam and Loosli (1959), mentioned above.

It should be emphasized that these considerations are of importance even beyond the time of a given lactation. This was shown by Everson et al. (1976), who compared the feeding of a constant 40:60 (C:R) ration throughout a lactation to a regime in which energy levels were varied in accordance with production (group feeding) over a 2 year period. Differences in average daily or total DM intakes, as well as actual milk or 308-day FCM production over the 2 year period were not observed to be significant. It was noted, however, that early in lactation when energy demands were greatest, group-fed animals achieved higher DM intakes (20.8 vs. 19.3 kg) earlier (16 vs. 19 weeks) in the first year's lactation, and therefore experienced the lowest peak negative net energy balance (-8.5 vs. -12.0 Mcal). Consequently, FCM production in control animals was reduced in year 2 compared to year 1, whereas that in experimental animals increased. In addition, the first postpartum estrus in the control cows was delayed, a common indication of nutritional stress.

Bath (1974) fed alfalfa cubes and concentrates in ratios to supply from 20 to 80% of the estimated net energy (ENE) in the ration

from concentrates, and concluded that milk production could best be maximized at about 50% ENE from concentrates, while MF% could be maximized at approximately 35% ENE from concentrates. This taken together with the other studies cited thus far suggests that in situations where group-feeding is not possible, rations should be formulated to approximate a 60:40 ratio of high-energy feedstuffs to roughage. Most fundamentally, roughage must be provided at levels adequate to maintain proper rumen function and overall health, while at the same time the ration must supply enough energy for high-producing cows to milk to their capacities, particularly in early lactation.

Crude Fiber or Acid Detergent Fiber Levels

Many reports, particularly in the popular literature, have recommended the use of crude fiber (CF) values as a more quantitative index of the fiber composition of feeds. Suggested CF levels for dairy rations have recently been revised upward by the National Research Council (NRC, 1971; 1978) from 13% to 17% in response to much research evidence. For example, Lofgren and Warner (1970) fed concentrates with hay, corncobs, beet pulp or oat hulls as CF sources to animals in which MF depression had been induced. Rations were all formulated to contain 15% CF (6% of the fiber from experimental sources) and 14% CP (both as-fed basis) and all rations raised the mean MF% from the depressed level of 2.3 to 3.3%. Correlation coefficients of change in CF and acid detergent fiber (ADF) intakes with fat percentage were .49 and .72, respectively. In comparing corn and barley silages in a production trial, Rock et al. (1974) found that for both silages, milk yield was reduced by .39 and .31 kg per day for each 1% increase in

CF and ADF, respectively, whereas MF% was increased .072 and .067 for each 1% increase in the same. Crowley (1974) reported that corn silage could be successfully used as the only roughage source for dairy cows provided a minimum of 17% CF was available in the complete ration on a DM basis. In dairy feeding programs generally, he suggested that cows receive a minimum of 5 lbs of dry hay daily with a particle size of at least 1" to maintain fat percentage. Guidelines of this order are not difficult to meet: 40 lbs daily DM intake of a 17% CF ration provides 6.8 lbs of crude fiber, well in excess of this minimum. As was seen for C:R ratios, however, it may be more realistic to set CF values within an optimum range, such as that proposed by Murley (1971) of 16-20%. Bath (1974) recommended 19% as providing an ample margin of safety.

It was intimated above with regard to the work of Lofgren and Warner (1970) that ADF may be a more valuable standard of comparison for fiber content among rations than CF. This fact is reflected in Derbyshire's (1973) conclusion that MF% was most closely related to the ADF component. Interestingly, he found the highest production as well as the highest fat percentage and greatest ADF intake (23.1 kg FCM/day, 3.32% and 3.62 kg/day, respectively) when his diets contained 30% wheat straw. Furthermore, work such as that by Spahr et al. (1966) indicates that CF analyses may not be very helpful in assessing potential milk fat production, as levels varying from 14.1 to 18.7% CF from alfalfa hay and corn silage fed in equal parts as roughage sources led to insignificant differences in milk production or percentages of milk constituents. Many writers have reported that increasing the

proportion of fibrous feedstuffs in cattle rations reduces the digestibility of DM, CP, ether extract, nitrogen-free extract and detergent solubles, while increasing that of the CF or ADF fractions only (Everson et al., 1976; Putnum and Loosli, 1959; McCullough and Sisk, 1972; Arroyo-Agiulo and Evans, 1972; and Colburn, 1973). However, again with reference to milk fat production, ADF values do not seem to be adequate, in themselves, as information upon which predictions may be based. For example, Kellogg and Miller (1972) found no changes in MF% associated with either CF or ADF in feeding varying levels of alfalfa straw with concentrates (CF% ranging from 14 to 21), although their reported mean fat percentage was an unusually low 2.78. In addition, MacGregor et al. (1976) found that when soybean mill run was substituted for corn in a concentrate mixture giving CF and ADF values of 13, 18 and 23%, and 20.4, 28.1 and 31.3%, respectively, there were no differences as to DM or NE intake, 4% FCM or milk fat percentage. Here, alfalfa was the main roughage source.

Fiber Form

Given the great diversity of feedstuffs which can be used as roughages in dairy rations, as well as the many possible systems by which rations may be fed, the importance of fiber form for fat production should not be surprising. Experimentally, perhaps the greatest amount of work has been done on the effects of feeding hay, offered in baled, chopped, cubed or pelleted form. Ronning et al. (1959) showed that cows fed pelleted hay consumed more DM than animals fed chopped hay, and they also produced more 4% FCM. Feeding concentrates

as 12% of the ration DM eliminated such differences, however, suggesting that animals receiving the chopped hay may not have been meeting their energy needs. Thomas et al. (1968) reported higher intakes with pelleted hay than for that which was either steamed or ground ("medium grind"). They also noted that when feeding alfalfa ground to be coarse, medium or fine with concentrates commensurate with production, animals tended to refuse the finely ground material, several to an extent sufficient to cause ketosis. Only the ration with the fine hay significantly reduced MF% (4.6 to 3.9), but since this ration resulted in the greatest decline in production and the lowest average increase in body weight late in lactation, one would suspect that insufficient energy intake was again an important factor. Conrad and Hibbs (1971) found that coarsely chopped (3/8" cut) alfalfa pellets could effectively substitute for long alfalfa when rations included corn silage, in that both normal milk yield and fat percentage were maintained. O'Dell et al. (1968) compared baled, ground and pelleted alfalfa and determined that pelleting reduced MF% from 3.5 to 3.0. They also failed to find any significant effect due to fineness of grind prior to pelleting, using .64, .36 and .16 cm as grinder settings. Anderson et al. (1975) compared baled and cubed alfalfa, each fed with and without corn silage. Animals receiving cubes consumed more dry matter as well as more hay as DM than bale-fed cows. They also produced more milk when corn silage was included. However, when cubes were fed without silage, butterfat percentage was reduced ($p<.05$) by .3%, a difference which did not appear when the silage was fed.

Clearly, when it is especially advantageous to prepare roughage from a particular source in a particular way, it is necessary to pay careful attention to the remainder of the roughage component in order to maintain desirable production levels and characteristics.

Timing of Feeding

While it may be of less practical importance than the aspects considered thus far, it should be noted that the frequency with which a given roughage is fed (when it is being offered ad libitum) as well as the timing of feeding with respect to other ration components (that is, when a complete feed is not being used) can affect the results obtained. For example, O'Dell et al. (1968) found that feeding alfalfa pellets two times daily significantly reduced fat percentage, from 4.0 to 3.6, while four times feeding actually produced an insignificant increase. Palmquist et al. (1964), in feeding pelleted alfalfa and concentrates either twice daily together or each twice but the concentrate 4 hours before the alfalfa pellets, observed that MF% was significantly higher when they were fed separately, although yield was not affected. Pellet intake was also greater when they were fed separately. The authors postulated that production effects were attributable to a more even pattern of fermentation in the rumen when the pellets and concentrates were fed separately. This conclusion would obviously not apply to a situation in which such materials were fed together in a thoroughly mixed complete ration.

Physiological Considerations of Fiber Feeding for Milk Fat Production

Numerous authors, Van Soest (1963) and Jorgensen et al. (1965) among them, have listed the changes in several physiological parameters which are most often associated with MF depression. Those reported by the latter included:

1) decreases in the proportion of acetic acid in the rumen,

2) increases in ruminal propionic and valeric acid proportions,

3) reductions in rumen pH,

4) decreases in the levels of blood lipids and ketones,

5) increases in blood glucose concentrations,

6) decreases in short-chain fatty acids and increases in long-chain (especially unsaturated) fatty acids in the milk fat,

7) increased rates of body weight gain.

In view of these kinds of changes, several physiological mechanisms to explain the condition have been described, although in the writer's opinion none necessarily excludes the others as a "first cause." In this section, the most important of the changes listed above are touched upon, with the greatest emphasis being given to those which were examined in our research.

Ruminal Volatile Fatty Acid Production

Undoubtedly the most commonly discussed physiological adjunct to butterfat depression in the literature is the shift in rumen fermentation to a pattern favoring propionate production over that of acetate and butyrate. This response has been readily induced for experimental purposes through the feeding of LR rations, as in the work of Davis

and Sachan (1966), who reported a change in the ratio of acetate to propionate (A:P) of from 3:1 to 1:1 on control versus HG rations. Similarly, Bickerstaffe et al. (1971) found acetate present in the rumen at 44.4 and propionate at 38.7 meq/liter on a HR ration, as compared to 21.3 and 46.3, respectively, when roughage was restricted. Beitz and Davis (1964) reported MF% and A:P ratios of 3.21 and 2.61, respectively, for control animals, while those on HG diets were measured to be 1.77 and 1.19. Stanley et al. (1975) found a mean A:P ratio for a HG ration to be 2.57 and that for HR 3.39 ($p<.05$). Jorgensen and Schultz (1963) suggested that an A:P ratio of 3.0 would typically give a normal test, a 2.0 ratio a definite depression, and 2.5 could be borderline.

Observations such as these have occasioned many investigations into the precise nature and course of these shifts, their immediate cause, and the extent to which they might explain reduced milk fat levels. Storry and Sutton (1969) fed cows a LR, fat depressing ration, then abruptly changed the diet to one with ample roughage. In the rumen, they observed a rapid rise in cellulolytic activity, decreases in lactate, valerate and propionate proportions, and increases in both pH and relative acetate concentration, all within 5 to 7 days. They further noted that while the rumen adapted relatively rapidly, blood and milk lipids continued to change in composition for as long as 3 weeks. Walker and Elliot (1973) conducted a study over 34 weeks in which mean daily milk and fat percentage were 23.2 kg and 3.36, respectively, for control animals. Comparable values for animals fed RR rations were 20.9 kg and 2.71%, both different ($p<.05$)

from the control animals. Volatile fatty acids were sampled at 3, 16 and 34 weeks, with results showing highly significant differences in both acetate and propionate in weeks 16 and 34 only. Butyrate was not different until the final sampling period. These two studies illustrate differences encountered in the literature as to the rate at which fermentation patterns in the rumen change, although the norm seems to be the relatively rapid shift to a new equilibrium, as seen by Storry and Sutton (1969).

As might be expected, there are indications that changing VFA ratios are a reflection of changing populations among the microorganisms in the rumen. Chalupa et al. (1967) reported that rumen protozoa were almost entirely absent during the secretion of low fat milk. Of the variables studied, rumen pH changes accounted for 36% of the variation in protozoa numbers. In noting the typical pH decline, accompanied by reduced molar percentages of acetate and butyrate and increased proportions of propionate and valerate, the authors determined that these five parameters accounted for 24, 45, 36, 40 and 42% of the observed MF% variation, respectively. In a similar study with fistulated animals, Latham et al. (1974) found that substitution of LR for HR diets reduced ruminal acetic acid proportions by from 6 to 15%. In cows with depressed MF%, the proportion of propionate increased markedly, while butyrate levels declined. However, animals on HR rations maintained normal MF production, propionate changed little, and butyrate increased. When the latter animals were shifted to the HG diet, responses similar to those noted for the experimental group were seen. In this work, 69% of MF% variation could be

attributed to shifts in the molar proportions of the three major VFAs. Substantial reductions in the numbers of ciliated protozoa were observed in animals with reduced MF%, by factors ranging from 10 to over 30%, and counts of viable bacteria were also increased on the LR ration. A likely consequence of these increased total bacterial populations is the frequently noted increase in total ruminal VFA concentration in cases where MF% is depressed (Davis, 1967; Stanley et al., 1975; Bauman et al., 1971).

