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Luis Carlos Solorzano

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Roy S. Emery
Major professor

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MAGNESIUM METABOLISM IN THE LACTATING HOLSTEIN COW

BY

LUIS CARLOS SOLORZANO

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ABSTRACT

MAGNESIUM METABOLISM IN THE LACTATING HOLSTEIN COW

By

Luis Carlos Solórzano

Three experiments were conducted to investigate different aspects of Mg metabolism. Experiment 1 determined the optimal dietary allowance of Mg during the first 84 days in milk (DIM). Twenty seven cows were fed either 0.25, 0.35, or 0.45% Mg (DM basis) in a 51% alfalfa haylage diet. There were no differences among treatments for DM intake (DMI, 21.7 kg/d), 3.5% fat corrected milk (FCM, 45.6 kg/d), milk fat % (4.01), or plasma Mg (2.33 mg/dl).

Two trials conducted for Experiment 2 determined the effects of intravenous infusion (IV) of Mg on lactational performance in cows fed a milk fat depressing diet (0.6 concentrate:0.4 corn silage) adequate in Mg content. In Trial 1, six cows (160 DIM) were IV infused isotonic solutions providing 0 or 12 g Mg/d, applied in a single reversal design. In Trial 2, twelve cows (34 DIM) were intravenously infused isochloric solutions providing 0, 6, or 12 g Mg/d, applied in a 2 X 3 cross-over design. In Trial 1, infusing Mg increased plasma Mg levels from 1.9 to 3.11 mg/dl, but resulted in no differences in DMI (22.9 kg/d), 3.5% FCM (26.7 kg/d), milk fat % (2.87), plasma triglycerides (TG, 8.2 mg/dl), and nonesterified fatty acids (NEFA, 206.2 μEq/l). In Trial 2,

infusing increasing levels of Mg resulted in serum Mg increasing from 2.84 to 2.91 and 3.11 mg/dl. There were no treatment differences in DMI (22 kg/d), 3.5% FCM (42.2 kg/d), milk fat % (4.37), serum TG (8.7 mg/dl), somatotropin (ST, 2.87 ng/ml) or insulin (INS, 0.47 ng/ml). There was a significant Treatment X Time interaction for serum ST. Cows infused 12 g Mg/d had a more constant ST concentration. Although blood Mg levels were increased within physiological range, it failed to improve the lactational performance.

Experiment 3 determined the effect of different levels of serum Mg on serum NEFA and Mg, the concentration of Mg in perirectal subcutaneous adipose tissue (SAT), and the activities of fatty acid mobilizing lipase (FAML), glyceride synthetase (GS), and lipoprotein lipase (LPL) of SAT when lipolysis was stimulated with 6 mg epinephrine (EPI) IV in early lactation cows fed a high-grain diet. Animal handling and treatments were as described for Trial 2, Experiment 2. Blood was sampled -10 to 60 min and a sample of SAT was obtained within 70 min of the EPI challenge. Increasing levels of serum Mg tended to increase serum NEFA response to the EPI challenge. Serum Mg decreased (up to 0.19 mg/dl) in response to the EPI challenge. The activities of FAML, GS, and LPL were not affected by treatment.

The results of experiments 2 and 3 suggest that Mg effect on milk fat is not post-ruminal.

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CHAPTER I

REVIEW OF LITERATURE

REVIEW OF LITERATURE

A) INTRODUCTION

Magnesium composes about 2.5% of Earth's crust. It is found as carbonate, dolomite, oxide, and chloride. It also occurs in silicate minerals. Sea water contains 1.27 g/kg of Mg.

Magnesium is one of the major minerals recognized to be essential for farm animals. The animal body contains about .05% Mg by weight, distributed among the skeleton, soft tissues, and extracellular fluids. Magnesium is an important cofactor of many enzymes. The two main areas of Mg activity are: 1) enzyme systems involved in carbohydrate metabolism and energy production, and 2) enzyme cofactor in the production and destruction of acetylcholine at the neuro-muscular junction (Wilson, 1981). Required by all farm animals, it is especially critical for ruminants. Magnesium deficiency occurs in calves, lambs, and in adult cattle. The dietary concentration of Mg required to maximize milk production in lactating dairy cattle is not well defined since Mg recommendations (NRC, 1989) are based on preventing "Hypomagnesemia". Recent evidence indicates that .2 to .25% Mg is too low and that the requirement may be closer to .48% of diet DM (O"Connor et al., 1988).

Magnesium digestibility is greater in dry cows than in lactating animals (Teller and Godeau, 1987). Higher crude

protein (CP) levels in the diet decrease Mg absorption in the stomachs of ruminant animals (Teller and Godeau, 1987). High intakes of K decrease Mg absorption from the rumen (Greene et al., 1983a,b). The inhibitory action of K appears to be dependent upon the Na:K ratio rather than the K concentration per se (Martens and Rayssiguier, 1980).

Magnesium absorption across the rumen wall is an active process linked to Na transport (Martens, 1985). It was demonstrated in vitro that Mg transport is reduced by ouabain, an inhibitor of the Na/K ATPase, and that there is a positive correlation between Na concentration in the buffer and the amount of Mg transported (Martens and Rayssiguier, 1980). Net Mg absorption is located in the digestive tract anterior to the duodenum (Greene et al., 1983a), and Mg absorption at the small intestine appears to be minor (Martens and Rayssiguier, 1980). There is uncertainty as to which part of the forestomach is the dominant site of Mg absorption (Martens and Rayssiguier, 1980).

Magnesium is excreted via the urine, and there is an endogenous fecal excretion in ruminants. Total fecal Mg includes dietary and endogenous Mg (Fontenot, 1980). Apparent Mg absorption increases with Mg intake (Fontenot, 1980). Urine Mg represents the excess of absorption over the body's requirements (Wilson, 1981). Urinary excretion involves a filtration-reabsorption mechanism in which Mg behaves as a threshold substance, appearing in urine when the Mg load

filtered by the kidney glomeruli exceeds that reabsorbed by the tubules (Wilson, 1981). Direct measurements indicate that the threshold Mg concentration in plasma of cattle is about .74 mM, and above this, urinary excretion and plasma Mg are linearly correlated (Wilson, 1981). Magnesium losses via milk are considerable. Colostrum contains .4 g/kg of Mg and milk contains .13 g/kg of Mg (Wilson, 1981). Based on current NRC nutrient requirements and an absorption of Mg of 30%, a cow producing 50 kg/d of milk secretes in milk about 30 to 40% of the Mg absorbed daily from the diet.

Most studies in the United States evaluating the effects of MgO on lactational performance were addressed to test the effects of MgO supplementation above NRC (1989) recommendations on cattle fed high-grain, low-forage, milk-fat depressing diets, and did not address the determination of optimal dietary allowances of Mg. It was suggested that oral MgO increases the uptake of endogenous acetate and triglycerides (TG) from the blood plasma into mammary gland tissues (Emery et al., 1965). Also, the ruminal acetate:propionate ratio increases with oral MgO supplementation (Thomas et al., 1984), although this is not always the case (Erdman et al., 1982). It was proposed that MgO could have alkalizing effects in the small intestine, thus potentially enhancing starch digestion (Beede and O'Connor, 1986).

Magnesium supplementation to corn silage based diets

improves lactational performance (Beede and O'Connor, 1986, Teh et al., 1985). Based on this, Beede and O'Connor (1986) concluded that .3 to .45% Mg in the diet DM is needed to maximize lactational performance. These authors also warned about the variable effects of Mg supplementation due to variability in availability caused by differences in particle size, chemical properties, and treatment of the Mg supplement.

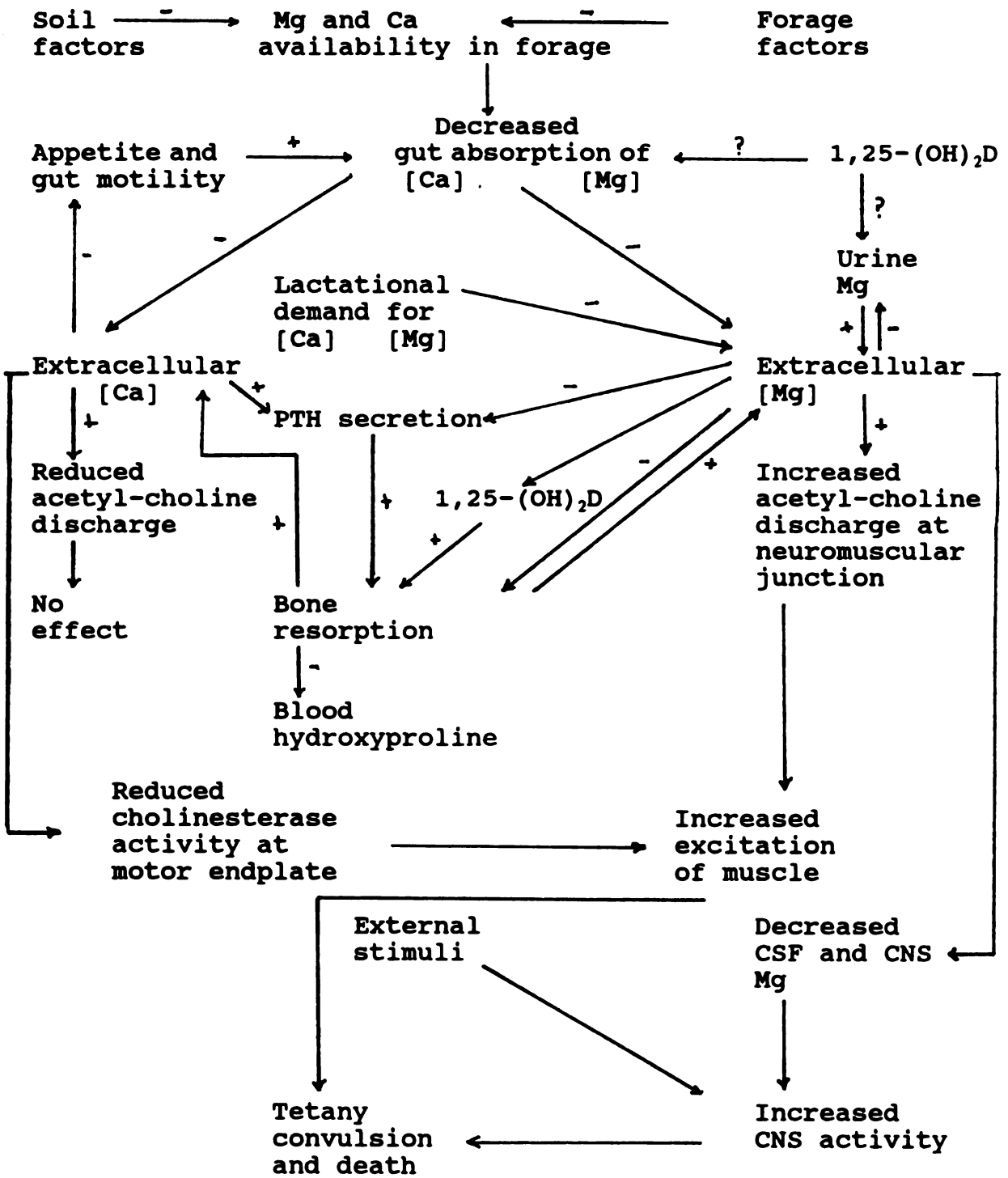
B) MAGNESIUM METABOLISM

B.1) HYPOMAGNESEMIA

Hypomagnesemic tetany of ruminants is a non-infectious metabolic disorder that occurs in a wide range of nutritional and management conditions (Harris et al., 1983). Morbidity is usually low, but can exceed 25% under certain environmental conditions (Harris et al., 1983), and is related to the breed of the animal (Smith and Edwards, 1988, Harris et al., 1983).