Thus far, VFA proportions have been given in relative terms. Such information, of course, leaves open important questions as to actual rates and quantities of their production, particularly of acetate and propionate. That is, given the A:P ratio declines where MF% is depressed, it would be helpful to know whether acetate production is actually diminished, or whether augmented propionate production adequately explains the shift resulting from HG feeding. While the literature pertaining to this specific question for the case of dairy cows on RR rations is not extensive, indications are that acetate production may be relatively uneffected, whereas that of propionate is increased substantially. Davis (1967) reported that total acetate production levels for cows on control and HG rations were 29.3 and 28.1 moles/24 hours, a difference which was not significant, although these values are uncorrected for the known condensation of acetate molecules to form butyrate which occurs in the rumen. This author concluded that a larger proportion of four-carbon fatty acids in the rumen were derived from acetate in the case of HG than control diets, and that this fact explained the significantly shorter

turn-over time for rumen acetate which he observed. For propionate, Bauman et al. (1971) found that production on normal rations was 13.3 moles/24 hours, while for RR:HG-fed animals it was 31.0 moles/24 hours. Comparable values for the total rumen propionate pool were .83 moles vs. 2.42 moles, respectively, and as the propionate turn-over times were not considered significantly different, the authors concluded that propionate was metabolized much more rapidly in the case of the HG diet. Based on his acetate studies mentioned above, Davis (1967) deduced similar relationships with regard to propionate.

Aside from manipulating the relative proportions of dietary roughage, a particularly valuable approach to assessing the influence of VFA proportions on butterfat production has been the experimental introduction of specific compounds into the rumen, either through intraruminal infusions (IR) or supplementation of the ration itself. For instance, Jorgensen and Schultz (1963) reported that butyrate, administered IR, caused increases in blood ketones, accompanied by a decrease in blood glucose. In a particularly interesting study, Rook et al. (1965) infused acetate and/or propionate into cow rumens in levels adjusted to maintain starch intake equivalence. Acetate caused increases ($p<.05$) in milk yield, whereas propionate and butyrate led to insignificant declines. As to MF%, acetate and butyrate produced increases ($p<.05$), and propionate a statistically insignificant reduction. Mean changes for MF% associated with some of the different infused materials were: propionate only, -.21%; acetate only, .28%; butyrate only, .39%; and acetate plus butyrate, .73%, with only the last two being different ($p<.01$).

Feeding Supplemental Buffers

The use of certain salts or buffers as supplements has been suggested as a means to offset the fat-depressing tendencies of restricted roughage diets, and has offered an important means of investigation, as well. The presumed sequence of events in which such materials are supposed to act was given by Davis et al. (1964):

1) HG feeding reduces rumination time, salivation, and the volume of natural buffers available to the rumen;

2) this reduces ruminal pH, encouraging a propionate-producing microflora;

3) increased propionate production in the rumen is accompanied by MF% depression, whatever its direct cause.

Thus, the supplemental buffering capacity was expected to prevent the initial reduction in ruminal pH. Davis et al. (1964) found that supplemental sodium or potassium bicarbonates, fed in equal parts to comprise 3% of the grain mixture, increased the ruminal molar percentage of acetate (55.6 for treatment vs. 47.8 for control) and decreased that for propionate (23.8 for treatment vs. 36.8 for control), leading to A:P ratios for treatment and control groups of 2.68 and 1.31, respectively. At the levels given, these bicarbonates prevented declines in MF% when animals were switched to RR rations, while those animals already giving low-fat milk responded, but to a lesser extent. When fed at only 1.5% of the concentrate allotment, differences were not significant. Jorgensen and Schultz (1965) obtained similar results with $NaHCO_3$, in addition to the predicted increase in the pH of the rumen contents. Emery et al. (1964) reported that $NaHCO_3$

increased MF% significantly, by .81 and .86 in two trials. Also increased were rumen pH, molar percentages of acetate and butyrate and blood fat levels. Interestingly, though effects upon yield and FCM were not significant, the treatment group consumed 12% less grain. Feeding sodium bicarbonate at either 2.83% or 5.66% of the concentrate mixture, increased both MF% and 4% FCM, according to Stanley et al. (1964). However, only the higher level raised the A:P ratio significantly above that seen with the control, HG ration. These authors suggested that the level of ruminal acetate necessary to raise MF% may depend somewhat upon the level of milk production. In a later study (1972) they also found that the roughage level of the ration played an important role. In their second trial in this particular experiment, cows produced more fat daily ($p<.05$) on 10.9 kg roughage than 7.28 kg, although when both roughage levels were supplemented with $NaHCO_3$, differences in milk yield and MF% were not significant. Still, the increase in fat percentage due to $NaHCO_3$ was 23.2% on the LR ration, compared to only 7.2% on the HR. In their first trial, the bicarbonate significantly raised the A:P ratio (1.9 to 2.3), fat production (.53 to .62 kg/day) and MF% (2.96 to 3.42) at all roughage levels. Overall, significant improvements were seen in MF% only when there was also a significant reduction in ruminal propionate.

MgO has also been evaluated experimentally for efficacy in counteracting fat-depressing effects of rations. Benson et al. (1972) fed animals normal, restricted roughage or MgO-supplemented rations, and found that while fat percentage was decreased from 3.0 to 2.5 by the HG diet, MgO restored normal fat production in 75% of the animals.

Emery et al. (1967) reported that the intravenous administration of Mg salts over a 4 day period caused a 15% increase in milk fat production as well as a 38% increase in its concentration ($p<.01$). This also indicates that MgO exerts its effects extraruminally. In an earlier paper, Emery et al. (1965) determined that both MgO and $NaHCO_3$ increased rumen pH, increased molar proportions of acetate and butyrate while decreasing that of propionate in the rumen, and reduced weight gains. They also suggested, given their conclusion that $NaHCO_3$ exerts its effects directly through reducing the molar percentage of ruminal propionate, whereas MgO does so by increasing mammary uptake of plasma acetate and triglycerides, that these two compounds could act synergistically. A very thorough review of the use of these two compounds and their physiological effects has been given by Thomas et al. (1969).

Having discussed the effects of supplemental buffers and the basis of their activity, it should be mentioned that the supposed high availability of salivary buffers under conditions of relatively high roughage feeding apparently is not, in itself, an adequate explanation for the resulting "normal" pattern of rumen fermentation, at least inasmuch as it is associated with the proportion of time an animal ruminates. Welch and Smith (1971) fed polypropylene ribbon as a roughage source to animals in which ruminating time had been reduced virtually to zero by a ration of alfalfa meal and concentrate, both as pellets. The ribbon restored the time spent ruminating to normal, as it did also in a later study conducted by the same authors (Welch and Smith, 1975). In the latter experiment, the ribbon was fed at 200 g per

animal for several weeks, but no change was seen in MF% levels as compared to controls. However, again in the later work, replacing ground hay with long hay returned both ruminating time and fat percentage to normal.

Thus, it appears that the nature of the feeding regime, not only as to chemical composition but also the way in which it is prepared and offered, is of the greatest importance in determining the levels of VFAs which are produced in the rumen, and that these levels subsequently influence, by some means, the production of milk fat by the mammary gland.

Lipid Status and Metabolism in Tissues

The processes by which volatile fatty acids are absorbed from the rumen, transported and metabolized, all with reference to milk fat synthesis, have been discussed extensively by many authors (Armstrong, 1965; Schmidt, 1971; Linzell, 1974). Having described changes in ruminal fermentation patterns which are typically associated with milk fat depression, their implications and consequences in other tissues may now be briefly reviewed.

As far as has been determined, transport of VFAs across the rumen epithelium into the hepatic portal circulation occurs along a concentration gradient; thus, their concentrations in the rumen are important in determining portal blood levels (Annison, 1965). According to Armstrong (1965), most of the acetate leaving the rumen passes unchanged to the liver, where relatively small amounts are either oxidized or converted to ketone bodies before the remainder moves through the

general circulation to the mammary gland. Ruminal butyric acid, on the other hand, is metabolized to ketone bodies either in the rumen epithelium or in the liver, where it may also be oxidized via the Krebs Cycle. Some ruminal propionate is metabolized to lactic acid in the epithelium, but most reaches the liver, where it is either oxidized or metabolized to glucose via the methylmalonyl-CoA pathway (gluconeogenesis), to be discussed further in the section on vitamin B_{12}. Thus, relative VFA proportions in the rumen and portal circulation are very different from those in the general circulation, with the liver being the primary intermediary.

As might be anticipated, however, changing patterns in rumen fermentation which are seen with fat-depressing rations are reflected in the levels of fat precursors which are carried to the udder. Bickerstaffe et al. (1971) reported substantial reductions in the mg% of blood acetate, β-hydroxybutyric acid and triglycerides in animals fed RR rations compared to controls, and Benson et al. (1972) noted a correlation of .89 between the arterial concentration of serum triglycerides and MF%. Rindsig et al. (1969) noted that along with significant increases in fat percentage, bentonite feeding with HG diets elevated the arteriovenous (a-V) difference across the mammary gland for acetate, although glucose, free fatty acids, triglycerides and other ketones were unchanged. Numerous other writers (Varnon and Schultz, 1968; Storry and Rook, 1965a and b; Storry and Sutton, 1969; Jorgensen and Schultz, 1965) have reported increased levels of blood ketones in cases where HR rations have been seen to increase fat percentage. For this reason, such rations are often said to be

ketogenic. A further observation in this regard is that intravenous infusions of ketogenic metabolites typically increase fat percentage (Storry and Rook, 1965a), although total fat production may not be increased. Qureshi et al. (1972) reported that these generalizations concerning blood lipids may apply only during the period prior to the final third of the lactation, however, and may thus depend somewhat upon the animals' level of production.

The necessary obverse of the relationships described for blood lipids or ketones, arises in consideration of glucose, propionate or other metabolites which are said to be glucogenic. In cases where milk fat is reduced by HG feeding, blood glucose levels are generally elevated, and these higher levels, as well as experimental glucose infusions, have acted to reduce blood lipids and ketones, along with fat percentage (Fisher, 1967; Fisher and Elliot, 1966; McClymont and Vallance, 1962; Storry and Rook, 1965a; Jorgensen and Schultz, 1963). This response is usually attributed to increased rates of gluconeogenesis in the liver from propionate, but the work of Jackson et al. (from Latham et al., 1974) with sheep showed that elevated ruminal propionate leads to increases in the passage of α-linked glucose polymers to the duodenum, adding to the absorbable glucose which is available to the bloodstream. This does, however, appear to be a relatively minor factor. Evans et al. (1975) also reported that both plasma glucose concentration and pool size were greater for animals receiving HG than HR rations. Interestingly, they also noted that the half-times for blood glucose on HR and LR were 40.0 and 30.4 minutes, respectively, indicating that higher glucose levels encourage its metabolism.

From the standpoint of lipid metabolism in body tissues as it is associated with milk fat percentage, an important consequence of increased blood glucose levels is seen in the similarly increased rate of insulin secretion into the bloodstream. A particularly significant study in this regard is that of Walker and Elliot (1973), who examined the relationship of serum insulin to MF% in cows fed restricted roughage or control rations during the first 34 weeks of lactation. By week 16, values in control and RR groups, respectively, were 3.3 and 2.3 for MF% and 19.5 and 24.9 for serum insulin (μU/ml), and both parameters were different ($p<.01$). The trend with regard to insulin is suggested, if not significant, by week 5. In week 16, correlations of MF% and rumen A:P ratio were -.55 and -.71, respectively.

Although the activity of enzyme systems may reflect control either through direct hormonal stimulation or substrate induction, many writers have related the above kinds of observations regarding insulin to such processes as a means of explaining fat depression and its physiological correlates. Most importantly, Varman and Schultz (1968) noted that insulin has been shown to stimulate formation of NADPH by the pentose cycle, thus making more H^+ ions available for fatty acid synthesis in adipose tissue. In the same study, the authors reported that the enzymes associated with fat synthesis there had twice the activity in cows on HG diets that they did in cows on HR rations, while the fatty acid synthetase system in the mammary glands from cows in the former group showed half the activity of that from cows fed normal diets. These results concerning mammary lipid uptake and synthesis seem to have been corroborated both by Bickerstaffe et al.