Although a similar clinical picture is shown in most cases of hypomagnesemia (HM), the pathogenesis may be affected by nutritional, environmental, animal factors and their interactions. Because of all the factors affecting HM, it is not surprising that Littledike et al. (1983) discuss more than six "types" of grass tetany. However, a marked decrease in serum Mg concentration seems to be the primary predisposing factor to all types of grass tetany (Harris et al., 1983). A summary of the pathophysiology of the clinical signs of HM is presented in Figure 1.

Figure 1. Proposed pathogenesis of the hypomagnesemic syndrome in cows. (Adapted from Littledike et al., 1983).



Increased animal production resulted in a higher incidence of HM. However, it is known that HM is not attributable to absolute Mg depletion, since Mg intake by hypomagnesemic animals is often sufficient to meet their daily requirements (Wilson, 1981). Harris et al. (1983) studied the factors predisposing dairy cows to grass tetany. The higher incidence of grass tetany was just prior to winter, with Shorthorns more prone to grass tetany than Jerseys or Holsteins. Disposition to HM increases with age.

Martens and Rayssiguier (1980) differentiate between two forms of HM: 1) chronic: affects calves reared on milk replacer, and cattle or sheep kept on pasture during winter or poor winter rations, and 2) acute: "Grass Tetany" affects animals in spring when turned out to pasture. These authors (Martens and Rayssiguier, 1980) also point out some possibilities for the cause of acute HM:

a) Reduced Mg absorption

b) Redistribution of Mg within the body

c) Reduced dietary intake of Mg

d) Increased excretion of Mg

e) Increased net Mg requirement

f) A combination of a-e

The possibilities for HM identified by Martens and Rayssiguier (1980) will be reviewed as two broad categories: a) Factors affecting Mg absorption at the gastro-intestinal (GI) tract, and b) factors affecting the utilization of

absorbed Mg.

B.2) Dietary allowances of Mg for dairy cows

The recommended dietary allowance of Mg is based on the prevention of grass tetany (NRC, 1989). However, the latest edition of the Nutrient Requirements of Dairy Cattle (NRC, 1989) increased its Mg recommendation for early lactation cows by a factor of 1.25 to a value of 0.25% Mg of the dietary DM. Few studies have determined the optimal dietary allowance of Mg for lactating dairy cows. This determination is difficult to ascertain for several reasons. The concept of optimal dietary allowance varies among researchers. During this review, optimal dietary allowance is defined as that amount of nutrient that will maximize lactational performance without any apparent negative effects on health. The most commonly used Mg supplement is MgO. It is well established that MgO has alkalizing effects in the forestomach of ruminants, therefore the effects of Mg <u>per se</u> and MgO as an antacid are usually confounded. Furthermore, most of the work conducted to date used lactating cows fed acidogenic diets. In general, supplementing MgO to dairy cows consuming high-grain, low-roughage diets improves their lactational performance. However, when MgO is supplemented to cows fed alfalfa based diets containing higher roughage content, milk yield is usually not affected (Solórzano, 1988). Supplementing Mg

salts to cows fed diets composed of corn silage and grain improves lactational performance (Teh et al., 1985, O'Connor et al., 1988, Solórzano et al., 1989). O'Connor et al. (1988) concluded that feeding .45 to .48% Mg improved milk production compared to feeding lower or higher levels of Mg. In contrast, Solórzano et al. (1990) concluded that supplementing above NRC (1989) recommendations up to .45% Mg did not improve the lactational performance of dairy cows fed alfalfa based diets. It is appropriate to point out that the maximum tolerable level of dietary Mg for cattle is 0.5% (NRC, 1980), which means that the maximum tolerable level is set too low, or that the optimal dietary allowance suggested by O'Connor et al. (1988) is very close to toxic levels, or that the dietary allowance of Mg varies according to the source of Mg. It appears that the optimal dietary allowance for Mg varies according to the nature of the diet, and therefore the allowance and toxic levels of Mg are not well established.

B.3) Factors affecting the absorption of Mg

An exhaustive list of factors interfering with Mg absorption and their relationships is presented by Martens and Rayssiguier (1980). Leonhard et al. (1989) proposed the following model for the transepithelial movement of Mg:

"Mg can move across the rumen epithelium through trans- and paracellular pathways of which the first form obviously provides two different mechanisms: an electrogenic and an electroneutral one.

The electrogenic pathway uses the potential difference of the apical membrane (PDa as driving force for the uptake of

Mg. The K conductance in the apical membrane and the K gradient across this membrane contribute to PDa. So that a change of the K gradient (high K concentration in the ruminal fluid) alters PDa and hence transcellular electrogenic Mg transport. Consequently the electrogenic Mg uptake must be considered as "K sensitive".

The electroneutral pathway for Mg is potential difference (PD) independent and not "K sensitive". It assumes a Mg/2 H exchange system rather than a Mg/2 anion cotransport.

Additionally there is a small passive and very likely paracellular Mg transport. The magnitude and direction of this transport is given by the chemical and/or electrical gradients and therefore changes with altered K concentrations. High ruminal K concentration elevates the transepithelial PD (by diminishing PDa) and by this way promotes Mg loss into the rumen fluid.

Both changes in Mg transport - the transcellular and the paracellular one - are additional and lead to the known reduction of Mg absorption at high K intake and/or Na deficiency."

A brief review of some of the factors affecting Mg absorption is presented below:

B.3.a) Na and K

It was suggested some 50 years ago that high K levels in pastures are involved in the pathogenesis of HM, however no mechanism was proposed at that time (Fontenot, 1980). Wyllie et al. (1985) using sheep cannulated in the abomasum and ileum, and with ruminal catheters, infused potassium bicarbonate into various segments of the GI tract and measured Mg absorption. Their results indicate that there is a net secretion of Mg at the large intestine, and that the stomach region is the main site of Mg absorption. Also, some absorption occurred at the small intestine. When K was infused in the rumen, total Mg absorption was decreased compared to similar infusions in the abomasum or ileum.

Martens and Rayssiguier (1980) reported in vitro studies

using isolated ruminal mucosa from sheep and showed that the inhibitory action of K was due to the Na:K ratio rather than the K concentration per se. Companion in vivo studies in sheep, in which the rumen was emptied, washed, and filled with a buffer solution containing different Na and K concentrations, showed that there was an almost linear increase of Mg absorption when the Na:K ratio increased from 0 to 5.

The mechanism by which K inhibits Mg absorption is not well understood. Evidence suggests that Mg transport across the rumen wall is an active process that involves the enzyme Na/K ATPase (Martens, 1985). It is speculated that the mechanism by which K inhibits Mg absorption involves competitive inhibition of Na by K at the receptor sites for the Na/K ATPase (Martens and Blume, 1986). The observation of competitive inhibition helps to explain why the Na:K ratio is more important than the K concentration per se. Recently, Leonhard et al. (1989) concluded that the enhancement in transmural potential difference of the forestomach (serosal side positive) and not K per se leads to the altered Mg flux. How K changes potential differences is not known.

High K or low Na levels in the diet of sheep depressed Mg absorption and supplementing Na counteracts these negative effects (Khorasani and Armstrong, 1990, Martens et al., 1987). Norgaand (1989) using dairy cows reported that as the Na:K ratio increased in the supernatant of rumen fluid from 3.2 to

4.7 the content of Mg decreased. Feeding an ionophore (which facilitates the passage of Na and K across cell membranes) improved the pre-intestinal absorption of Mg (Greene et al., 1988). These reports show the practical application of understanding the role of Na:K ratios in Mg absorption.

B.3.b) Potassium fertilizations of forages

Heavy fertilization of pastures with K is related to an increase in the incidence of HM. Potassium fertilization follows recommendations given for hay and silage crops. Hay and silage crops remove more K from the soil than do grazing animals from pastures. The high use of K fertilizers results in imbalances among cations in both soil and plant. A high K/(Ca+Mg) ratio in the plant is related to HM (Fontenot, 1980). Corn absorbs soil K preferably to soil Mg (Bertié et al., 1989) resulting in plants with high K concentrations and low Mg concentrations. The content of Mg in the ear leaf of corn is highly inheritable (Kovacevic and Vujevic, 1989), a characteristic that could be employed in the development of forages with high Mg contents and reduce the incidence of HM. Reviews on the effects of soil K on Mg utilization by the plant and the herbivore animal are available (Fontenot, 1980, Mayland and Wilkinson, 1989 and Mayland et al., 1990).

B.3.c) Rumen ammonia concentrations

The incidence of HM is highest during early spring, when pastures are young and lush. These pastures are high in protein relative to energy content. Consumption of these pastures may result in high rumen NH_3 concentrations. High rumen NH_3 concentrations have a negative effect on Mg absorption (Martens and Rayssiguier, 1980). Ammonia infused intra-ruminally to sheep decreased Mg absorption by 30%, but there was a compensatory mechanism over time (Gaebel and Martens, 1985). Based on this observation, they concluded that an acute rise in rumen NH_3 concentration impaired Mg absorption. Feeding isonitrogenous diets containing natural protein or NPN had no effects on Mg absorption due to N source. However there was a negative relationship between dietary crude protein (CP) level and Mg absorption (Teller and Godeau, 1987). Feeding ammoniated straw to beef cows had no effect on Mg absorption (Grings and Males, 1987). The effects of NH_3 on Mg metabolism appear to be confined to the pre-absorptive stage. Intravenous infusion of NH_3 to sheep had no effects on the concentrations of Mg in blood or urine (Gaebel and Martens, 1985).

It is important to consider that diets containing high levels of CP are fed to animals with high feed intakes. High feed intake increases the ruminal rate of passage and may decrease Mg absorption even further. On the other hand, the synchronization of protein and starch degradation could have

beneficial effects on Mg absorption, since rumen ammonia is used more efficiently.

B.3.d) Energy deficit

Excess nitrogen relative to readily degradable carbohydrate content of forages may give a rise to an energy deficit, which in turn impairs protein utilization (Martens and Rayssiguier, 1980). The consequences are a decrease in microbial protein synthesis while NH_3 is still being produced, and a decrease in levels of VFA and CO_2. House and Mayland (1976) reported that feeding decreasing amounts of sucrose to sheep resulted in increasing ruminal NH_3 levels. Urinary excretion and apparent absorption of Mg were higher in those lambs fed supplementary sucrose, however no differences could be detected in blood Mg levels. Feeding substantial quantities of readily digested carbohydrates increased Mg absorption, but the mechanism of action is not clear (Fontenot et al., 1989). Magnesium absorption increases with increasing concentration of VFA's in the rumen (Martens and Rayssiguier, 1980). The effects of carbohydrate supplementation and VFA concentration on Mg absorption could be mediated by accompanying changes in ruminal pH, and not by carbohydrate supplementation and its metabolism per se.