(1971) and Storry and Sutton (1969). Benson et al. (1972) in an especially thorough-going study of the activities of several enzymes, found that switching cows to RR from normal feeding caused the mean activity of lipoprotein lipase to increase by about three times, from 5.6 ± 1.5 to 17.8 ± 5.1 μeq fatty acid/hr/g tissue. When one atypical cow was eliminated, these differences were significant. In addition, palmitate esterification in adipose tissue proceeded at a rate eight times greater for the RR group than the control one. It is interesting in connection with comments made previously that Benson's group reported adipose lipoprotein lipase activity for cows fed MgO to be insignificantly different from that seen in the RR group. However, they also indicated that MgO seemed to increase the uptake of triglycerides by the mammary gland at the expense of adipose tissue, even though no differences as to the activities of lipoprotein lipase and fatty acid esterifying enzymes in the mammary gland could be attributed to ration effects.

The notion that the glucogenic response to HG diets, including elevated insulin secretion which was envisioned to encourage adipose deposition of fat and inhibit its mobilization for milk fat synthesis, is the heart of a theory to explain fat depression which was first proposed by McClymont and Vallance (1962). Their explanation did not make explicit reference to the changes that have been described in ruminal A:P ratios, although it is not inconsistent with them.

The magnitude and mechanisms of the uptake of milk precursors by the mammary gland has been reviewed thoroughly in publications by Linzell (1967, 1974). It is well known that the ruminant udder relies

heavily upon acetate, β-hydroxybutyrate and blood triglycerides for fat synthesis, and is able to extract each from arterial blood with a high degree of efficiency. As would be expected in view of the changing blood levels of these precursors in cases of MF% depression as it has been documented thus far, not only the amounts but the chemical composition of the fats secreted are altered. Tha nature of these alterations may now be briefly touched upon.

The greatest proportion of short-chained fatty acids, considered to be those containing 10 or fewer carbons, is synthesized in the alveolar epithelial cell itself from acetate and β-hydroxybutyric acid (Schmidt, 1971). Thus, it is commonly noticed that milk triglycerides from cows experiencing fat depression contain reduced proportions of short-chained fatty acids relative to longer ones (Chalupa et al., 1967; Benson et al., 1972; Brown et al., 1962), which are derived largely from blood triglycerides. Stull et al. (1966) superimposed the IV administration of acetate, propionate or butyrate on groups of animals fed LR or HR rations, and examined the distribution of fatty acids in the milk. Treatment with acetate increased concentrations for C_4, C_6, C_{16}, and $C_{18:0}$ fatty acids for both diets, while butyrate increased C_4 and $C_{18:2}$ content, also for both diets. Propionate, however, raised C_{12}, C_{16}, $C_{16:1}$, $C_{18:1}$ and $C_{18:2}$ concentrations on the LR diet and reduced C_4, C_6, C_8 and $C_{18:0}$. On the normal or control diet, it increased C_{16} and $C_{18:1}$ levels, and caused reductions in C_4, C_6, C_8, C_{10}, C_{12} and $C_{18:0}$. Another common observation is that milk fatty acids obtained from fat-depressed cows are unsaturated to a much greater extent than those from normal cows (Storry and Sutton, 1969;

Nicholson and Sutton, 1971). Storry and Rook (1965a) noted large relative declines in the amounts of "major" saturated acids, to levels as low as 20% of normal, while "major" unsaturated acids such as oleic, linoleic and linolenic, were reduced only to about 70% of normal. Davis et al. (1964) reported that feeding bicarbonates resulted in MF which was more highly saturated than controls, mainly due to increases in stearic and decreases in oleic acid levels. Similarly, Stanley et al. (1972) observed that $NaHCO_3$ feeding caused significant increases in $C_{14:0}$ and $C_{16:0}$ in milk fat triglycerides, with significant decreases in $C_{16:1}$ and $C_{18:1}$. There are instances in the literature where the feeding of HG rations which depressed milk fat percentage did not change the molar percentage of any major milk fatty acid (Fisher, 1967; Brown et al., 1962; Stanley et al., 1975).

Overall, as of the mid-1970s, researchers had reached a stand-off of sorts with the problem of milk fat depression. It was understood to the extent that it could generally be avoided in practical situations. Its physiological adjuncts had been combined into an ad hoc theory which seemed able to explain most instances of the problem, stated succinctly by Bickerstaffe et al. (1971).

> "Many workers have suggested that the increased production of propionate is the critical factor, and that its effect is mediated through the increased availability of glucose which leads directly (or indirectly, via increased insulin secretion) to a change in the balance of fatty acid uptake and release in adipose tissue in favor of triglyceride synthesis. This results in the reduced availability of fatty acids for the synthesis in the liver of lipoproteins which account for 50-60% of milk fat. A concurrent reduction in the availability of acetate, a major fatty acid precursor in adipose tissue and mammary tissue in the ruminant, acts in the same direction."

However, in 1977, Frobish and Davis published a paper suggesting that vitamin B_{12} could be involved in the syndrome by way of its role in propionate metabolism. It is to the explication of the basis for this theory that we now turn.

Vitamin B_{12} Status and Its Implications for MF Production

During the approximately 50 years since its existence was first suspected in 1926 (Scott et al., 1976), investigations into the occurance, structure and biochemical roles of vitamin B_{12} have consumed the interest of innumerable researchers. Not until 1973 was the complete synthesis of this complex organometallic compound achieved (Maugh, 1973), and the history of that and other important inquiries have been reviewed frequently and extensively (Scott et al., 1976; Underwood, 1977; Smith, 1965; Smith and Loosli, 1957). In this section brief mention of the chemistry, techniques for analysis, tissue distribution in animals with particular reference to ruminants, and deficiency syndromes will be made. Special attention will be given to the role of vitamin B_{12} in propionate metabolism, as this is the means whereby its effects on MF synthesis are presumed to take place.

The chemical structure of cyanocobalamin, the form in which vitamin B_{12} is generally isolated, is given in Figure 1. More descriptively, its name 5,6-dimethylbenzimidazolyl cobamide cyanide (Gawthorne, 1969) indicates the presence of a particular nucleotide bonded at right angles to a corrin nucleus. This nucleus contains a central, trivalent cobalt atom surrounded by four pyrrole rings. In

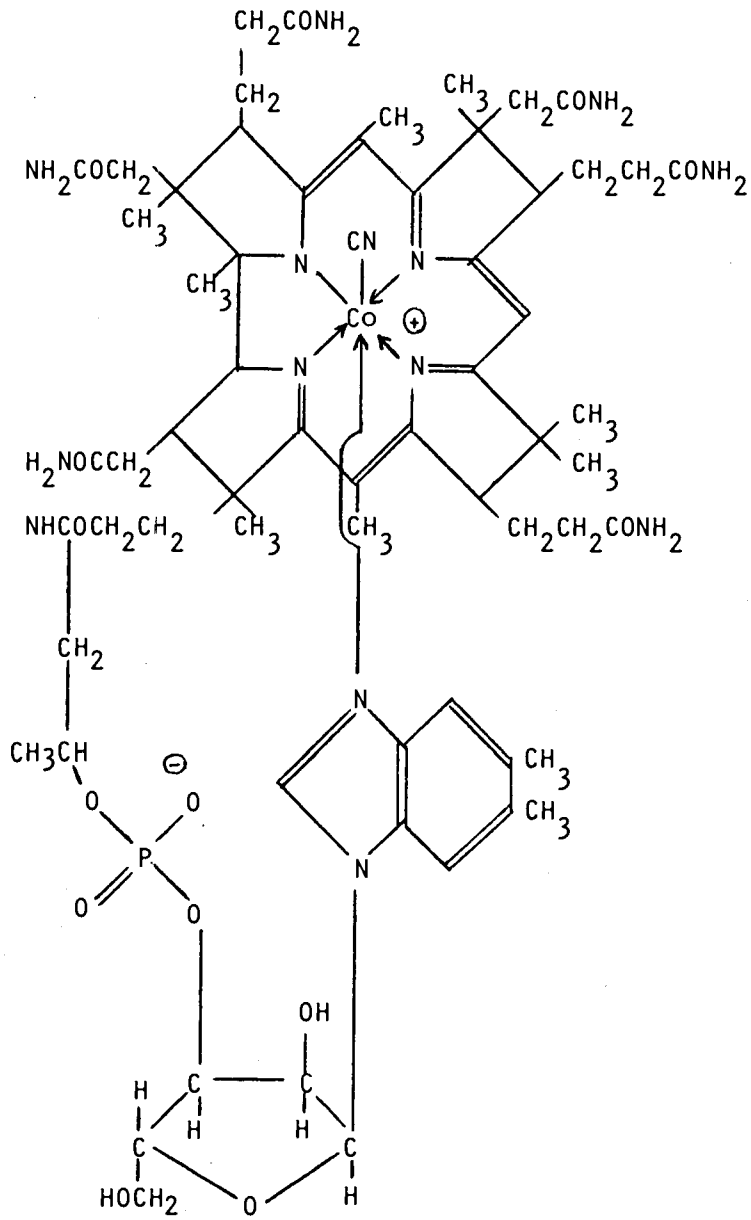


Figure 1. Structure of Vitamin B_{12} (from Skinner, 1976).

this case, a cyanide (CN^-) anion is also bonded to the cobalt, although other cobalamins occur in which this group is replaced by such anions as chloride, bromide, hydroxide and others. These kinds of compounds are given designations such as B_{12a} (hydroxycobalamin) and B_{12c} (nitrocobalamin). Another class of related compounds, the so-called pseudovitamins B_{12} or vitamin B_{12} analogues, contain nucleotide moieties replacing the 5,6-dimethylbenzimidazole. Examples of these include pseudovitamin B_{12} (or B_{12f}, containing adenine), Factor A, containing 2-methyladenine (2-MAC) and Factor C, containing guanine. Factor B, frequently encountered in the literature, is an acid degradation product of cyanocobalamin and lacks a nucleotide. Finally, in the cobamide coenzyme of vitamin B_{12}, important both in its biochemical activity and as a storage form in animal tissues, a 5'-deoxyadenosine group replaces the anion (usually CN^-) in a bond to the Co atom. Again, this information is reviewed in the general literature (Scott et al., 1976).

In view of the great steric specificity of enzyme and, in many cases, nutrient absorption systems, the existence of such a variety of chemically distinct forms of the vitamin might be expected to have broad implications, as indeed it has. Among cobalamins other than cyanocobalamin, the work of Hogan et al. (1973), and Frobish and Davis (1977) suggests that at least hydroxycobalamin, injected parenterally, has biological activity in animals. With regard to the B_{12} analogues, Dryden et al. (1962) noted that those "...containing nucleotides with benzimidazole derivatives tend to possess some vitamin activity for animals, while those containing purines tend to have activity only for micro-organisms." Conversely, Ford (1953)

determined that "animal materials" contained B_{12} mainly as cyanocobalamin, while those from bacterial fermentation contained large proportions of other forms, although so far as is known, vitamin B_{12} found in any tissue was originally a product of microbial synthesis. It is important to note that rumen micro-organisms produce substantial quantities of analogues. Both the extent and consequences of this fact will be discussed further below.

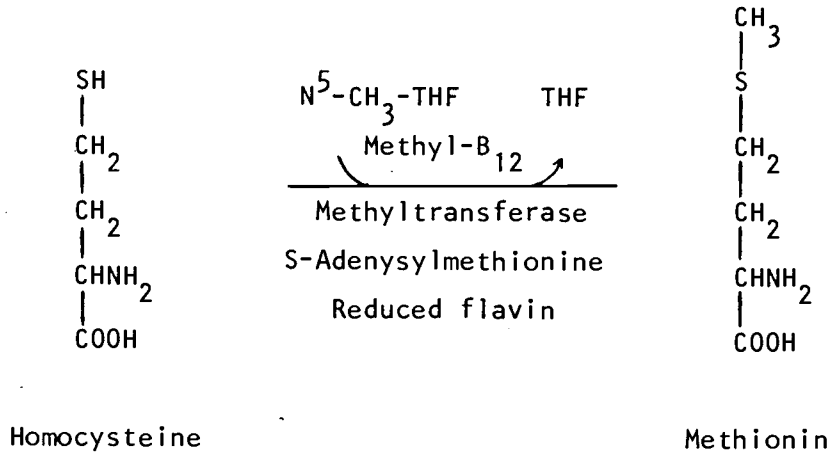
That such a diversity of forms of the vitamin could be present in a sample to be analyzed for B_{12} has presented researchers with the challenge of developing methods to suit particular needs. Dawbarn et al. (1957), Booth and Spray (1962) and Gawthorne (1969) discussed a paper ionophoresis technique which has been useful in separating B_{12}-like compounds, and it has been possible to analyze quantitatively for them, following extraction from the resulting spots, using any of several microbiological procedures. Dawbarn and Hine (1955) compared the four most commonly used of these assays in a study of B_{12} levels in the feces and urine of sheep. In relative terms, estimations were greatest for the Escherichia coli plate method, followed by the E. coli tube, Lactobacillus leichmannii and Ochromonas malhamensis methods. This list indicates also the relative specificity of these assays: that is, the protozoan Ochromonas is able to utilize only cyanocobalamin for growth, while Lactobacillus is somewhat less fastidious, and E. coli still less so (Ford, 1953; Scott et al., 1976; Skinner, 1976). Still another important method of analysis has involved radioisotopes of cobalt, such as ^{60}Co or ^{57}Co. This technique has been especially useful in elucidating the relationships between Co and

vitamin B_{12} and the distributions of each in tissues. This procedure is demonstrably the least discriminating among forms of the vitamin, as all involve cobalt, so a ratio of Ochromonas to radioisotope (O:R) estimations of B_{12} activity has been used commonly to indicate the percentage of animal-active vitamin in the sample (Hedrich et al., 1973; Sutton and Elliot, 1972; Elliot et al., 1971). As Dawbarn and Hine (1955) noted, in cases where a sample is known to contain cyanocobalamin almost exclusively, these various assays would be expected to yield comparable results. This was known, for example, by Lichtenstein et al. (1959), who found that both Ochromonas and Lactobacillus were able to recover added cyanocobalamin at from 93.5 to 96.9 percent, with neither method being more precise than the other. Thus, a researcher's confidence as to the nature of his samples would tend to determine which assay(s) would be selected for use.

The two enzyme-catalyzed reactions in which vitamin B_{12} is known to be involved are shown in Figure 2. General discussions of these processes are provided by Skinner (1976), Scott et al. (1976), and a particularly thorough treatment is given in Weisbach and Taylor (1968).

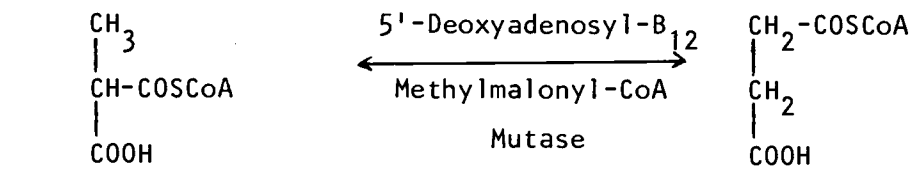
Cobalt and Vitamin B_{12} in Ruminant Nutrition

Underwood (1977) has described historical developments in the understanding of the interrelationships between cobalt and vitamin B_{12}. Much progress was made early in this century through studies of syndromes characteristic of sheep and cattle in certain areas of New Zealand and Australia. In such conditions as bush sickness and coast disease, animals typically showed signs of listlessness, anorexia,



Homocysteine

Methionine



L-methylmalonyl-Coa

Succinyl-CoA

Figure 2. Reactions requiring vitamin B_{12} coenzymes (from Skinner, 1976).

and emaciation, as well as the blanched skin and mucous membranes characteristic of anemia. Ultimately, these conditions were found to be associated with the grazing of cobalt-deficient pastures, with the resulting cobalt deficiency manifesting itself physiologically as a deficiency of vitamin B_{12}.

Documentation of the effect of cobalt intake on vitamin B_{12} status is extensive, particularly with reference to ruminants. Hedrich et al. (1973) fed sheep rations supplying .06, .50 or 1.02 ppm Co, and over these levels noted a "linear component" ($p<.01$) to the response in duodenal cobalamin levels. Hine and Dawbarn (1954) and Smith and Marston (1970) fed sheep cobalt-deficient diets and supplemented them experimentally with oral doses of 1 mg Co/day. Withdrawal of this supplementation caused a rapid decline in the B_{12} activity of rumen contents in both experiments. Marston et al. (1961) observed that when Co fell below 40 ng/g of rumen contents (or 2 µg/ml of bacteria-free supernatant), B_{12} production fell from 600 to 1000 µg daily to 50 µg/day. Smith and Loosli (1957) reviewed earlier studies of the vitamin B_{12} production and tissue activity of sheep under a variety of conditions. A great deal of more recent research has corroborated the statement of Gawthorne (1970a) that cobalt is the "primary limiting factor" for the synthesis of cobamides and cobinamides in the rumens of Co-deficient sheep (Marston, 1970; Gawthorne, 1970b). Marston (in Smith and Loosli, 1957) suggested that a cobalt content in the diet of .04 ppm would probably be adequate for cattle, while sheep require a somewhat higher level, of .08 to .10 ppm, both dry basis.

It is generally believed that oral supplementation of cobalt by

some means (top-dressing pasture, cobalt bullets, or as part of a mineral mix) is most efficacious in ruminants as compared to parenteral administration (Underwood, 1977; Smith and Loosli, 1957). Using lambs receiving a basal diet which contained .04 ppm cobalt, Kercher and Smith (1956) provided supplementary Co at 1 mg/day by oral, intravenous or subcutaneous means. Blood vitamin B_{12} analyses from sheep on the oral treatment averaged .62 ng/ml, greater ($p<.01$) than the other two treatments. Their interesting observation that parenteral treatments resulted in increased B_{12} contents in the cecum and large intestine was rationalized to be a consequence of bacterial synthesis following excretion of Co into the gut through the bile. This supply of the vitamin would be unavailable for absorption and use by the animal, however (see below). A similar process is suggested by the results of Tseng et al. (1976), in which dietary supplementation with inorganic cobalt caused significant increases in tissue B_{12} stores in hamsters. That is, cobalt passing through the gut was presumably utilized by the intestinal microflora to synthesize the vitamin, excreted in the feces and subsequently ingested and absorbed by means of coprophagy. Again in sheep, Marston (1970) determined that a ration supplying about 30 µg Co/day necessitated an additional .5 to 1.0 mg/day of cobalt orally for "maximum vitamin B_{12} status" of 3 ng/ml in serum and 1.4 µg/g in liver tissue. In a study of 11 herds of cattle which were considered at least marginally Co-deficient, Skerman et al. (1959) noted a response ($p<.02$) in body weight gains to the use of 20 g cobalt pellets.

Given that cobalt is used by micro-organisms in the host to synthesize cobalamines, and indeed that its availability can be the

primary limitation of the process, it is also clear that a large proportion of the compounds produced have no metabolic activity for the animal. Gawthorne (1969) identified nine different cobamides in sheep rumen fluid by ionophoresis, and in later (1970a) *in vitro* studies found that addition of particular nitrogenous bases to cobalt-adequate cultures of rumen micro-organisms stimulated the synthesis of the corresponding cobamides "at the expense" of others. Dawbarn *et al.* (1957) made similar observations during the course of *in vitro* studies.

The distribution of analogues in rumen contents under normal conditions of feeding has been variously reported. For example, Dryden *et al.* (1962) found that cyanocobalamin itself made up 10% of the activity in *E. coli* assays of cow rumen samples, Factor A, 60%, and Factors B, C and pseudovitamin B_{12} accounted for the remainder. Dryden and Hartman (1971) indicated that an average of 36% of the normal B_{12} activity in the rumens of heifers was from cyanocobalamin (ranging from 28 to 42%).

From the *in vitro* work noted above, shifts in the relative proportions of cobalamins would be expected to follow changes in diet. Gawthorne (1970b) reported that the proportion of B_{12} activity from cyanocobalamin was increased in sheep from 35 to 63% following a decline in cobalt intake from .34 ppm to .04 ppm, while that due to Factor A was unaltered at about 44%. Similarly, Dawbarn and Hine (1955) found that the ratio of activity given by *E. coli* and *Ochromonas* was reduced from 14:1 in the feces of sheep receiving supplemental Co to 7:1 in those of sheep fed Co-deficient rations. However, Hedrich *et al.* (1973)

determined from duodenal samples that when cobalt availability was inadequate, the proportion of cyanocobalamin to analogues was significantly reduced. Comparisons may also be made as to ruminal B_{12} levels seen in feeding HG vs. HR diets. In samples of rumen contents analyzed using L. leichmannii, Hayes et al. (1966) found that levels of all B-vitamins except riboflavin and biotin were significantly increased by all-concentrate rations. On the other hand, Sutton and Elliot (1972) reported that total ruminal B_{12} levels in sheep were reduced with increasing proportions of dietary concentrates. Both these workers and Walker and Elliot (1972) noted that there were reduced proportions of Ochromonas-sensitive B_{12} in the rumens of sheep fed HG rations compared to controls. Elliot et al. (1971), also in samples of digesta from sheep, found much higher proportions of analogues when pelleted concentrate, versus pelleted grass meal, was fed.

From the standpoint of the host animal, ruminal B_{12} levels or rates of production are not nearly so important as the amounts of metabolically-active vitamin available in the tissues, particularly in the liver. Crucial in establishing these levels is the relative efficiency of absorption from the gut, which most research indicates is remarkably low. For example, Elliot et al. (1971), using sheep fitted with abomasal and ileal re-entrant cannulas, reported an average percentage absorbance for cobalamines of 9.3%. Numerous other authors have given values of well below 10% (Kercher and Smith, 1955; Marston et al., 1961; Smith and Marston, 1970). Because these reports refer to the proportion of total cobamide production which is absorbed and

cyanobobalamin accounts for perhaps 10 to 35 percent of that, it has often been supposed that vitamin B_{12} absorption, mediated by gastric intrinsic factor, is quite specific for cyanocobalamin. Smith (1965) noted a much lower affinity of intrinsic factor for analogues or other B_{12} derivatives than for cyanocobalamin, and Gawthorne (1970b) found that B_{12} activity in plasma was due to cyanocobalamin "almost entirely." On the other hand, Booth and Spray (1962) identified four different cobalamins in the serum of rats by chromotgraphy. Sutton and Elliot (1972) reported that in sheep the absorption of various cobalamins was relatively high between duodenal and ileal cannulas, and that the proportion of serum analogues was increased by HG feeding. Similarly, Walker and Elliot (1972) noted a correlation of .48 ($p<.01$) between radioisotope assays of rumen and serum B_{12} activity, while there was no such relationship when Ochromonas was used. Overall, questions relating to the absorption of analogues from the gut and their levels in the blood or other body tissues are best considered open, as is the possibility of interconversions among forms of the vitamin following absorption.

Levels of vitamin B_{12} in several tissues, determined under a wide variety of circumstances, are given in Table 1 as a convenient basis for comparisons. From this, it is clear that the tissue, cobalt status, species and assay used all bear importantly on the results obtained.