B.3.e) Rumen pH

Smith and Horn (1976) examining the distribution of ^{28}Mg in rumen contents of steers found that the fraction of total Mg that is ultrafilterable increases with decreasing pH and peaks at a pH in the range of 4 to 6. This observation is important because that Mg in solution is the Mg that is absorbed. For ruminants it means that acidogenic diets (i.e., high-grain, low-roughage diets) will favor Mg absorption. The negative effects of high K on Mg absorption may be mediated by increases in rumen pH (Khorasani and Armstrong, 1990). Norgaand (1989) reported that the supernatant of rumen fluid of dairy cows was characterized by high pH and had the lowest content of Mg. Thus, it appears that these factors do not act independently in their effects on Mg absorption. The inter-relationships among the factors that inhibit Mg absorption are not understood.

4) Factors affecting the utilization of absorbed Mg

Lipolysis was reported to increase during HM because high concentrations of free fatty acids (FFA) are found in blood when the disease is provoked experimentally (Cseh et al., 1984). In ruminants, stimulation of lipolysis induces a drop in blood Mg levels (Rayssiguier and Gueux, 1979, Rayssiguier and Larvor, 1976). Hypomagnesemia could result from the sequestration of Mg by adipocytes. In vitro, adipocyte membranes from rats can take up Mg (Elliot and Rizack, 1974).

Also, it was suggested that the formation of chelates between Mg and FFA in blood could result in HM (Flink et al., 1979).

C) EFFECTS OF Mg ON BLOOD LIPID METABOLISM

C.1) Mg deficiency

The effects of Mg deficiency on blood lipids are subjects of research because of the role of abnormal blood lipid concentration in cardiovascular diseases in humans. Dietary Mg deficiency results in increased total blood lipids (Rayssiguier, 1986a), blood very low density lipoproteins (VLDL) and low density lipoproteins (LDL) (Rayssiguier et al., 1981), blood triglycerides (TG) (Luthringer et al., 1988, Gueux et al., 1984, Rayssiguier et al., 1981, 1991, and Rayssiguier, 1986a), and blood cholesterol (Luthringer et al., 1988, Rayssiguier et al., 1991). The increases in blood cholesterol and TG can be partially explained by the increases of the lower density lipoproteins. High levels of blood cholesterol have gained much attention in recent years because of its linkage to increased health risks. Dietary Mg restriction increases blood oleic and linoleic acids and their proportions in blood TG and phospholipids, respectively (Rayssiguier, 1986b).

In contrast, dietary Mg deficiency decreases blood concentrations of high density lipoproteins (HDL) (Rayssiguier et al., 1981), HDL-cholesterol (Luthringer et al., 1988), esterified cholesterol (Rayssiguier, 1986a, Rayssiguier and

Gueux, 1979), stearic and arachidonic acids (Rayssiguier, 1986a), and the activities of lecithin carnitine acyl transferase (LCAT) (Rayssiguier, 1986a) and lipoprotein lipase (LPL) (Rayssiguier et al., 1991). The decrease in HDL-cholesterol (so called "good cholesterol") may be of concern, since this is part of the cholesterol that will be cleared from the organism. The decrease in LCAT may explain the decrease in cholesterol esterification, and it may also contribute to the impaired transport and disposal of TG (Gueux et al., 1984). The rate of TG secretion is not modified during Mg deficiency and the increase in TG may be due to decreased clearance (Rayssiguier, 1986b). The enzyme LPL is a key step in the removal of circulating TG. Therefore, a reduction of LPL activity may account for the observed increases in TG and lower density lipoproteins. Serum LPL activity was not correlated to low blood Mg levels in hypomagnesemic cows (Cseh et al., 1984). There is a small amount of lipase in post-heparin plasma in cattle which is partially inhibited by salt and slightly activated by plasma (Liesman et al., 1984). These workers concluded that the lipase measured in blood of cows is predominantly LPL.

The increase in linoleic acid and the decrease in arachidonic acid in total plasma lipids suggests a blockage of the synthesis of arachidonic from linoleic acid (Rayssiguier, 1986a). Disturbances in the metabolism of arachidonic acid or other essential fatty acids will have implications in the

synthesis of prostaglandins and also may result in immunosuppression.

C.2) Mg supplementation

The main problem in nutrition appears to be a deficiency in Mg and not its toxicity. Therefore, most of the work conducted on the effects of Mg on lipid metabolism has been with Mg deficiency models. In humans, Mg is supplemented for clinical purposes to patients with cardiovascular disease and hyperlipidemia.

Increasing blood Mg levels increase lecithin and the lecithin:cholesterol ratio, decrease cholesterol and β-lipoproteins, with no effects on the concentration of α-lipoproteins (Seelig, 1980). Although these effects prove significant in improving the condition of these patients, their significance in bovines is undetermined. Decreases in cholesterol and β-lipoproteins could have negative effects in the reproductive efficiency of bovines.

Seelig (1980) in her review of Mg and cardiovascular diseases indicates that when South African or Australian aborigines were compared to their compatriots from European origin, the aborigines had higher blood Mg and lower cholesterol levels. When patients from the same genetic population (i.e., within Europeans) were given parenteral administration of Mg, there were no correlations between low serum Mg and lipid levels. This comparison suggests that

blood Mg, blood lipids, cardiovascular diseases, and genetics are interrelated. This also supports the observation by Harris et al. (1983) that HM is related to the breed of the cow.

D) EFFECTS OF Mg ON MILK LIPID METABOLISM

D.1) Synthesis of milk lipids

Milk fat consists of approximately 98% triglycerides, the remainder being di and monoglycerides, phospholipids, free fatty acids, sterol esters and hydrocarbons (Dils, 1986). The fatty acids in milk fat arise from two sources: circulating blood lipids and de novo synthesis in the mammary gland (Bauman and Davis, 1974). It is generally accepted that short chain fatty acids (4 to 10 carbons) are synthesized in the mammary gland from acetate and betahydroxy butyric acid (BHBA). Intermediate chain fatty acids (12 to 16 carbons) may be synthesized in the mammary gland from acetate or BHBA or are extracted from blood plasma. Long chain fatty acids (>16 carbons) are mainly derived from blood lipids contained in VLDL and chylomicrons (Bauman and Davis, 1974), although small amounts of plasma non-esterifed fatty acids are also used (Bauman and Davis, 1974).

Fatty acids extracted by the mammary gland reflect the fatty acid composition of the dietary fat and of the fatty acids synthesized by adipose tissue and released into the blood stream (Dils, 1986, Rindsig and Davis, 1974).

D.2) Dietary effects on milk lipids

Increased genetic potential for milk production led to feeding high-energy diets in an attempt to meet the cow's energy needs. In order to increase the energy density of the diet, the concentrate portion of the diet was increased. This often results in diets containing less than adequate fiber for optimal rumen function. The increase in the grain portion of the diet is associated with a higher incidence of management problems, the most significant among them, being displaced abomasum and a condition known as "the low milk-fat syndrome" (Emery, 1975). Addition of fat to the diet of lactating cows is another way of increasing the energy density of the diet. However, fat supplementation to dairy rations, particularly vegetable oils, may decrease milk fat synthesis (Selner, 1978). A review of the theories of milk-fat depression is presented by Solórzano (1988). We must keep in mind that there are non-nutritional factors that can influence milk composition (Emery, 1988), but their discussion is beyond the scope of this review.

D.3) Effects of Mg salts supplementation on milk lipids

The effects of mineral salts, commonly referred to as buffers (a more proper designation would be antacids), were reviewed within the last three years (Erdman, 1988, Stapples and Lough, 1989, and Solórzano 1988), and therefore only a brief discussion is appropriate in this review.

Emery et al. (1965) supplemented MgO to cows fed high-grain diets and successfully alleviated milk-fat depression. Mixtures of mineral salts high in Mg content also have alleviated milk-fat depression (Solórzano et al., 1989). Emery et al. (1965) suggested that Mg increases the uptake of endogenous acetate and TG from blood plasma into the mammary gland tissues. However, intra-venous single pulse doses of Mg did not alleviate milk-fat depression in a short term experiment (Emery and Thomas, 1967) or in a second report in which cows were infused with isotonic solutions of saline or $MgCl_2$ intravenously (Solórzano et al., 1990). These later reports may suggest that any beneficial effects of Mg supplementation on the alleviation of milk-fat depression are at the GI tract level and not at the tissue level.

E) THE ROLE OF Mg IN LIPOLYSIS

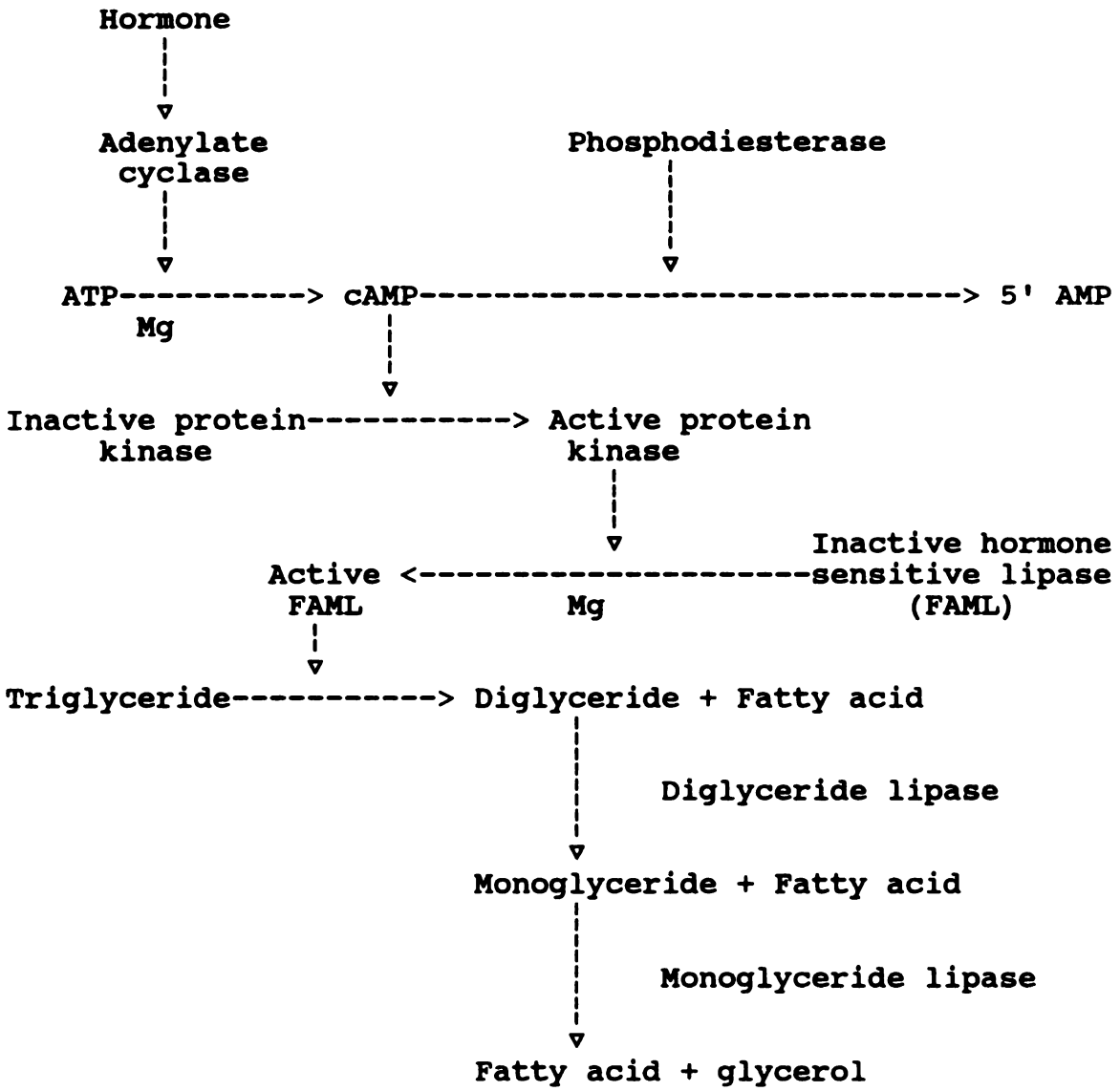
The primary site for triglyceride (TG) storage is the adipose tissue. The specialized cell in this tissue is referred to as adipocyte. When an energy deficit occurs the animal responds to the deficiency with a hormonal signal. Lipolytic hormones or agents bind to receptors located in the plasma membrane of the cell and activate the enzyme adenylate cyclase (Figure 2). Cyclic AMP (cAMP) is formed from ATP by the action of adenylate cyclase. This formation of cAMP requires Mg as a cofactor. The cAMP activates a protein kinase which phosphorylates and activates fatty acid

mobilizing lipase (FAML). The activation of FAML requires Mg as a cofactor. This enzyme converts TG to diglycerides, and this step is considered rate limiting in lipolysis (Vance, 1988). Diglycerides and monoglycerides are converted to FA and glycerol.