Several additional comments should be made concerning the distribution of B_{12} among tissues of the body, as well as the effects of its supplementation directly. Cyanocobalamin is concentrated,

Table 1. Tissue vitamin B_{12} Content - Representative Literature Values

Reference	Species and Tissue	Concentration	Assay	Comments
Smith and Marston (1970)	Sheep rumen production	400-700 µg/day	O. malhamensis	Full feed, Co-deficient diet + 1 mg Co/day drench.
Smith and Marston (1970)	Sheep rumen production	50-110 µg/day	O. malhamensis	Same diet, unsupplemented.
Sutton and Elliot (1972)	Sheep rumen production	603 µg/day	O. malhamensis	60:40 C:R ratio.
Sutton and Elliot (1972)	Sheep rumen production	1195 µg/day	O. malhamensis	All roughage diet.
Hedrich et al. (1973)	Sheep rumen production	37 µg/day	O. malhamensis	0.06 ppm Co in ration.
Hedrich et al. (1973)	Sheep rumen production	1006 µg/day	O. malhamensis	0.50 ppm Co in ration.
Hedrich et al. (1973)	Sheep rumen production	1553 µg/day	O. malhamensis	1.02 ppm Co in ration.
In Smith and Loosli (1957): Hoekstra	Sheep rumen contents	9 µg/100 g DM	L. leichmannii	Co-deficient.
Hoekstra	Sheep rumen contents	130 µg/100 g DM	L. leichmannii	Co-supplemented.
Kercher & Smith	Sheep rumen contents	15 µg/100 g DM	L. leichmannii	Co-deficient.
Kercher & Smith	Sheep rumen contents	104 µg/100 g DM	L. leichmannii	Co-supplemented.
Elliot et al. (1971)	Sheep abomasum	1.019-4.067 µg/g DM	O. malhamensis	---
Elliot et al. (1971)	Sheep ileum	1.405-6.674 µg/g DM	O. malhamensis	---
Elliot et al. (1971)	Sheep abomasum	4.199-12.372 µg/g DM	radioisotope	---
Elliot et al. (1971)	Sheep ileum	4.615-20.657 µg/g DM	radioisotope	---

Table 1. (Continued)

Reference	Species and Tissue	Concentration	Assay	Comments
Smith et al. (1969)	Sheep liver	0.015 µg/g wet	L. leichmannii	B_{12}-deficient diet for 56 weeks.
Smith et al. (1969)	Sheep liver	0.78 µg/g wet	L. leichmannii	B_{12}-deficient diet for 56 weeks but supplemented w/IM inj.
Tseng et al. (1976)	Hamster liver	0.11 µg/g wet	E. coli	B_{12}-deficient.
Tseng et al. (1976)	Hamster liver	0.97 µg/g wet	E. coli	B_{12}-supplemented w/1 mg/kg diet.
Frenkel et al. (1973)	Rat liver	.172 µg/g wet	not given	Normal animals.
Frenkel et al. (1973)	Rat liver	.045 µg/g wet	not given	B_{12}-deprived.
Frenkel et al. (1973)	Rat liver	.185 µg/g wet	not given	B_{12}-supplemented with cyanocobalamin
Anthony et al. (1951b)	Calf blood	ca 1 ng/ml	L. leichmannii	From before birth to after first colostrum.
Anthony et al. (1951a)	Cow blood	.72 ng/ml	L. leichmannii	Jersey
Anthony et al. (1951a)	Cow blood	1.03 ng/ml	L. leichmannii	Holstein
Corse and Elliot (1970)	Cow serum	.043 ng/ml	radioisotope	Dry period.
Corse and Elliot (1970)	Cow serum	.061 ng/ml	radioisotope	2 weeks postpartum.
Corse and Elliot (1970)	Cow serum	.068 ng/ml	radioisotope	4 months postpartum.
Elliot et al. (1965)	Cow whole blood	0.25-0.35 ng/ml	L. leichmannii	Values lowest around freshening.

Table 1. (Continued)

Reference	Species and Tissue	Concentration	Assay	Comments
Hogan *et al.* (1973)	Lamb plasma	0.14 ng/ml	not given	Cobalt deficient.
Hogan *et al.* (1973)	Lamb plasma	0.63 ng/ml	not given	Following hydroxo-cobalamin dose (IM) totalling 3 mg.
In Smith and Loosli (1957): Hoekstra	Sheep blood	0.47 ng/ml	not given	Cobalt-deficient.
Hoekstra	Sheep blood	2.3-4.3 ng/ml	not given	Cobalt-supplemented.
Smith *et al.* (1969)	Sheep serum	0.25 ng/ml	not given	B_{12}-deficient for 56 weeks.
Smith *et al.* (1969)	Sheep serum	7.2 ng/ml	not given	B_{12}-deficient for 56 weeks but supplemented w/IM injections.
Somers (1969)	Sheep plasma	0.1 ng/ml	not given	B_{12}-deficient.
Somers (1969)	Sheep plasma	1.7 ng/ml	not given	B_{12}-deficient but supplemented w/IM injections.
Sutton and Elliot (1972)	Sheep serum	4.4 ng/ml	radioisotope	All roughage diet (adequate Co).
Sutton and Elliot (1972)	Sheep serum	6.9 ng/ml	radioisotope	60:40 C:R ratio.
Kercher and Smith (1956)	Sheep blood	0.62 ng/ml	*L. leichmannii*	Given Co-supplement orally.
Hedrich *et al.* (1973)	Sheep serum	ca 6.0 ng/ml	radioisotope	Over 90% was *Ochromonas*-active.

Table 1. (Continued)

Reference	Species and Tissue	Concentration	Assay	Comments
Frenkel et al. (1973)	Rat serum	1.106 ng/ml	not given	Normal animals.
Frenkel et al. (1973)	Rat serum	0.142 ng/ml	not given	B_{12}-deficient.
Frenkel et al. (1973)	Rat serum	1.386 ng/ml	not given	B_{12}-supplemented.
Tseng et al. (1976)	Hamster serum	1.6 ng/ml	*E. coli*	B_{12}-deprived.
Tseng et al. (1976)	Hamster serum	21.6 ng/ml	*E. coli*	B_{12}-supplemented, 1 mg/kg diet.

largely in its coenzyme form, in the liver, kidney, spleen and pancreas (Smith, 1965; Skinner, 1976; Scott et al., 1976). In ruminants, a critical level in the liver appears to be about .1 µg/g (Marston et al., 1972; Skerman et al., 1959). Below this level, animals show typical indications of B_{12} deficiency, while a level of .4 µg/g seems to be approximately optimum. As Table 1 shows, blood levels are more problematical, as is the relationship between the B_{12} concentration in body organs and the blood. Wilson et al. (1967) carried out a study on lactational trends in the liver B_{12} status of dairy cattle, and noted that "like blood levels," liver B_{12} tends to be lower earlier in lactation than later. A similar conclusion was reached by Elliot et al. (1965), and suggests that vitamin B_{12} could tend to be depleted during periods of heavy production. In the work of Wilson et al. (1967), liver and blood B_{12} content were not significantly correlated with one another, but the authors mentioned great within-group variation and surmised the likely occurence of relatively consistent B_{12} distribution patterns within individual cows. Sinnett and Spray (1962) conducted similar work in rabbits, and found highly significant correlations between serum B_{12} content and that in the liver ($r=.885$, $p<.001$) and kidney ($r=.734$, $p<.01$). Regression equations indicated that if the serum became indetectably low, so would the level in the kidney, while the liver retained its stores tenaciously. These authors noted that similar relationships have been observed in rats, but no reference was made concerning ruminants.

We have discussed the importance of adequate dietary cobalt in maintaining proper vitamin B_{12} status, and the effectiveness of oral

cobalt supplementation in ruminants. Provided in this way, cyanocobalamin itself is considerably less effective. Dawbarn and Hine (1955) administered 500 µg cyanocobalamin daily by mouth, 450 µg of which was excreted daily in the feces, as determined by E. coli. Using the Ochromonas assay, only 115 µg/day was excreted. From this, the authors concluded not only that the supplement was inefficiently absorbed, but that approximately 75% of it was metabolized into forms having no animal activity, presumably by the rumen micro-organisms. Smith and Marston (1970) also mentioned indications of degradation of cyanocobalamin in the rumen. In studies with Co-deficient lambs, Kercher and Smith (1955) fed 500 µg B_{12}/day for 5 weeks, and noted responses in appetite, body weight and hemoglobin levels comparable to those seen with injections of 500 g B_{12} intramuscularly over 2 weeks' time. Feeding 100 µg B_{12} daily was ineffective. They calculated that the total effective oral dose was about 35 times that of parenteral administration. Oral supplementation is, however, relatively more effective in non-ruminants. Tseng et al. (1976) included 40 µg B_{12}/kg or 1 mg/kg in an otherwise B_{12}-deficient diet, and noted significantly increased tissue stores of the vitamin at both levels, as well as remission of many typical B_{12} deficiency signs.

Many examples can be found of the efficacy of parenteral B_{12} supplementation in ruminants (Underwood, 1977). Smith et al. (1969) found good responses in B_{12}-deficient sheep to IM injections of 250 µg/week (see Table 1). Other writers have obtained better results using injections of either hydroxycobalamin (Hogan et al., 1973; Frobish and Davis, 1977) or 5'-deoxyadenosylcobalamin (Smith and

Marston, 1970), due to slower absorption from the site of injection, increased utilization and less excretion than with cyanocobalamin. From a practical standpoint, of course, these results are of limited value due to the economy of dietary cobalt supplementation for ruminants.

Vitamin B_{12} and Propionate Metabolism

As was mentioned above in the section on "Physiological Considerations of Fiber Feeding for Milk Fat Production," feeding milk fat-depressing, high concentrate diets to dairy animals typically leads to increased production of propionate in the rumen and subsequently to elevated levels of glucogenic metabolites in the bloodstream. Having also shown that vitamin B_{12} is required for the normal metabolism of propionate, evidence tending to associate faulty propionate metabolism with MF synthesis may now be presented.

Numerous studies relating the clearance of VFAs, especially propionate, to vitamin B_{12} status have been carried out, once again most frequently using sheep. Marston *et al.* (1961) infused B_{12}-deficient, fasted sheep with 3.5 mmoles/kg body weight of formate, acetate or propionate and observed that only propionate clearance was impaired. For example, 1 hour after propionate loading, the blood of B_{12}-adequate animals contained less than 1 mmole propionate/liter, while that from deficient sheep contained over 7 mmoles propionate/liter. In subsequent *in vitro* studies, these authors showed that liver homogenates from deficient animals could not effect the normal conversion of propionate to succinate: however, adding 5'-deoxyadenosyl

cobalamin restored this activity. Somers (1969) reported that half-times for both acetate and propionate clearance in sheep became increasingly longer as B_{12} deficiency heightened. Marston et al. (1972) noted increases in total VFA concentrations ($p<.05$) and propionate content ($p<.01$) in the blood of cobalt-deficient sheep, but no differences for acetate. Corse and Elliot (1970) compared propionic acid utilization and B_{12} status in dairy cows during three stages of production: dry period, 3 weeks postpartum, and 4 months postpartum. They found no significant differences in serum B_{12} levels (see Table 1), although the rate of propionate utilization was lower ($p<.01$) for dry than lactating cows. A particularly interesting observations was that during lactation, higher rates of glucose increase were correlated with higher serum B_{12} levels ($r=.42$), a fact which may relate to the conclusion of Bauman et al. (1971)(see "Ruminal VFA Production" section above) regarding increased rates of propionate utilization in response to HG feeding. However, Corse and Elliot (1970) did not find that the ability to metabolize propionate was significantly correlated with serum B_{12} levels. Unfortunately, they did not attempt to determine liver vitamin B_{12} status. Their results also do not indicate stress resulting from any real B_{12} inadequacy.

A good deal of this work has been done in laboratory animals as well. Williams and Spray (1972) injected 1 mmole of sodium propionate intraperitoneally into rats, and observed that concentrations of propionate in the blood of B_{12}-deficient rats were markedly raised even 2 hours later as compared with controls. The deficient rats also excreted seven times the amount of propionate in the urine that their normal littermates did. Also from studies with rats, the results of

Frenkel et al. (1973) on serum and liver vitamin B_{12} levels (see Table 1) and propionate catabolism suggest the importance of their relationship. Using CO_2 consumption as an index of propionate catabolism, they found that 618, 273 and 575 mmoles of $^{14}CO_2$/100 g were used by liver tissue from B_{12}-supplemented, B_{12}-deprived and normal animals, respectively. Venkataraman et al. (1967) fed sodium formate and sodium propionate at 5% of the diet to chicks, and found that the growth-depressing effect of vitamin B_{12} inadequacy was intensified in deficient chicks, while no such effect was seen when chicks received supplementary B_{12}. They obtained similar results in rats with Na-propionate at 2% of the diet.

Smith and Marston (1971) noted derangements in the utilization of propionate and other VFAs similar to those discussed above following IV Na-propionate administration to B_{12}-deficient sheep. Their conclusion was that, "All the effects (seen in this research) may be attributed to depletion of 5'-deoxyadenosyl cobalamin in the tissues, with a consequent loss of activity of methylmalonyl mutase," the enzyme responsible for catalyzing the conversion of methylmalonyl-CoA to the succinyl-CoA from which glucose is ultimately derived in the liver (see Figure 2). Its activity has been found to be highly correlated with vitamin B_{12} levels in the liver (Reed and Tarvey, 1970), and it has been reported to limit the rate of propionate utilization in cows (Mathias and Elliot, 1967).