Almost all reactions involving ATP hydrolysis occur in the presence of Mg ions (Ingraham, 1988). The effect of Mg ions on the free energy of hydrolysis of ATP depends on pH and the ionic strength (Ingraham, 1988). In vitro, a combination of Mg, ATP, and cAMP are needed as cofactors in the stimulation of FAML. These cofactors together are more effective than combinations of two in stimulating FAML activity (Tsai et al., 1970).

Upon stimulation of lipolysis there is a drop in blood Mg concentration, which suggests redistribution of Mg within the body. There is an accumulation of Mg in rat adipocytes when lipolysis is stimulated by catecholamines or adrenocorticotropin (Elliot and Rizack, 1974). There is an increase of 470% in Mg accumulation in the fat tissue of cold treated rats provided with insufficient dietary Mg supply (Meyer et al., 1982). Stimulating lipolysis in sheep by fasting results in increased blood FFA and decreased blood Mg (Rayssiguier and Larvor, 1976). Similar results were shown in dogs following ethanol withdrawal (Flink et al., 1979). Declines in plasma Mg were observed in calves and ewes fed low Mg diets and exposed to low ambient temperatures (Meyer et

Figure 2. The adenylate cyclase cascade.



al., 1982, Terashima et al., 1984). An increase in serum FFA and a concomitant decrease in serum Mg were found after acute myocardial infarction in humans (Cohen et al., 1984). Possible explanations for the decreases in blood Mg are: 1) Mg is sequestered by adipocytes (Elliot and Rizack, 1974), 2) Mg is chelated by FFA and sequestered by cell membranes other than in adipocytes (Flink et al., 1979), or 3) a combination of both. Most data indicate that changes in lipid metabolism result in alterations in Mg status. Conversely, Mg has a role in the stimulation of lipolysis, therefore, alterations in Mg status may result in changes in lipid metabolism.

F) THE ROLE OF Mg IN PEPTIDE HORMONE METABOLISM

F.1) Role of Mg in protein synthesis

The biosynthesis of nucleotides is a vital process because nucleotides serve as precursors for the synthesis of DNA and RNA. Protein synthesis is halted if synthesis of RNA is stopped, and without DNA cells can not divide.

Magnesium serves as a cofactor in reactions catalyzed by pyrophosphatases, amidotransferases, synthases, and kinases in nucleotide synthesis (Blakley, 1988). Magnesium stimulates the activation of amino acids to form the various amino acyl adenylates (Heaton, 1980). Alterations in RNA metabolism are reflected in alterations in protein metabolism. Magnesium is involved in maintaining the structural conformation of ribosomes and nucleic acids (Heaton, 1980). The concentration

of Mg in mammalian cells that is essential for protein synthesis is in the range of 1 to 10 mM (Cameron and Smith, 1989). So a deficiency of Mg can result in alterations in protein synthesis. In fact, liver slices from Mg deficient rats had lower protein synthesis than liver slices from normomagnesemic rats (George and Heaton, 1978). Therefore, the status of Mg can potentially affect the synthesis of peptide hormones such as growth hormone and insulin.

F.2) Growth hormone

There is very little information on the relationship between Mg and growth hormone (GH). Supplementing the ration of lactating dairy cows with $NaHCO_3$ decreased the concentrations of plasma Mg and GH (Dussault et al., 1983). No data on feed intake were provided. The addition of $NaHCO_3$ to the ration of lactating cows decreased the concentration of GH, independently of restricted or ad lib feed intake (Vicini et al., 1984). No data were provided for blood Mg levels. However, based on the report of Dussault et al. (1983), blood Mg levels can be assumed not affected or decreased. Supplementing Mg salts to the diet of lactating cows decreased feed intake, did not affect serum Mg concentrations, and increased the concentration of GH (Emery et al., 1986). To further examine the relationship among Mg, feed intake, and GH, Emery et al. (1986) computed a linear regression of GH on serum Mg and feed intake. Their results suggested that at low

serum Mg concentration, the increase in GH concentration due to decreased feed intake is less than the increase observed at higher levels of serum Mg. There is a positive relation between level of blood Mg and concentration of GH. The effects of serum Mg on GH can be both, at the synthesis level or at the target tissue for GH. The effects of different levels of blood Mg on GH concentrations when intake is not affected remains to be established.

F.3) Insulin

Increased blood insulin concentration was implicated in the development of milk-fat depression (McClymont and Valance, 1960). The supplementation of Mg salts alleviates milk-fat depression, therefore, it is possible that Mg has a post-absorptive effect on insulin. Omission of Mg from the medium perfusing rat pancreas inhibited the release of insulin (Curry et al., 1977). Insulin was decreased during HM in rats (McNeill et al., 1982). On the other hand, high Mg levels can suppress insulin production (Durlach et al., 1990). Intracellular concentration of Mg appears to have an optimum range for insulin secretion from the pancreas. It was suggested that the content of Mg in β-cells of the pancreas might be a more important factor than the serum Mg level in modifying insulin secretion (Wallach, 1980) Also, the Ca:Mg ratio in β-cells can influence the release of insulin (Atwater et al., 1983). Insulin takes part in glucose absorption by

the tissues, and Mg is indispensable for maintaining the effect of insulin (Aikawa, 1981).

Feeding low Mg/high K diets to hypomagnesemic sheep depresses the glucose-induced insulin secretion and the insulin-induced glucose utilization (Matsunobu et al., 1990). Feeding a low Mg diet or a low Mg/high K diet for 1 week to normomagnesemic sheep resulted in no changes in insulin response (Terashima et al., 1984). It is possible to alter insulin concentrations in blood by dietary means in the long run. Insulin sensitivity can be altered by changes in Mg status, but the effects are too variable to draw any conclusions (Durlach et al., 1990).

G) IMPLICATIONS TO THE DAIRY COW

G.1) Dietary allowances

The Mg requirement of the lactating dairy cow is not well established. As long as this requirement is not established it will be difficult to asses any detrimental effects of moderate Mg deficiency or beneficial effects due to Mg supplementation. It is well documented that Mg supplementation to acidogenic diets improves the lactational performance of dairy cows. The dietary allowance of Mg for lactating cows fed alfalfa based diets (normal roughage-non acidogenic diets) remains to be established.

G.2) Lipid metabolism in early lactation

Lipid metabolism during early lactation is extremely important. Since the cow can not consume enough energy to meet her energy needs, she attempts to compensate by mobilizing body fat. A Mg deficiency or over supplementation could result in impairment of adipose tissue mobilization, therefore negatively affecting lactational performance. Because HM occurs rapidly, it has not been possible to determine the effects of acute HM on lipid metabolism, and since the Mg requirements are not well defined it is difficult to define what constitutes moderate HM. It would be interesting to determine if FFA are chelating Mg or if there is Mg sequestration by adipocytes in the dairy cow. This information would be useful in determining if Mg redistribution is sufficient to cause HM, and to gain understanding of the chelation and/or sequestration process.

Since Mg is a cofactor in the stimulation of lipolysis, could we manipulate body fat mobilization by supplementing different levels of Mg in the diet? If the answer to this question is yes, we should be able to manipulate milk production and composition, body weight loss or gain, both of which are of economic importance to the dairy industry.

What is the mechanism for Mg alleviating milk-fat depression? There is some evidence that Mg acts at the rumen level and not at the tissue level as previously suggested. However, if Mg has an effect at the tissue level, it would be

interesting to know what the effects are.

G.3) Relationship between Mg and peptide hormones

Emery et al. (1986) suggested that serum Mg has an effect on serum bovine somatotropin (BST) concentrations. If BST acts through diverting nutrients from adipose tissue into the mammary gland, then it is likely that BST is affecting LPL and FAML, two of the hormones involved in the control of lipolysis. If this hypothesis proves to be correct, then information regarding the effects or the mechanisms of Mg for alleviating milk-fat depression, or the mechanism for BST increasing milk production may be explained.

H) SUMMARY

Magnesium requirements for the dairy cow are not well defined. Many factors contribute to the variability in recommendations of optimal Mg concentrations in the diet. Hypomagnesaemia can result from either decreased absorption of Mg or from factors affecting the utilization of absorbed Mg. Absorption can be affected by rumen concentrations of Na and K, K fertilizations of pasture, rumen ammonia concentrations, energy deficit, and rumen pH, among other factors. Absorbed Mg can be chelated by FFA, or sequestrated by adipocytes. In ruminants, a stimulation of lipolysis results in a drop of blood Mg level. No correlation was found between plasma LPL and low serum Mg concentrations.

A deficiency of Mg affects serum lipids. In general, TG are increased and cholesterol esterification is decreased. Among the enzymes that Mg affects are Lpl and LCAT. A shortage of Mg also results in changes in TG pattern of lipoproteins and changes in the size of lipoproteins. Genetics seem to play a role in HM and in the effects of HM on serum lipids.

Magnesium oxide supplementation and other antacid mixtures high in Mg partially alleviate milk-fat depression. The proposed mechanism for Mg alleviating milk-fat depression involving increases in acetate and TG uptake by the mammary gland is questioned. Effects of Mg on LPL activity at the adipose tissue of dairy cows is unknown. The implications of defining the effects of Mg on lipolysis and milk-fat depression were discussed.

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CHAPTER II

DIETARY ALLOWANCES OF Mg FOR EARLY LACTATION COWS FED AN ALFALFA BASED DIET

L. C. Solórzano, P. E. Naasz[1], J. S. Liesman, and R. S. Emery

Department of Animal Science
Michigan State University
East Lansing 48824

Key words: magnesium, dairy cow, lactation, mineral supplementation

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[1] Upper Peninsula Experiment Station, Michigan State University, Chatham, MI 49816.

INTRODUCTION

Magnesium is one of the major minerals recognized to be essential for farm animals, being especially critical for ruminants (Fontenot, 1980). It was proposed that currently held Mg recommendations are based on preventing "Grass Tetany" and do not maximize milk production (O'Connor et al., 1988).

Most studies conducted in the United States evaluating the effects of Mg supplementation as MgO were addressed to test the effects of MgO on cattle fed high-grain, low forage, milk-fat depressing diets, and did not address the determination of optimal dietary allowances of Mg. Magnesium supplementation to corn silage based diets improves lactational performance (Teh et al., 1985, O'Connor et al., 1988). Based on this, it was suggested that .3 to .45% Mg in the diet is needed in order to maximize milk production (O'Connor et al., 1988). However, it is not clear what is the mechanism of action for Mg improving lactational performance.