Several enzyme-catalyzed reactions are involved in the pathway leading from propionate to succinyl-CoA, which is then available for glucose synthesis, and these are diagrammed in Figure 3. This figure

also outlines the possible influences of propionate on fat metabolism in the mammary gland and elsewhere. Flavin and Ochoa (1957) and Frenkel et al. (1973) have provided particularly useful discussions of the biochemistry of propionate utilization, while that given here must be restricted to the means whereby MF synthesis could be effected.

As presented in the general literature (Schmidt, 1971; Larson and Smith, 1974; Linzell, 1967), acetate is a highly important precursor for the synthesis of fatty acids in ruminants by the cytoplasmic pathway. Malonyl-CoA is an intermediate of this pathway and, as Figure 3 indicates, there is evidence that intermediates of propionate catabolism, which accumulate in vitamin B_{12} deficiency, interfere with its utilization. Cardinale et al. (1970) studied the activities of enzymes extracted from rat liver tissue, and found that adding ^{3}H-methylmalonyl-CoA to fatty acid synthesizing systems reduced the amounts of ^{14}C-acetyl-CoA and 1,3-^{14}C-malonyl-CoA included in fatty acids. Fatty acids derived from the labelled methylmalonyl-CoA were structurally unusual in that most were saturated and branched. They concluded that methylmalonyl-CoA was able to compete directly with malonyl-CoA for incorporation into fatty acids. Forward and Gompertz (1970) also studied these processes in rat liver and brain extracts, and found their results to be consistent with competitive inhibition of malonyl-CoA conversion. Methylmalonyl-CoA could not substitute for malonyl-CoA as a substrate for the microsomal FA elongation system, thus indicating a major difference between de novo FA synthesis and chain elongation. These authors further observed that, at least prior to its activation by citrate, acetyl-CoA

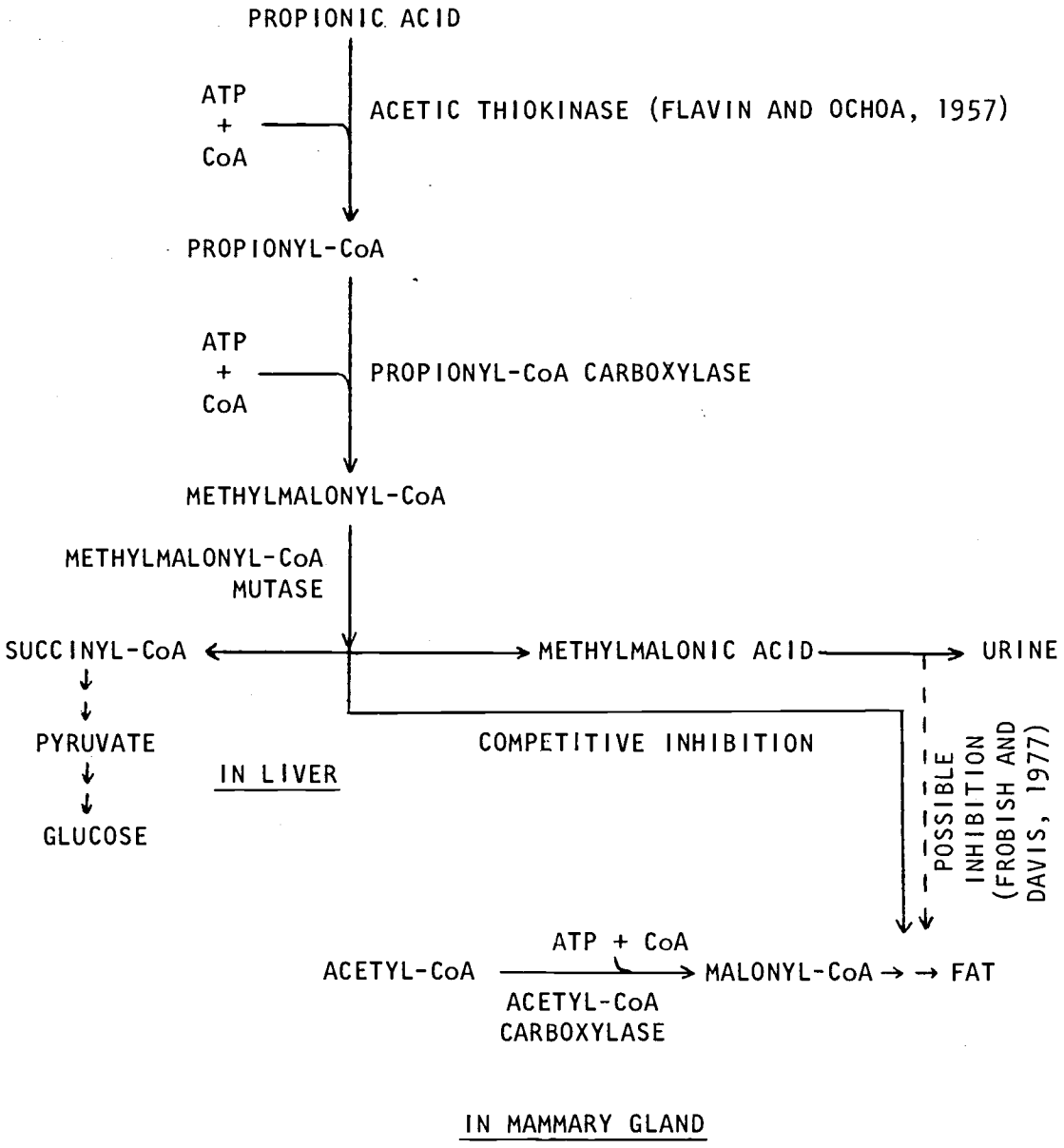


Figure 3. Propionic Acid Metabolism
(after Frobish and Davis, 1977).

carboxylase can be inhibited by methylmalonyl-CoA, a finding which also was made by Frenkel et al. (1973). Interestingly, these workers also found that propionyl-CoA could be a substrate for acetyl-CoA carboxylase, leading to the synthesis of methylmalonyl-CoA: thus, the inhibition of acetyl-CoA carboxylase mentioned above would be that exerted by an end product.

In addition to the competitive inhibition of malonyl-CoA conversion by methylmalonyl-CoA, Frobish and Davis (1977) have suggested that methylmalonic acid, a derivative of methylmalonyl-CoA, could have some inhibitory effect on fat synthesis. There is ample evidence that it does accumulate in B_{12} deficiency: indeed, elevated methylmalonate excretion is considered diagnostic of the condition (Williams et al., 1969; Gawthorne, 1968; Hogan et al., 1973; Tseng et al., 1976). Experimentally, increased methylmalonate excretion has been correlated with hepatic methylmalonyl-CoA activity and its reduction by over 50% in rats fed B_{12}-deficient diets (Reed and Tarver, 1970), and it has also arisen frequently as a consequence of propionate loading (Williams and Spray, 1972; Hogan et al., 1973). Whether or not it has any direct activity itself, however, seems to be unclear at this time.

By whatever metabolic means such an effect might be exerted, if vitamin B_{12} deficiency in dairy cows is an important factor leading to MF depression, two kinds of evidence would clearly be helpful in establishing that fact: 1) injections of some form of the vitamin should lead to the correction of derangements in propionate metabolism and thereby in milk fat synthesis. Several authors (Marston et al., 1972; Smith and Marston, 1971; Somers, 1969) have demonstrated the former,

while only Frobish and Davis (1977), using doses of up to 150 mg hydroxycobalamin/injection, have noted any appreciable response in MF% to B_{12} therapy, and 2) it should be possible to experimentally correlate cases of fat depression with reduced B_{12} content in either blood or liver tissue. Our research was directed at the latter prospect.

EXPERIMENTAL DESIGN

The design of this production trial was that of a latin square having four periods, four rations and eight replications. Thirty-two Holstein cows were selected from the Oregon State University dairy herd on the basis of two criteria: 1) all had an expected capability (based on records of previous lactations) of producing 70 lbs or more of actual milk per day at the peak of lactation, and 2) they had all been in milk for 136 days or less at the start of the trial. Animals were assigned to replications according to days in milk, and were distributed among groups by using a random numbers table. Average days in milk for groups 1 to 4 were 69.6, 69.9, 67.6 and 72.2, respectively, and most-probable producing ability (MPPA) values for 4% FCM in lbs $\pm$ herd average were 92.4, -408.0, -350.6 and 554.1 for groups 1 to 4, respectively. No first-calf heifers were used in the experiment. The four periods were planned to last approximately 28 days each, and the experiment was conducted between January 9 and April 28, 1978.

Rations were based upon four lots of alfalfa hay which had originated in the Klamath Basin of Oregon. The initial analysis of these hays is given in Table 2. Each lot of hay was chopped and stored in a stack under cover prior to being mixed into a complete feed with corn silage and concentrates and fed. Grab samples were taken from each stack twice weekly as it was fed, and a composite analysis of these samples from each lot is shown in Table 3.

Rations were formulated to be isocaloric and to provide a minimum nitrogen content with the least amount of variation possible. On a DM basis they consisted of about 30% hay, 20% corn silage and 50%

Table 2. Original analyses of hay lots (DM basis)

Lot ID #	Hay #	CP %	ADF %	Ca %	P %	Class	RFV*
66187	1	15.8	35.1	.82	.30	#3 low	109
66188	2	15.2	32.1	.82	.28	#3 high	121
66184	3	22.0	29.9	.80	.27	#2 low	127
66152	4	19.8	33.7	.93	.39	#2 high	132

*RFV - Relative Feed Value - Rohweder et al. (1978).
RFV = (estimated digestible dry matter ÷ 40) X 100.

Table 3. Composite analyses of hay lots (DM basis)

Lot ID #	Hay #	CP %	ADF%	Ca %	P %	NDF %
66187	1	15.4	40.3	.90	.32	48.0
66188	2	14.1	35.7	.91	.34	47.1
66184	3	19.0	36.4	.82	.34	42.2
66152	4	18.1	35.6	.88	.38	39.8

concentrate. Quantities of each of these materials to be fed were adjusted daily in an effort to offer enough feed to exceed consumption slightly. Table 4 shows the calculated compositions of the four rations as well as mean values from laboratory analyses of samples of the complete rations taken periodically throughout the trial.

Rations were fed once daily before 8:30 am. Record was kept of feed offered, and that rejected was collected and weighed each week so that the dry matter intake for each group could be calculated.

Animals were housed under cover in one aisle of a free-stall barn bedded with wood shavings. The loafing area was cleaned regularly with a water flush system. Cows were milked twice daily in the treatment groups of eight animals. Milk yields were recorded for each milking using calibrated DeLaval receiving jars. Milk samples were collected every week from two consecutive milkings and the composite from each animal was used to determine percent milk fat.

Fat percentage was analyzed using the Banco Fat Determination Method[1]. Average daily actual milk, 4% FCM and average weekly milk fat percentage were calculated for each cow during each period. Average persistency during each period was also calculated for all cows by dividing the actual average daily milk yield of a given week by that of the week before.

Cows were weighed at the beginning of the trial and thereafter at the end of each period. An average was taken from measurements made on two consecutive days, and average daily body weight changes were calculated for every animal during each period.

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[1]Banco Laboratory Chemicals-Anderson Laboratories, Inc., Fort Worth, TX.

Table 4. Calculated* or proximate composition of experimental rations

Composition in kg of dry matter	High CP hay rations (3 and 4)		Low CP hay rations (1 and 2)	
Feedstuff	Kg DMI/day	Kg DM/100 kg	Kg DMI/day	Kg DM/100 kg
Alfalfa hay**	5.40	29.59	5.40	29.71
Corn silage	3.60	19.73	3.60	19.81
Barley, Pacific	3.97	21.76	3.67	20.19
Wheat, Pacific	3.97	21.76	3.67	20.19
Cottonseed meal	1.01	5.54	1.50	8.25
Urea	0.05	0.27	0.10	0.55
Iodized salt	0.09	0.49	0.09	0.50
Dicalcium phosphate	0.044	0.24	0.017	0.09
Limestone	0.113	0.62	0.129	0.71

Proximate analyses, DM basis:

Rations	% DM	% CP	% ADF	% Ca	% P
1	55.5	15.80	27.10	0.53	0.38
2	52.7	15.18	24.60	0.48	0.38
3	56.1	16.07	22.80	0.50	0.39
4	52.4	15.50	23.60	0.53	0.40
Corn Silage	26.3	9.10	23.60	0.04	0.26

*Church (1977) - Rations formulated to meet requirements of 1350 lb (614 kg) cow producing 70 lbs (32 kg) of 3.5% MF milk daily. Intake Estimated at 3% of body weight daily (dry basis) or 18 kg DM/day.