Magnesium is required by cellulolytic microorganisms and Mg supplementation improves cellulose digestion in vitro (Durand and Kawashima, 1980). The ruminal acetate:propionate ratio increases with MgO supplementation (Thomas et al., 1984), although this is not always the case (Erdman et al., 1982). It was proposed that MgO could have alkalizing effects in the small intestine, thus potentially enhancing starch digestion. Also, it was suggested that MgO increases the uptake of milk fat precursors from blood plasma into mammary

gland tissue (Emery et al., 1965). Since: 1) Mg content of milk does not decline with declining levels of Mg intake, 2) Mg plays an important role in enzyme and neuro-muscular activation, and 3) Mg alters the ruminal environment and affects feed digestibility, it is important to establish the optimum dietary allowance for high producing dairy cows in order to maximize lactational performance.

Objectives of this study were to determine: 1) if currently held Mg recommendations are adequate for high producing cows fed an alfalfa based diet during the first 84 d of lactation, and 2) the optimal dietary allowances of Mg for high producing cows during the first 84 d of lactation when fed an alfalfa based diet.

MATERIALS AND METHODS

Twenty seven Holstein cows, 15 multiparous and 12 primiparous, from the Upper Peninsula Experimental Station herd located in Chatham, MI, were blocked by parity and expected calving date and randomly assigned at d 15 post-partum to one of three dietary treatments: NOR) .25% Mg, MED) .35% Mg, or HI) .45% Mg (DM basis) until d 84 post-partum. Cows were individually fed ad lib a total mixed ration (TMR) consisting of alfalfa silage, grain mix, vitamins, and minerals balanced to meet or exceed NRC (1989) nutrient recommendations for dairy cattle (Table 1). The first 14 d post-partum served as a covariate period during

Table 1. Ingredient composition of diets (g DM/100 g diet DM).

	COVARIATE	EXPERIMENTAL[1]		
		NOR	MED	HI
Ingredient				
Alfalfa haylage	52.1	51.1	51.0	50.9
Ground corn	29.5	36.0	36.0	35.9
Maxi-Tech[2]	15.2			
Soybean meal	2.6	10.8	10.8	10.7
NaH_2PO_4	0.32	0.60	0.60	0.60
Limestone		0.87	0.87	0.87
Magnesium oxide	0.03	0.09	0.27	0.46
TMS[3]	0.23	0.19	0.19	0.19
Selenium 600[4]	0.03	0.05	0.05	0.05
Vitamin E premix[5]	0.04	0.03	0.03	0.03
Vitamin ADE[6]	0.03	0.23	0.23	0.23

[1] NOR = 0.25% Mg, MED = 0.31% Mg, and HI = 0.36% Mg.

[2] A commercial product containing 204.6 Mcal NE_l/kg, 32% CP, 2.8% Ca, 0.95% P, 0.5% Mg, 1.5% K, 0.56% Na, 0.84% Cl, 0.5% S, 416 ppm Fe, 332 ppm Zn, 84 ppm Cu, 332 ppm Mn, 4.2 ppm I, 1.8 ppm Se, 32 IU vitamin A/kg, and 6.6 IU vitamin D/kg.

[3] Trace mineralized salt contains 0.1% Mg, 38% Na, 58% Cl, 0.04% S, 2000 ppm Fe, 3500 ppm Zn, 330 ppm Cu, 2000 ppm Mn, and 70 ppm I.

[4] Contains 600 mg Se/kg.

[5] Contains 44,000 IU/kg.

[6] Contains 6,600 KIU vitamin A/kg, 3,300 KIU vitamin D/kg, and 13.2 IU vitamin E/kg.

which cows were fed the regular herd TMR balanced to contain .23% Mg (DM basis, Table 1). The alfalfa silage was sampled weekly and analyzed for DM, CP, ADF, Ca, Mg, K, and P at the Wisconsin DHIA Laboratory (Bonduel, WI) and rations adjusted weekly if needed. Water was provided ad lib. Water contained (mg/dl) 2.07 Mg, 3.31 Ca, 0.54 K, and 1.51 Na. Feed offered and weighbacks were measured daily and sampled weekly. Composites of feed offered and orts were analyzed for DM by drying in a forced air oven at 60 °C for 72 h and ground in a Wiley Mill to pass a 1 mm screen. Feed and orts were analyzed for organic matter (OM) by combustion at 600 °C (AOAC, 1975), CP by the Kjehldal method (AOAC, 1975), ADF (Goering and Van Soest, 1970), and Ca, Mg, K, and Na by atomic absorption spectroscopy (Perkin Elmer, model 5200, Norwalk, CT). Chemical composition of diets is presented in Table 2. Blood samples were obtained weekly from the tail vessel and analyzed for minerals as above.

Cows were milked twice a day and milk weights recorded at each milking. Milk was sampled on two consecutive milkings weekly and analyzed for protein, fat, lactose, total solids, and solids non-fat (SNF) by near infrared spectroscopy at the Michigan DHIA Laboratory (East Lansing, MI). Cows were weighed and body condition scored weekly. The scale used to condition score was from 0 (thin) to 5 (fat).

Data collected were analyzed by analysis of variance (SAS, 1985) as a split plot design on time with repeated

measurements (Gill, 1978). Analysis was conducted according to Milliken and Johnson (1984), therefore the models used were:

$Y_{ijk} = \mu + + T_i + B_j + T_ixB_j + W_k + W_kxT_i + E_{ijk}$

Where:

T_i is the average effect of treatment,

B_j is the average effect of block,

W_k is the average effect of week,

E_{ijk} is the random residual, assumed normally and independently distributed.

This model served to determine the effects of week and the week by treatment interaction. Means and the mean square error are presented.

$Y_{ij} = \mu + COV + T_i + B_j + E_{ij}$

Where:

COV is the covariate,

T_i is the average effect of treatment,

B_j is the average effect of block,

E_{ij} is the random residual, assumed normally and independently distributed.

This model served to determine the effects of treatment. Single degree of freedom orthogonal contrast compared the linear (NOR vs HI) and curvilinear (MED vs the combined effect of NOR and HI) effects of Mg. Least square means and the mean square error are presented.

Table 2. Chemical composition of diets[1].

	COVARIATE	EXPERIMENTAL		
Constituent		NOR	MED	HI
DM	54.3	55.0	55.5	55.1
OM[2]	91.8	92.1	91.8	92.1
CP	17.2	16.4	16.1	15.5
ADF	21.3	19.7	20.2	20.7
Ca	0.71	0.73	0.72	0.74
Mg	0.23	0.25	0.31	0.36
K	1.69	1.61	1.66	1.67
Na	0.20	0.15	0.16	0.16

[1] g/100 g ration DM, except DM expressed as g/100 g of ration as fed.
[2] Organic matter.

RESULTS

Three multiparous cows suffered a displaced abomasum during the covariate period and their data were deleted. Cows fed HI consumed more Mg ($P<0.001$) and Na ($P<0.06$) than cows fed NOR (Table 3). There were no differences for any other intake variables measured ($P>0.18$). There was a Treatment X Week interaction for the intake of ADF ($P<0.004$, Figure 1). Cows fed MED consumed less ADF after the 6th week post-partum compared to cows fed NOR or HI. Cows fed NOR consumed less Na after the 10th week post-partum compared to cows fed MED or HI, and the Treatment X Week interaction was significant ($P<0.01$, Figure 2). There were no differences in body weight or condition score ($P>0.50$).

Feeding MED compared to the combined effect of NOR and HI decreased the yield of milk ($P<.03$) and fat ($P<.05$) and tended to decrease the yield of protein ($P<.13$), lactose ($P<.06$), SNF ($P<.08$), and 3.5% FCM ($P<.09$, Table 4). There were no differences ($P>.27$) between NOR and HI on any of the milk production variables measured. Treatment had no effects on milk composition (Table 4) and averaged 4.01% fat ($P>.85$), 2.99 % protein ($P>.53$), 4.99% lactose ($P>.37$), and 12.75% SNF ($P>.65$). There was no Treatment x Week interaction ($P>.28$) for any of the lactational performance variables measured.

There were no linear or quadratic effects of treatment on plasma Ca, Mg, K, and Na. Feeding MED compared to the combined effect of feeding NOR and HI decreased plasma K

(P<0.02, Table 5). The Treatment x Week interaction tended to be significant for plasma Ca (P<0.06) and plasma Mg (P<0.11, Figures 3 and 4).

DISCUSSION

Magnesium oxide is the most commonly used Mg supplement. It could be argued that since MgO has alkalizing effects in the alimentary tract, the effects of MgO as a Mg supplement or as an antacid are confounded. However, in this study the alkalizing effects of MgO can be assumed to be negligible because: 1) cows were fed a high-roughage, alfalfa based diet which should result in a higher ruminal pH than if cows were fed a corn silage based diet (DePeters et. al., 1984); 2) no milk-fat depression occurred as evidenced by the high milk fat concentration (4.01%); and 3) the levels of MgO supplemented were either below or at the lowest level recommended for MgO as an antacid agent (Thomas and Emery, 1984).

Magnesium supplementation had no effects on feed intake. The effects of Mg supplementation on feed intake are variable. Our results are in agreement with those reported for similar levels of Mg supplemented as MgO to multiparous cows in early lactation (Teh et. al., 1985) or Mg supplemented as $MgSO_4$ to cows in mid-lactation (O'Connor et. al., 1988) when fed a diet

Table 3. Intake of dietary constituents, body weight and body condition.

	TREATMENT[1]			
	NOR	MED	HI	MSE[2]
N, cows	9	7	8	
	kg/d			
DM	22.3	20.6	22.1	2.5
OM[3]	20.5	18.8	20.4	2.3
CP	3.55	3.41	3.37	0.36
ADF	4.35	4.04	4.37	0.65
	g/d			
Ca	160.9	150.2	153.8	17.1
Mg[4,5]	53.4	65.3	77.9	7.0
K	345.5	336.4	364.8	33.9
Na[6]	30.2	33.4	35.4	4.6
BW, kg[7]	580.2	571.4	573.9	24.6
BCS[7]	2.1	2.1	1.9	0.4

[1] NOR = 0.25, MED = 0.31, and HI = 0.36% Mg of dietary DM.
[2] Mean square error.
[3] Organic matter.
[4] Overall treatment effect ($P<0.001$).
[5] Probability that NOR vs. HI are among treatments that do not differ ($P<0.001$).
[6] Probability that NOR vs. HI are among treatments that do not differ ($P<0.06$).
[7] BW = body weight; BCS = body condition score.

Figure 1. Effects of Mg supplementation on the intake of ADF. Mean square error is 0.55.

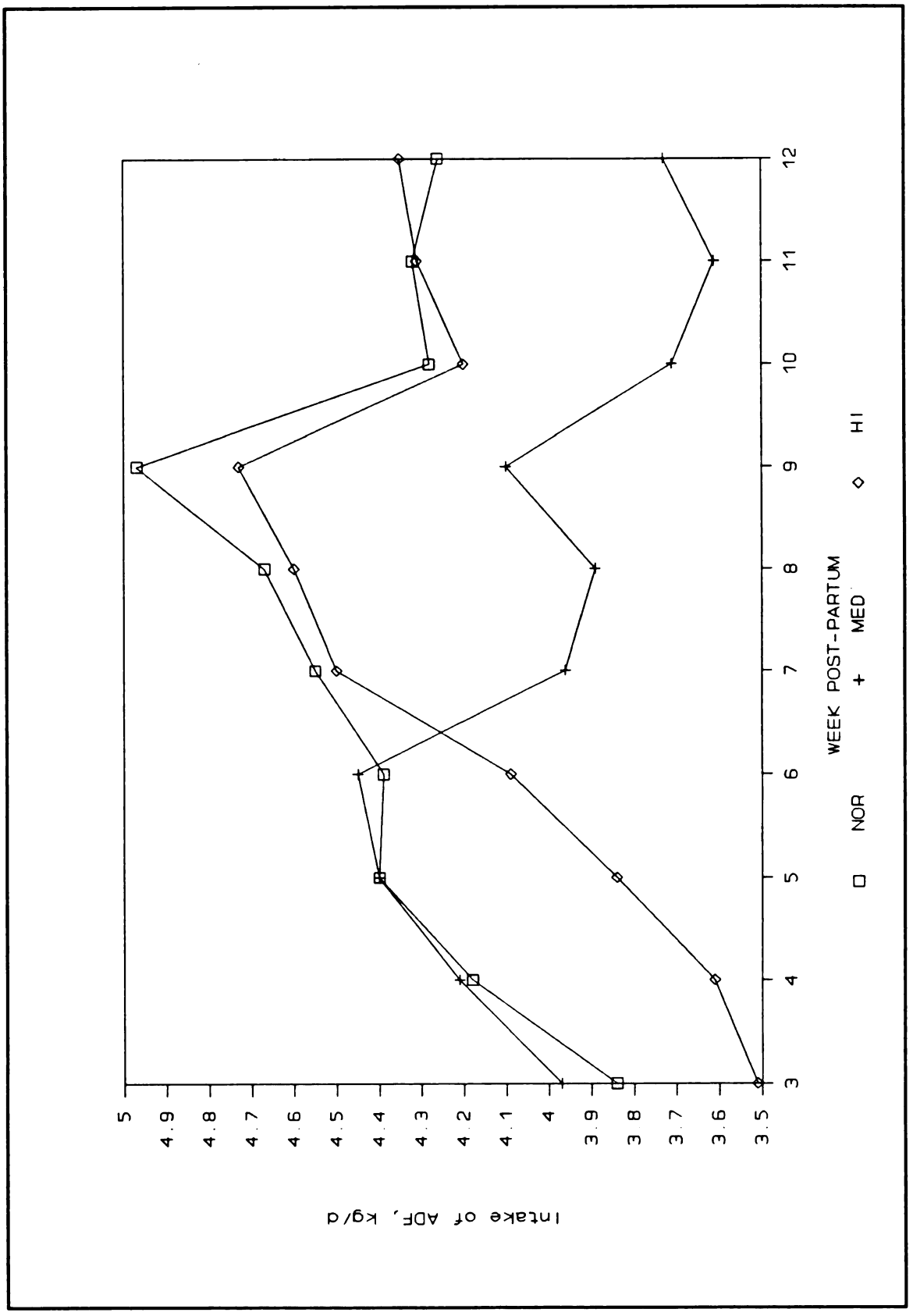

Intake of ADF, kg/d
5
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
4
3.9
3.8
3.7
3.6
3.5
3
4
5
6
7
8
9
10
11
12
WEEK POST-PARTUM
□ NOR
+ MED
◇ HI

Figure 2. Effects of Mg supplementation on the intake of Na. Mean square error is 6.4.

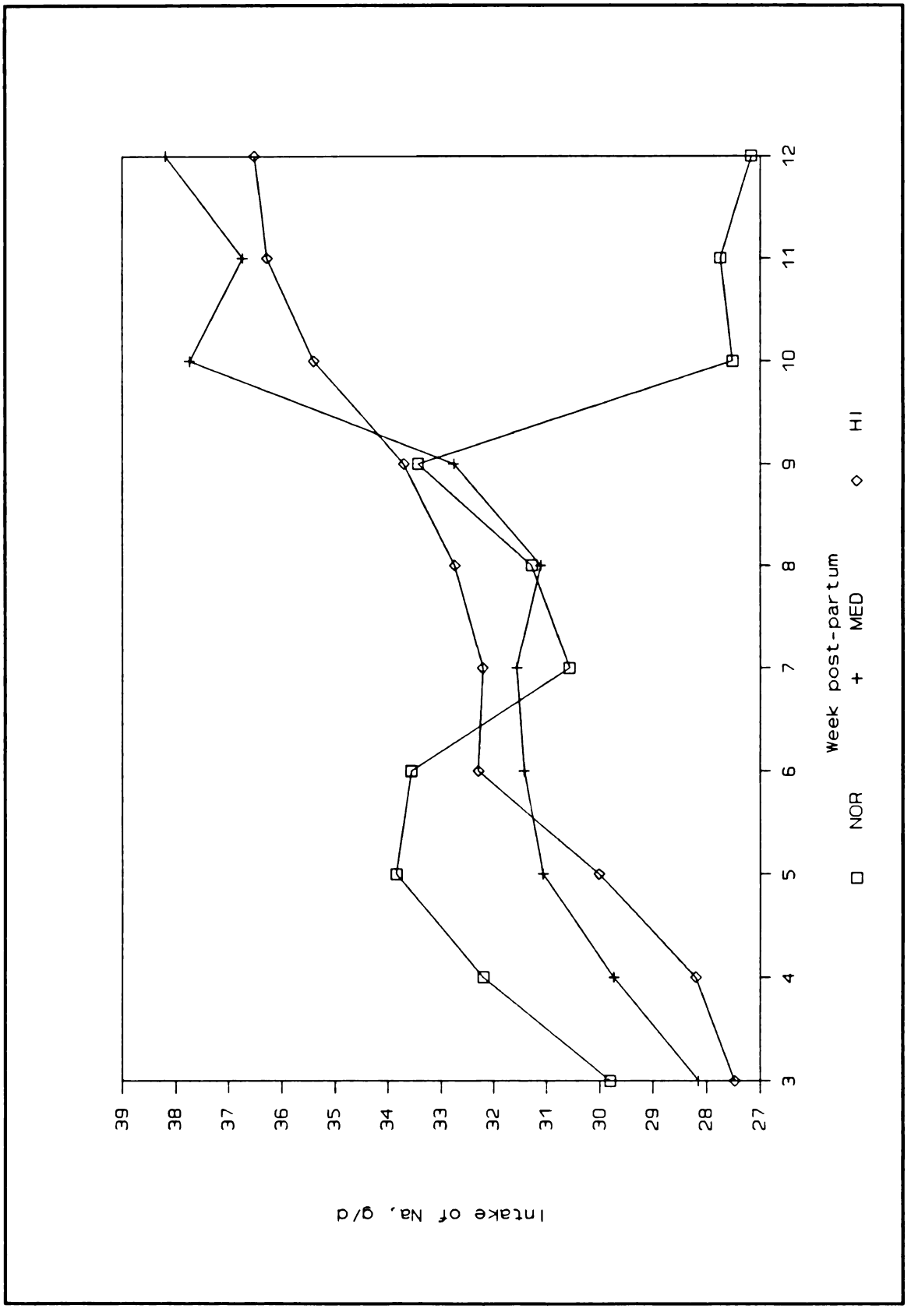

Intake of Na, g/d
39
38
37
36
35
34
33
32
31
30
29
28
27
3
4
5
6
7
8
9
10
11
12
Week post-partum
□ NOR
+ MED
◇ HI

Table 4. Effects of Mg supplementation on milk yield and composition.

	TREATMENT[1]			
VARIABLE	NOR	MED	HI	MSE[2]
N, cows	9	7	8	
Yield, kg/d				
Milk[3]	37.5	34.3	38.1	3.0
3.5% FCM[4]	46.3	44.5	46.0	1.9
Fat[3]	1.50	1.36	1.51	0.14
Protein[4]	1.14	1.04	1.11	0.11
Lactose[4]	1.85	1.71	1.92	0.18
SNF[4]	4.74	4.37	4.79	0.41
Concentration, g/100 ml milk				
Fat	4.04	3.98	4.02	0.19
Protein	2.98	3.03	2.97	0.11
Lactose	4.95	5.02	5.00	0.09
SNF	12.7	12.8	12.7	0.27

[1] NOR = 0.25, MED = 0.35, and HI = 0.45% Mg of dietary DM.
[2] Mean square error.
[3] Probability that MED vs NOR and HI is among treatments that do not differ ($P<.05$).
[4] Probability that MED vs NOR and HI is among treatments that do not differ ($P<.13$).

Table 5. Effects of Mg supplementation on plasma cations (mg/dl).

	TREATMENT[1]			
	NOR	MED	HI	MSE[2]
N, cows	9	7	8	
Ca	9.62	9.71	9.92	0.46
Mg	2.32	2.30	2.38	0.16
K[3]	22.49	21.12	22.51	0.96
Na	332.0	331.6	327.9	17.0

[1] NOR = 0.25, MED = 0.35, and HI = 0.45% Mg of dietary DM.
[2] Mean square error.
[3] Probability that MED vs NOR and HI is among treatments that do not differ (P<.02).

Figure 3. Effects of Mg supplementation on plasma Ca. Mean square error is 0.49.