**Alfalfa hays used in rations 1 to 4 were those given in Tables 2 and 3 as hays 1 to 4, respectively.

Four animals from each of the four groups were chosen, largely on the basis of temperment, to provide samples of rumen fluid and blood. These samples were collected, always following the afternoon milking, during the final week of each period, the former for VFA and the latter for serum vitamin B_{12} analysis.

Approximately 200-500 ml of rumen fluid was collected from each animal by applying vacuum to a 5/8 inch diameter high pressure hose guided through a speculum into the rumen. After straining through cheesecloth, 5 ml of the fluid was added to 1 ml of 25% metaphosphoric acid in a polyethylene centrifuge tube and stored in a refrigerator. Within 48 hours they were spun in a high-speed centrifuge at 12,000 rpm for 30 minutes, and the supernatant was decanted into a small glass vial, capped and frozen until all samples had been collected. VFA analyses were made using a Varian Aerograph Series 1200 gas chromatograph with a flame ionization detector. The method of Carlsson (1973) was adapted to allow the use of a glass column packed with Chromosorb 101[2]. Peak areas were automatically computed by a Spectra-Physics Minigrator™, and concentrations of acetate and propionate were calculated in μmoles/ml. From these data, A:P ratios were obtained for each cow in each period.

Approximately 10 ml of blood was collected by tail puncture, allowed to coagulate, and at least 3 ml of serum was drawn by pipette into a glass vial. Samples were frozen and kept in storage until time permitted analysis.

It was initially planned to use the Ochromonas malhamensis method

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[2]Johns-Mansville, Inc., Denver, CO.

for analysis of vitamin B_{12}. However, the standard medium used for this assay (Difco Ochromonas Medium--0665-15[3]) was found to be unavailable, and the organism could not be successfully maintained on media prepared in our laboratory. For this reason, and because many studies of B_{12} levels in ruminants reported in the literature involved its use, the Lactobacillus leichmannii (A.T.C.C. 4797[4]) assay was selected. The procedure described by Matthews (1962) for serum vitamin B_{12} analysis was used. Preliminary experiments on samples taken from milking cows not involved in the trial indicated that 4 ml of serum extract, 1 ml water and 5 ml of assay medium (Difco Lactobacillus Medium--0360-15[5]) led to growth in culture tubes well within the range covered by standards: the latter contained from 50 to 500 µµg cyanocobalamin/tube. The turbidometric analysis was carried out using a Bausch and Lomb Spectronic 20 at a wavelength of 700 nm, following a 24 hour incubation period at 37°C in a water bath. Ten samples were assayed in duplicate in each experimental series, with each tube being read twice. The resulting four values were used to compute an average for each sample, and these data were plotted on a standard curve from which concentrations could be read in µµg/tube. The standard dilution factor was then used to calculate the B_{12} content of serum in ng/ml.

A digestibility evaluation was also made of the four hays used in the production trial (Tables 3 and 4). This experiment involved

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[3]Difco Laboratories, Detroit, MI.

[4]American Type Culture Collection, Rockville, MD.

[5]Difco Laboratories, Detroit, MI.

four Holstein steers fed hay only, and was also designed as a latin square having four rations and four periods. Steers were considered appropriate for use in this trial, since their digestive physiology is not known to differ appreciably from that of a lactating cow.

The steers were housed under cover in large pens bedded with wood shavings. They were fed three times daily throughout the trial, and at feeding time they were given ample access to water.

At the beginning of each period animals were fed their respective hays in excess of intake for a minimum of 5 days. During this preliminary phase, refused feed was collected and weighed each morning so that the maximum voluntary intake by each animal could be determined. Feed offered during the subsequent collection period was adjusted to 85% of this maximum intake.

For 5 days during each period, animals were fitted with bags for fecal collections. In the mornings, the bags were removed, cleaned, and 100 g samples of feces were collected and placed in large, sealed plastic jars containing 100 g of 5% HCl. Samples from each animal in a given period were composited, so at the end of each collection phase jars contained 500 g of feces. These composite samples were then air-dried, ground and oven-dried to 100% DM for analysis of % digestible CP, % digestible ADF, and % digestible DM.

RESULTS AND DISCUSSION

Digestibility Trial

Results from the digestibility study are given in Table 5. It will be noted that in none of the four parameters examined were there significant effects due to treatment. Differences for digestible crude protein approached the 5% level, however ($p<.058$), and reference to Tables 2 and 5 shows that the digestibility of this fraction was somewhat higher for hays 3 and 4, which were originally determined to have substantially higher CP content than hays 1 and 2. Visual inspection corroborated this finding, in that hays 3 and 4 were distinctly superior in leafiness and color. It is likely that the frequently observed (Church, 1977) relationship between CP content and DCP explains the differences which were suggested.

As Table 2 illustrates, the hays used in this experiment varied in class from #3 low to #2 high and RFV from 109 to 137. Given a lack of differences in digestibility due to rations, however, it might be anticipated that production effects in milking cows would be minimal, especially where rations were formulated to differ only in the hay component.

Production Trial

Physiological and production responses of the cows to treatments are given in Table 6. Of the nine parameters examined, only ruminal propionate concentration and acetate: propionate ratio showed significant treatment effects. Significant differences attributable to sources of variation other than treatments may often be understood as

Table 5. Digestibility trial - measurements of steers fed four alfalfa hays

Measurement means	Hay 1	2	3	4	$S_{\bar{x}}$	P
Feed intake, lbs DM/100 lbs BW	2.10	2.27	2.05	2.20	.083	.32
Digestible DM, %	62.7	67.6	68.2	67.4	1.779	.20
Digestible ADF, %	47.8	52.6	50.3	56.2	3.046	.35
Digestible CP, %	71.8	76.5	79.3	78.9	1.64	.058

Table 6. Production trial - measurements of cows fed rations based on four alfalfa hays

Measurement means	No. of cows/ group	Ration 1	2	3	4	$S_{\bar{x}}$	P
Feed intake, lbs DM/100 lbs BW (group average)		2.73	2.97	3.13	2.64	.228	.4764
Body wt change, lbs/day	8	1.55	1.27	1.18	1.50	.3269	.4764
Milk yield, lbs 4% FCM	8	48.88	48.02	48.38	48.24	.7001	.10
Milk fat, %	8	3.13	3.12	3.05	3.01	.0616	.303
Persistency, %	8	99.13	97.98	97.70	98.78	.5009	.1624
Rumen acetate conc., µmoles/ml	4	46.11	46.08	43.84	48.50	1.6574	.2826
Rumen propionate conc., µmoles/ml	4	18.14[a]	17.56[a]	19.44[a]	23.03[b]	1.0476	.0028**
Acetate:propionate ratio	4	2.82[b]	3.00[b]	2.36[a]	2.23[a]	.1356	<.001 **
Serum B_{12} conc., ng/ml	4	6.75	6.91	6.40	5.31	.8069	.5020

[a]Means with different superscripts are different ($p<.05$).

**Means significantly different at 1% level.

typical of the variability one would expect among cows in different stages of lactation (i.e., squares and period effects), as well as that between individual animals (i.e., within squares effects).

Feed Intake

Differences in DM intake, expressed in Table 5 as a percent of body weight, were not significant. Overall, intakes were slightly lower than our original estimates of 3% BW, although all rations were readily consumed and no unexpected problems with refusal or digestive upsets were seen. The 26.3% DM of the corn silage used was slightly lower than published values (Church, 1977), and this may have tended to reduce ration intakes somewhat.

Body Weight Changes

Differences in body weight changes were significant only for periods, where mean changes in lbs/day for periods 1 through 4 were 1.75, 2.15, 1.47 and .415, respectively. No satisfactory explanation for these results is apparent, particularly in view of the fact that cows may lose weight for the first 5 or 6 months of lactation (Schmidt, 1971). Gains are typically greatest not long before the dry period begins. Differences due to stage of lactation would be expected to appear under squares as a source of variation, in any case. Jorgensen et al. (1965) noted that increased weight gains accompany MF depression in HG diet feeding. As will be discussed below, however, there was no indication that MF was reduced by any of the rations.

4% FCM/Day

Highly significant differences for FCM production could be attributed to all sources of variation except treatments, indicating that the four rations used in this trial did not differ appreciably in their ability to support milk production. Rations were formulated to be approximately equivalent in both nitrogen and energy content, and the absence of treatment effects shows that these fractions did not differ in availability among the four alfalfa hays. This conclusion was anticipated in the results of the digestibility study.

Differences seen among cows in squares reflects differences in production capabilities among cows, and those observed for squares and periods again indicate stage of lactation effects. Means for periods 1 through 4 were 58.19, 50.18, 43.7 and 44.19 lbs/day, respectively, a pattern which conforms clearly to be a typical lactation curve (Schmidt, 1971).

Milk Fat Percentage

Once again, highly significant differences were observed for all sources of variation except treatments. Although the MF% was lower on the two rations having the lowest ADF values, these differences were not significant. It is evident that none of the four hays had the effect of depressing MF% when included in our experimental rations, which can be considered typical of those used by dairymen in the Pacific Northwest. This conclusion can be extended to situations in which similar hays are chopped and mixed for feeding with concentrates and corn silage, but presumably not beyond. That is, results might

well differ if the hay is pelleted or cubed, even if it is included at 30% of ration DM and mixed with silage and grain. Similarly, results could differ for cases where rations formulated to be identical to those used in this study were offered in a self-fed system where ration ingredients are offered separately.

The normal trend for dairy cows is for MF% to increase gradually during the course of a lactation as milk yield declines (Schmidt, 1971). Although differences in both squares and periods were highly significant, this pattern did not emerge in this study. For example, period means were 3.20, 3.21, 2.77 and 3.14% for periods 1 to 4, respectively. It may be that the cows put on the experiment very early in lactation kept such a gradual increase in fat percentage with time from becoming apparent, or it may have been included as part of the cows in squares source of variance.

Persistency

No significant differences in persistency were observed in this experiment, and the means given in Table 6 are not remarkable in any way.

Rumen Acetate Concentration

The only differences seen here were in cows in squares ($p<.003$). Again, this is an indication of individual variation among cows, or perhaps differences in water intake during the hours prior to sampling. The latter could also be a factor in other aspects of rumen fermentation discussed below.

Shifts in the pattern of rumen fermentation characteristic of

MF depression were discussed in the Literature Review. There was no sign of reduced milk fat in our work, but if there had been it is questionable whether any substantial change would have been noted in ruminal acetate concentration. Bickerstaffe et al. (1971) and Latham et al. (1974) did see such a change in response to feeding MF%-depressing diets, for example, but Davis (1967) and Bauman et al. (1971) did not. The literature as a whole suggests that changes in propionate concentration in the rumen, and thereby the acetate:propionate ratio, are of over-riding importance.

Rumen Propionate Concentration

Highly significant differences were found in propionate content for all sources of variation. Cows in squares includes random individual differences, but the difference in periods ($p=.003$) cannot be explained on the basis of any apparent factor: period means were 18.31, 20.97, 22.12 and 16.77 moles/ml for periods 1 through 4, respectively.

Means for rations were also different, and are shown in Table 6 with an L.S.D. analysis. Substantial increases in ruminal propionate production and concentration have been described in the literature as the invariable consequence of feeding restricted roughage rations, one which is often followed by MF% reductions. Bickerstaffe et al. (1971) found that rumen propionate content was increased by nearly 20% with LR feeding. Storry and Sutton (1969) also reported higher propionate following the feeding of HG diets, and Latham et al. (1974) found that cows showing fat depression had elevated ruminal propionate

content. Bauman et al. (1971) observed that propionate production and pool size in the rumen both had more than doubled in cows on RR rations vs. normal rations. The rations used in this experiment cannot be described as having restricted proportions of roughage. Nevertheless, it is to be expected that in cases of marginal (statistically insignificant) MF% depression arising from ration effects, significant differences in propionate production should appear which, if intensified, would lead to significant fat depression.

These relationships are discussed further in the next section.

Acetate:Propionate Ratio

Differences in the ratio of rumen acetate to propionate were highly significant for all sources of variation. Since results for this parameter were calculated from those obtained for ruminal acetate and propionate contents, it embodies differences in both and is a more complete biological description of possible changes in fermentation patterns. It has also received more frequent consideration in the literature of fat depression than the relative concentrations of individual fatty acids.

Comments made above concerning sources of variation other than treatments apply here. In particular, it is not clear why there are significant variations among periods. However, with regard to treatment effects, comparison of Tables 4 and 6 shows that the two rations with the lowest %ADF values (rations 3 and 4) also had significantly lower mean A:P ratios. Table 3 indicates that these two rations also included hays having the lowest % NDF. An analysis was made in which

A:P ratio data were regressed against several Feed characteristics, and the results are given in Table 7. It can be seen that while the correlation coefficients for these comparisons were quite low, that for hay % NDF and A:P was significant (p=.012). Unfortunately, values for complete ration % NDF are not available.

On the basis of present evidence, it appears that lower ration or hay fiber levels may have been associated with reduced A:P ratios for rations 3 and 4. Although this shift in rumen fermentation is of the type generally observed to accompany MF% depression, it apparently was not sufficiently great to influence significantly the process of butterfat synthesis.

Serum Vitamin B_{12} Concentrations

The content of Vitamin B_{12} in the serum did not respond significantly to treatments. The only difference (p .001) was with periods, one which has caused us considerable anxiety. Wilson et al. (1967) and Elliot et al. (1965) both carried out studies on lactational trends in the B_{12} status of dairy cows, and reported that liver and blood B_{12} levels were lower earlier in lactation than later, and the latter authors suggested that the vitamin could be depleted by heavy periods of production. Our data do not point to such a trend, given means of 5.50, 14.07, 3.22 and 3.03 ng/ml for periods 1 through 4, respectively. The inordinately high values for period 2 are presumed to be an artifact, although similar procedures were maintained throughout so far as was possible. Indeed, assays for 10 samples from period 2 were repeated and gave much the same results. Thus, no justification for

excluding these values from the statistical analysis could be found. In the literature, only the results of Tseng et al. (1973) from hamster serum are of this magnitude (see Table 1).

If our elevated values from period 2 may be set aside for purposes of discussion, our results are comparable to those of other authors studying ruminants. Remembering that results of B_{12} analyses vary somewhatwith the assay used, Table 1 shows that Corse and Elliot (1970) and Elliot et al. (1965) with cattle, and Hogan et al. (1973) in lambs found levels ranging from .25 to .63 ng/ml in animals which were either supplemented with the vitamin or normal. On the other hand, authors including Smith et al. (1969), Sutton and Elliot (1972) and Hedrich et al. (1973) reported values of 4.4 to 7.2 ng/ml in B_{12}-adequate sheep. It is difficult to determine how much of this variation is actual versus that which might arise from differences of method. The critical level in the blood below which the vitamin is clearly deficient is not well defined, although it appears to fall between .1 and .5 ng/ml in ruminants (Hogan et al., 1973; Smith et al., 1969; Somers, 1969). It is evident that animals in this experiment were not under the stress of vitamin B_{12} deficiency.

The partitioning of vitamin B_{12} among various tissues was discussed in the Literature Review section. It was suggested that stores of the vitamin in tissues other than the liver (and possible the kidney in some species) could act as labile reserves tending to minimize short-term depletion at the primary sites of utilization. Results of our work point to the possible occurence of such interactions. In Table 6 it can be seen that while ration effects for serum B_{12} content were not significant, the mean level for ration 4 was substantially lower than

the others. Recalling that rumen propionate content was significantly increased in response to the feeding of ration 4, reduced serum B_{12} levels might be expected to follow transient liver B_{12} depletion caused by an elevated rate of propionate metabolism. However, again noting that the A:P ratio is the most complete indication of rumen fermentation patterns, Table 7 shows that the correlation between A:P and serum B_{12} was very low and not significant. Interestingly, correlations given in Table 7 for both ration and hay % ADF and serum B_{12} were significant at the 5% level. Since such a relationship can only be understood as involving rumen fermentation as an intermediary, the absence of a significant correlation between A:P and serum B_{12} becomes difficult to rationalize, although the small number of observations may be a factor.

The hypothesis concerning MF depression proposed by Frobish and Davis (1977) rests on the notion that elevated production of pripionate in the rumen in response to HF feeding leads to levels in the liver which exceed that organ's capacity for its utilization. The primary limitation upon the rate at which the process occurs is presumed to be the availability of coenzyme-B_{12} (see Figure 2), which in turn depends on ruminal synthesis and intestinal absorption of the vitamin itself. Our results indicate that A:P ratios above those usually associated with fat depression do not supply propionate in levels beyond those which can be readily metabolized. Thus, they are consistent with the hypothesis of Frobish and Davis (1977).

Table 7. Coreelations among several ration components and rumen A:P ratio and serum B_{12} content; n=50

Variables	Correlation (r=)	t	p
Hay NDF and hay ADF	.632	5.65	<.001**
Hay NDF and ration ADF	.800	9.26	<.001**
Hay NDF and A:P	.347	2.56	.012*
Hay NDF and serum B_{12}	.354	9.26	.641
Hay ADF and ration ADF	.867	12.07	<.001**
Hay ADF and A:P	11.03	.767	.447
Hay ADF and serum B_{12}	.312	2.28	.027*
Ration ADF and A:P	.246	1.76	.0851
Ration ADF and serum B_{12}	.354	2.62	.012*
A:P and serum B_{12}	.192	1.357	.182

* Significant at 5% level.

**Significant at 1% level.

CONCLUSIONS

This study has shown that alfalfa hays varying in % ADF within the range of the four lots used (29.9 to 35.1) will not cause depression of milk fat when they are fed to cows, in chopped form, as 30% of the ration dry matter. Also, they will not cause differences in other animal performance measurements, such as DM intake, body weight changes, 4%-FCM, MF% and persistency.

However, changes in rumen fermentation were observed which, if exacerbated by increasing the percentage of hay in the rations, could lead to MF% reductions. In this experiment, these changes have been related most successfully to the % NDF in the hay, although other indices of the fiber fraction of the diet may be equally suggestive in general practice.

No evidence has been obtained in this experiment that would tend to disprove the hypothesis that vitamin B_{12} is involved in the milk fat depression syndrome. Equivocal indications are that serum vitamin B_{12} levels respond to dietary fiber levels, presumably through changes in the levels of propionate made available by the rumen. A conclusive description of these relationships in dairy cattle remains to be made.

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APPENDICES

Table 1. Digestibility trial - analysis of variance table for DM intake

ANOVA

Sources	SS	df	MS	F	P
Steers	1.23212	3	.4107	14.902	.0034*
Hays	.11932	3	.03977	1.443	.32
Periods	62522	3	.2084	10.305	.0088*
Error	.16533	6	.02756		
Total	2.14199	15			

* Significant at 5% level.

Table 2. Digestibility trial - analysis of variance table for percent digestible dry matter

ANOVA

Sources	SS	df	MS	F	P
Steers	85.9450	3	28.6483	2.262	.18
Hays	77.7375	3	25.9125	2.046	.20
Periods	294.3375	3	09.1125	7.748	.017*
Error	75.9794	6	12.6632		
Total	533.9994	15			

*Significant at 5% level.

Table 3. Digestibility trial - analysis of variance table for Percent digestible crude protein

ANOVA Sources	SS	df	MS	F	P
Steers	163.4825	3	59.994	5.058	.044*
Hays	142.4875	3	47.496	4.408	.058
Periods	118.7325	3	39.578	3.673	.082
Errors	64.6469	6	10.774		
Total	489.3494	15			

* Significant at 5% level.

Table 4. Digestibility trial - analysis of variance table for percent digestible ADF

ANOVA Sources	SS	df	MS	F	P
Steers	79.2525	3	26.418	.712	.58
Hays	149.3925	3	49.798	1.342	.35
Periods	343.0525	3	114.351	3.081	.11
Errors	222.7069	6	37.118		
Total	794.4044	15			

Table 5. Production trial - analysis of variance table for DM intake

ANOVA Sources	SS	df	MS	F	P
Rations	.5874	3	.1958	.9436	.4764
Periods	2.7902	3	.9301	4.4824	.0563
Groups	.4644	3	.1548	.7460	.5629
Error	1.2452	6	.2075		
Total	5.0872	15			

Table 6. Production trial - analysis of variance table for body weight changes

ANOVA Sources	SS	df	MS	F	P
Squares	33.338	7	4.762	1.3929	.218
Cows in squares	52.7273	24	2.1970	.6426	.891
Periods	44.3870	3	14.7957	4.3277	.007**
Rations	3.0204	3	1.0068	.2945	.829
Error	307.6946	90	3.4188		
Total	441.1673	127			

** Significant at 1% level.

Table 7. Production trial - analysis of variance table for 4% FCM/day

ANOVA

Source	SS	df	MS	F	P
Squares	7,849.4934	7	1121.3562	71.499	<.001**
Cows in squares	11,086.5238	24	461.9385	29.4563	<.001**
Periods	3,445.6381	3	1148.5460	73.2323	<.001**
Rations	12.7378	3	4.2459	.2707	.10
Error	1,411.5269	90	15.6836		
Total	23,793.1822	127			

** Significant at 1% level.

Table 8. Production trial - analysis of variance table for milk fat production

ANOVA Sources	SS	df	MS	F	P
Squares	8.0560	7	1.1509	9.4802	<.001**
Cows in squares	29.6232	24	1.2343	10.1672	<.001**
Periods	4.3026	3	1.4342	11.8138	<.001**
Rations	.4488	3	.1496	1.2323	.303
Error	10.9297	90	.1214		
Total	53.3603	127			

** Significant at 1% level.

Table 9. Production trial - analysis of variance table for persistency

ANOVA Sources	SS	df	MS	F	P
Squares	34.8591	7	4.9799	.6202	.7780
Cows in squares	170.3044	24	7.0960	.8837	.6222
Periods	12.7582	3	4.2527	.5296	.6631
Rations	42.1663	3	14.0554	1.7503	.1624
Error	722.7075	90	8.0301		
Total	982.7955	127			

Table 10. Production trial - analysis of variance table for acetate concentrations

ANOVA Sources	SS	df	MS	F	P
Squares	235.53535	3	78.5118	1.7863	.1645
Cows in squares	1,650.0263	12	137.5022	2.1284	.003**
Periods	186.37865	3	62.1262	1.4138	.2522
Rations	173.20936	3	57.7365	1.3136	.2826
Error	1,846.0249	42	43.9530		
Total	4,091.1746	63			

** Significant at 1% level.

Table 11. Production trial - analysis of variance table for propionate concentrations

ANOVA Sources	SS	df	MS	F	P
Squares	326.1418	3	108.7139	.0014**	
Cows in squares	736.1022	12	61.3419	3.4937	.0013**
Periods	286.4873	3	95.4958	5.4389	.003**
Rations	289.4316	3	96.4772	5.4948	.0028**
Error	737.4239	42	17.558		
Total	2,375.58929	63			

Treatment	Mean
Ration 1	18.14[a]
Ration 2	17.56[a]
Ration 3	19.44[a]
Ration 4	23.03[b]

$S_{\bar{x}} = 1.0476$

$L.S.D._{.05} = 2.994$

[a]Means with different superscripts are different.

**Significant at 1% level.

Table 12. Production trial - analysis of variance table for acetate:propionate ratio

ANOVA Sources	SS	df	MS	F	P
Squares	9.7742	3	3.2581	11.0782	<.001**
Cows in squares	11.9461	12	.9955	3.3849	.002**
Periods	5.0408	3	1.6803	5.7134	.002**
Rations	6.4895	3	2.1632	7.3553	<.001**
Error	12.3541	42	.2941		
Total	45.6047	63			

Treatment	Mean
Ration 1	2.82[b]
Ration 2	3.00[b]
Ration 3	2.36[a]
Ration 4	2.23[a]

$S_{\bar{x}} = .1356$

$L.S.D._{.05} = .3875$

[b]Means with different superscripts are different.

**Significant at 1% level.

Table 13. Production trial - analysis of variance table for percent serum B_{12} concentration

ANOVA

Sources	SS	df	MS	F	P
Squares	42.575	3	14.1917	1.3622	.2674
Cows in squares	59.6857	12	4.9738	.4774	.9167
Periods	1,315.7492	3	438.5831	42.0986	<.001**
Rations	24.9370	3	8.3123	.7978	.5020
Error	437.5542	42	10.4180		
Total	1,880.5011	63			

**Significant at 1% level.