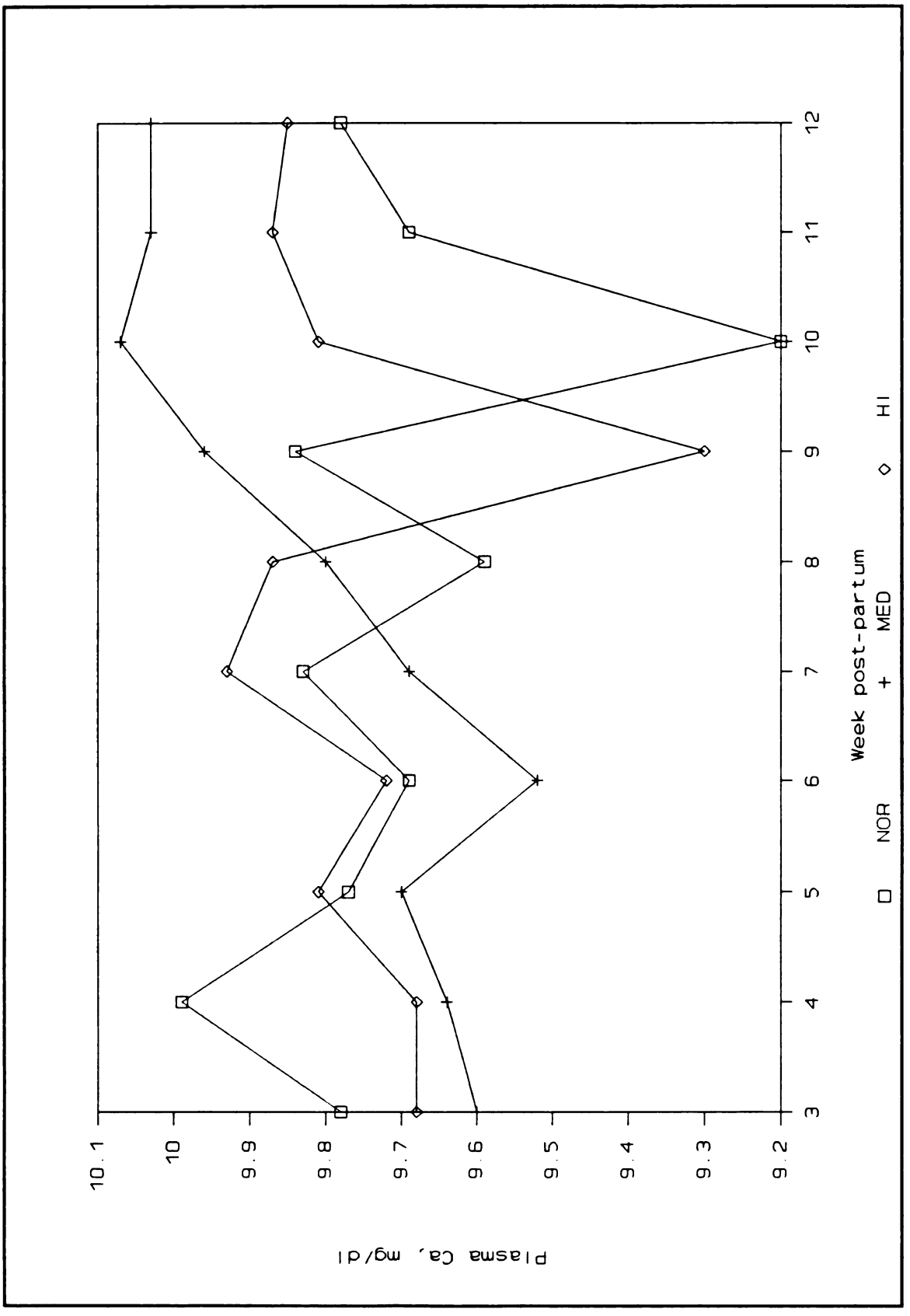
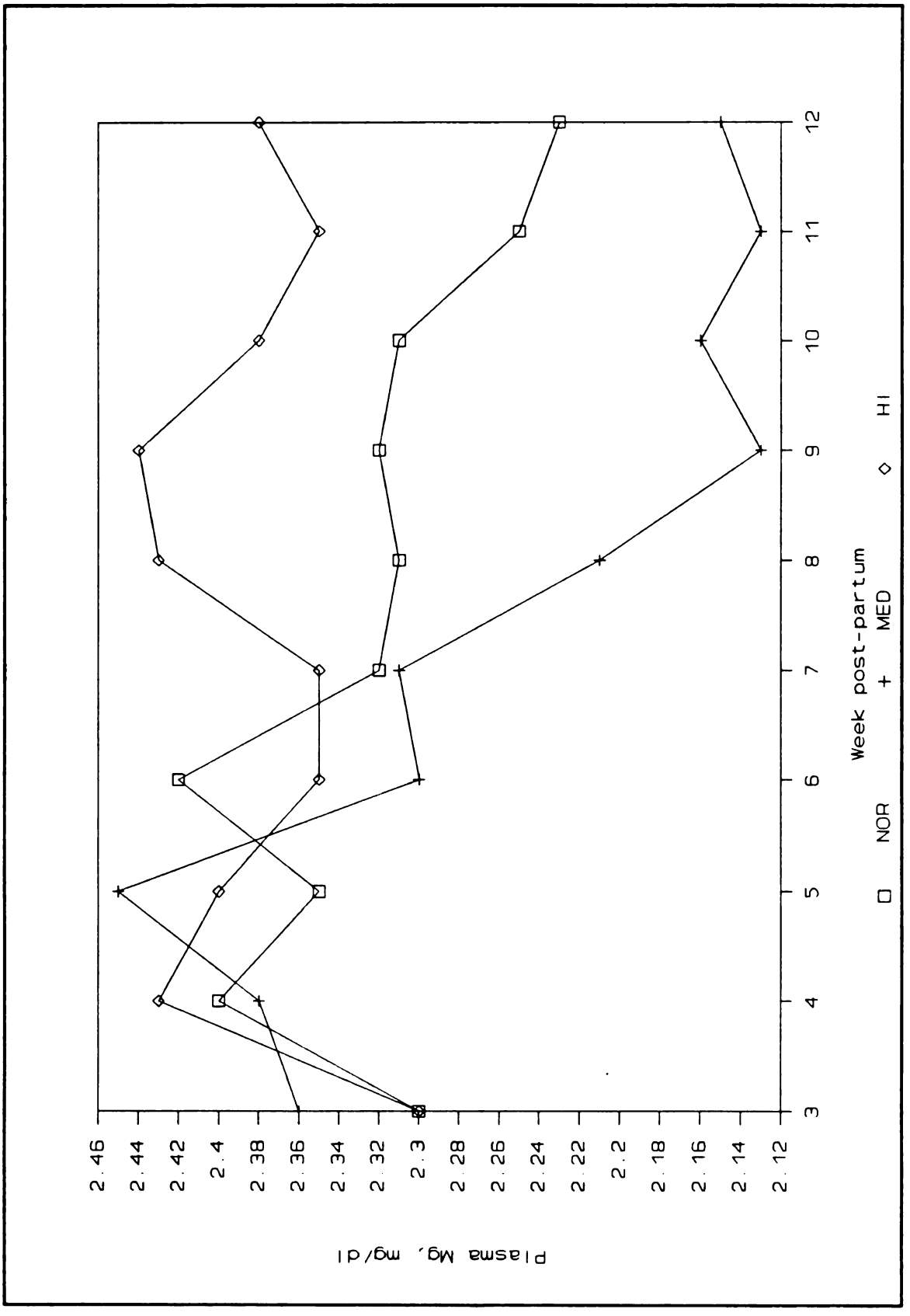

Plasma Ca, mg/dl
10.1
10
9.9
9.8
9.7
9.6
9.5
9.4
9.3
9.2
3
4
5
6
7
8
9
10
11
12
Week post-partum
NOR
MED
HI

Figure 4. Effects of Mg supplementation on plasma Mg. Mean square error is 0.16.


Plasma Mg, mg/dl
2.46
2.44
2.42
2.4
2.38
2.36
2.34
2.32
2.3
2.28
2.26
2.24
2.22
2.2
2.18
2.16
2.14
2.12
3
4
5
6
7
8
9
10
11
12
Week post-partum
NOR
MED
HI

containing 50% corn silage (DM basis). Supplementing a commercial buffer mixture high in Mg to lactating cows fed a milk-fat depressing, corn silage based diet did not affect feed intake (Solórzano et al., 1989). On the other hand, supplementing .5% MgO of differing particle sizes to cows in mid-lactation fed a high concentrate, restricted roughage diet decreased feed intake (Thomas et al., 1984). Askew et al. (1971) reported that supplementing 1% MgO to cows in various stages of lactation decreased feed intake. These results suggest that Mg per se does not affect feed intake when it is included up to .48% of diet DM, and that any negative effects of Mg supplementation on feed intake are due to factors such as chemical properties, particle size, palatability, and level of inclusion of the Mg supplement.

Feeding HI failed to improve milk production in this trial. Furthermore, feeding MED depressed milk yield when compared to the combined effect of feeding NOR and HI. The effects of feeding MED on milk production can be partially explained by the decreased intake of ADF observed after the 6th week post-partum. Other workers (Teh et al., 1985, O'Connor et al., 1988) concluded that .45 and .48% Mg improved milk production compared to feeding lower levels of Mg when cows were fed corn silage based diets.

Concentration of milk components was unaffected by increasing levels of Mg supplementation. The high concentration of milk fat in this trial can be attributed to

the feeding of the high-roughage, alfalfa based diet and not to the Mg supplement. Alkalizing agents and buffers will alleviate milk-fat depression but will not raise milk fat concentration in cows that are not milk-fat depressed. Supplementing levels of Mg similar to the ones used in this trial had no effects on milk fat concentration (Teh et al., 1985, O'Connor et al., 1988). Solórzano et al. (1989) reported a linear increase in milk fat concentration, milk fat yield, and 3.5% FCM when Mg supplemented as a commercial buffer mixture (Rumen-Mate) was increased from 0.24 to 0.87% of the dietary DM. Feeding high levels of Mg (>.6%) results in decreased milk protein concentration and milk protein yield (O'Connor et al., 1988, Solórzano et al., 1989). Feeding MED in this trial decreased the yield of milk fat, milk protein, milk lactose, and milk SNF compared to feeding the other two levels of Mg. The effects of feeding MED on the yield of milk components can be explained by the decrease in the yield of milk.

The primary difference between this trial and those reported by (Teh et al., 1985, O'Connor et al., 1988, and Solórzano et al., 1989) is the forage fed. Thus, it appears that the lactational performance response to Mg supplementation is dependent on the environmental conditions encountered within the lumen of the alimentary tract as influenced by diet, more specifically the source of roughage. Differences in the types of diets (i.e. acidogenic vs. normal

diets) can account for differences in Mg solubility, absorption, alkalizing effects, and effects in the reticulo-rumen.

Whether Mg exerted its beneficial effects on improving lactational performance at the alimentary tract or post-absorptive level in the studies reported by Teh et al. (1985), O'Connor et al. (1988), and Solórzano et al. (1989) is not clear. However, since Mg supplementation seems to improve the lactational performance of cows fed corn silage based diets and not of those cows fed alfalfa based diets, it is tempting to conclude that Mg effects are at the alimentary tract level and not at the post-absorptive level.

Supplemental Mg failed to increase plasma Mg concentrations. Our results agree with those of Emery et al. (1986) and Thomas et al. (1984) when supplementing $Mg(OH)_2$, and O'Connor et al. (1988) who supplemented up to .48% Mg as $MgPO_4$. Increasing blood Mg levels by supplementing MgO not always results in improved lactational performance (Jesse et al., 1981, Thomas et al., 1984). In this trial, cows fed MED had the poorest lactational performance, lowest overall plasma Mg concentration, and their plasma Mg level dropped after the 7th week post-partum. These results suggest that a minimum level of blood Mg must be maintained in order to avoid a decrease in lactational performance. This level of plasma Mg is around 2.32 mg/dl, and is well above the plasma Mg level at which signs of hypomagnesemia are observed.

In summary, supplementing Mg up to .36% of dietary DM did not improve the lactational performance of early lactation cows and had no effects on feed intake. Feeding .31% Mg decreased milk production and milk fat yield and tended to decrease the production of FCM, milk protein, milk lactose, and milk SNF. We conclude that the current NRC recommendation for Mg maximizes the lactational performance of early lactation Holstein cows fed an alfalfa based diet.

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CHAPTER III

EFFECTS OF INTRAVENOUS Mg INFUSION ON HOLSTEIN COWS FED A HIGH-GRAIN DIET

L. C. Solórzano, J. S. Liesman, R. S. Emery

Department of Animal Science

Michigan State University

East Lansing 48824

and

L. E. Armentano and R. R. Grummer

Department of Dairy Science

University of Wisconsin-Madison

Madison 53706

Key words: magnesium, high-grain diet, dairy cow, milk-fat depression

INTRODUCTION

Magnesium is a nutrient required by all farm species due to its participation in fundamental biochemical events at the tissue level. Additionally, Mg is required for activity by ruminal microbes (Durand and Kawashima, 1980). Supplemental Mg can increase ruminal digestion of forages, although excess ruminal Mg may decrease fermentation (Wilson, 1980). Supplementing Mg above NRC (1989) recommendations improves the lactational performance of dairy cows fed corn silage based diets (O'Connor et al., 1988, Teh et al., 1986, Solórzano et al., 1989). Therefore, the dietary allowance of Mg required to maximize milk production is not well defined. Alterations of dietary Mg may affect milk production by direct effects of Mg at the tissue level or by alterations in nutrient supply from the forestomach.

Feeding high-grain diets to lactating dairy cows can lead to a condition known as the "low milk-fat syndrome". Several theories are available to explain the causes of milk-fat depression (Solórzano, 1988). The glucogenic theory proposes that increases in blood insulin mediate milk-fat depression (McClymont and Vallance, 1962, Jenny et al., 1974). Emery et al. (1965) supplemented MgO and successfully alleviated milk-fat depression. Antacid mixtures high in Mg also alleviate milk-fat depression (Solórzano et al., 1989). Feeding MgO may increase the ruminal acetate:propionate ratio and rumen pH, changes generally associated with increased milk fat

concentration (Emery, 1983). Emery et al. (1965) suggested that Mg increases the uptake of endogenous acetate and triglycerides (TG) from blood plasma into mammary gland tissue. Another post-ruminal effect of Mg is to modulate blood somatotropin (ST) concentrations. At low serum Mg concentration, the increase in ST concentration due to decreased feed intake is less than the increase observed at higher levels of serum Mg (Emery et al., 1986). The effects of different levels of blood Mg on ST concentration when feed intake is not affected remains to be established.

Objectives of these studies were to determine: 1) if increasing blood Mg supply would improve the lactational performance of dairy cows fed a milk-fat depressing diet adequate in Mg content (NRC, 1989), 2) if increasing blood Mg supply would alleviate milk-fat depression, and 3) the effects of increasing blood Mg levels on the concentration of blood somatotropin and insulin.

MATERIALS AND METHODS

Trial 1

Six multiparous Holsteins, 150 to 170 d post-partum, were fed a milk-fat depressing diet. The diet consisted of 60% grain mix and 40% corn silage (DM basis), formulated to meet or exceed nutrient recommendations (except for fiber) for a 625 kg cow producing 35 kg milk/d and consuming 20 kg DM/d

(NRC, 1989). Composition of the diet is in Table 1. Cows were offered a total mixed ration (TMR) ad lib twice daily at 1100 and 2230 h. Feed offered and refused was weighed daily.

Cows received each treatment according to a single reversal design with two periods. Treatments were the aseptic, intravenous drip infusion of saline solution (control, 9 g NaCl/l) or an isotonic magnesium solution (20.86 g $MgCl_2 \cdot 6\ H_2O$/l). Our goal was to infuse, via the jugular vein, 5.5 l/d of either solution. Cows were fed the experimental diet for 21 d prior to the start of infusions, and for 9 d after the end of infusions. Infusion periods lasted 9 d each, with 10 d between the two infusion periods. Jugular catheters were installed on d 1 of each infusion period. Cows were disconnected from the infusion tubing twice daily for each milking for approximately 1 h each time. At this time, catheters were flushed with 3 to 5 ml of saline containing 91 units sodium heparin/ml (LyphoMed Inc., Melrose Park, IL). All cows were treated prophylactically with 10 million units penicillin (Cristicillin, E. R. Squibb and Sons, Princeton, NJ), administered by intramuscular injection, from the start of each infusion until 5 d post-infusion.

Grain mix and corn silage were analyzed for DM by drying at 105 °C, NDF (McQueen and Nicholson, 1979), CP by the Kjeldahl method (AOAC, 1975), and Mg and K were determined by inductively coupled plasma emission spectrophotometry (Schulte et al., 1987). The day samples were taken, feeds were

Table 1. Nutrient composition of ration ingredients for Trial 1[1].

Feed	DM	CP	NDF	K	Mg
Concentrate[2]	89.0	22.0	12.0	1.35	0.36
Corn silage	45.0	8.1	45.6	1.05	0.14
Mixed ration[3]	64.0	16.4	25.4	1.23	0.27

[1] g/100 g DM except DM is g/100 g as fed.
[2] Contained (per kg as fed): 354 g soybean meal, 604 ground corn, 26 g dicalcium phosphate, 6.55 g iodized salt, 8.75 g $MgSO_4$, 5,100 IU vitamin A, 960 IU vitamin D, and 225 mg trace mineral mix. Trace mineral mix contained (per 100 g as fed): 0.32 g $CoSO_4 \cdot 7H_2O$, 0.43 g KI, 17.87 g $CuSO_4 \cdot 5H_2O$, 40.69 g $MnSO_4 \cdot H_2O$, and 40.69 g $ZnSO_4 \cdot 7H_2O$.
[3] Calculated from 40% corn silage and 60% concentrate, based on toluene DM.

analyzed for DM by toluene distillation (AOAC, 1975) and daily feed offerings adjusted accordingly.

Milk was sampled 10 d prior to and after feeding the experimental diet, when cows were fed an alfalfa based diet. Milk production was recorded and milk sampled at each milking starting 5 d prior to the start of infusions until 7 d after the end of infusions. Milk fat and protein concentration were analyzed by near infrared spectrometry (Wisconsin DHIA Laboratory, Appleton, WI).

Blood was sampled from an internal ileac artery or tail vessel at 1300 h on d 9 of each infusion period. Blood was collected into glass tubes containing sodium heparin to provide 25 units heparin per ml of blood, kept on ice until centrifuged at 1200 x g for 15 min at 4 °C. Plasma was stored at -20 °C until analyzed for Ca and Mg by atomic absorption (Perkin Elmer Model 403, Norwalk, CT), non-esterified fatty acids (NEFA, McCutcheon and Bauman, 1986), and TG (Patton, 1989, Appendix A).

Data were analyzed by analysis of variance using the generalized linear model procedure (SAS, 1985). Milk production and components, and DM intake for the last 6 d of each period, and data for plasma Ca, Mg, NEFA, and TG concentration were analyzed by a model including terms for cow, period and Mg infusion effects. Period was dropped from the model for plasma mineral analysis as it had no effect on these variables ($P>.80$). In addition, daily production of

milk and milk components from the 40 d sampling period were analyzed using a split plot in time analysis to test for an interaction between treatment sequence and the day to day pattern of milk secretion. Terms included in this model were treatment sequence, cow, day, and treatment sequence by day.

Trial 2

Six multiparous and six uniparous Holstein cows averaging 34 d in milk were fed a milk-fat depressing diet as for Trial 1 (Table 2). Cows were fed ad lib a TMR twice daily at 0330 and 1300 h. Feed offered and refused was weighed daily. Cows received the experimental diet for 21 d before the start of infusions. Data obtained during this period were used to pair cows. Cows continued on the experimental diet for 10 d after the end of infusions.

Treatments were applied in a 2 x 3 cross over design (Gill, 1979). Treatments were the aseptic, intravenous infusion of LO) 287.7 g NaCl/l (0 g Mg/d), MED) 416.7 g $MgCl_2 \cdot 6 H_2O$/l (6 g Mg/d), and HI) 833 g $MgCl_2 \cdot 6 H_2O$/l (12 g Mg/d). We expected LO and MED to be isochloric, but because of a mistake in calculations, LO provided an extra 3.5 g Cl/d compared to MED. Our goal was to infuse via jugular vein 120 ml/d using an Autosyringe pump (Model AS*2BH, Hooksett, NH) that was calibrated to dispense .3125 ml every 1/16 h. Infusion periods lasted 9 d each, with 10 d between the two infusion periods. Jugular catheters were installed on d 1 of

Table 2. Ingredient composition of experimental diet for Trial 2.

Ingredient	g/100 g ration DM
Corn silage	40.1
Soybean meal	22.9
High moisture corn	34.2
Dicalcium phosphate	0.67
Limestone	1.26
MgO	0.10
Trace mineralized salt[1]	0.46
Selenium 200[2]	0.15
Vitamin ADE premix[3]	0.20

[1] Contains (per 100 g as fed): 0.593 g Zn, 0.796 g Mn, 0.314 g Cu, 0.017 g I, 0.013 g Se, 175,330 IU vitamin A, 51,982 IU vitamin D^3, and 729 IU vitamin E.

[2] Contains 200 mg Se/kg.

[3] Contains (per 100 g as fed) 593 mg Zn, 314 mg Cu, 796 mg Mn, 17 mg I, 13 mg Se, 175.1 KIU vitamin A, 51.9 KIU vitamin D, and 727.8 IU vitamin E.

each infusion period, and on d 8 of each infusion period in the contralateral jugular vein for blood sampling. Rectal temperature was monitored twice daily, and penicillin applied only if rectal temperature exceeded 39.4 °C. Beginning 2 d prior to the start of each infusion period cows were milked in their stanchions. Therefore, there was no need to interrupt the infusions for milking. At any other time, cows were milked in the parlor.

On d 9 of each period, jugular blood was sampled at 20 min intervals for 8.67 h, from 950 to 1830 h. Blood was allowed to clot overnight at room temperature, then at 4 °C for 3 h, and then centrifuged at 3,000 X g for 10 min. Serum was stored at -20 °C until analyzed for ST (Zinn et al., 1989), insulin (Villa-Godoy et al., 1990), Ca, Mg, K, Na by atomic absorption spectroscopy (Perkin Elmer Model 5000, Norwalk, CT), and creatinine (Sigma Kit 555-A, St. Louis, MO). Milk and urine were sampled on d 7 of each infusion period. Milk was stored at -20 °C until analyzed for minerals as above. Urine was stored at -20 °C until analyzed for minerals and creatinine as above.

Corn silage and high moisture corn were sampled weekly and dried at 105 °C. Accordingly, feed offerings were adjusted weekly. The TMR was sampled weekly throughout the trial. The TMR was analyzed for DM by drying at 60 °C for 48 h, organic matter (OM) by combustion at 600 °C (AOAC, 1975), CP by the Kjeldahl method (AOAC, 1975), ADF (Goering and Van

Soest, 1968), Ca, Mg, K, and Na by atomic absorption spectroscopy (Perkin Elmer Model 5000, Norwalk, CT). Additionally, TMR and orts were sampled on 3 consecutive d each period, and 10 fecal grab samples taken at 7 h intervals. Feed, orts and feces were analyzed as above, and for lignin (Goering and Van Soest, 1968), which was used as a digestibility marker.

Milk was sampled 10 d before and 10 d after feeding the experimental diet, when cows were fed an alfalfa based diet. Milk production was recorded and milk was sampled at each milking while the cows were fed the experimental diet. Milk was analyzed for fat, protein, lactose, and SNF by near infrared spectrometry (Michigan DHIA Laboratory, East Lansing, MI).

Data were analyzed as for Trial 1. The model used for lactational performance, feed intake and digestibility, creatinine, and kidney filtration ratio measurements was:

$$Y_{ijkl} = \mu + A_i + C_j(A_i) + P_k + T_l + (A_i*T_l) + (A_i*P_k) + (P_k*T_l) + E_{ijkl}$$

Where A_i is the average effect of the age of the cow (primiparous vs. multiparous),

$C_j(A_i)$ is the average effect of cow within age,

P_k is the average effect of period,

T_l is the average effect of treatment,

E_{ijkl} is the random residual, assumed normally and independently distributed.

Table 3. Chemical composition of experimental diet in Trial 2.

Constituent	g/100 g DM
DM[1]	54.8
Organic matter	94.8
CP	16.5
ADF	13.0
Ca	0.68
Mg	0.20
K	0.81
Na	0.16

[1] DM expressed as g/100 g of ration as fed.

When an interaction term was not significant (P>0.20), that term was deleted and data for the dependent variable was reanalyzed with the reduced model. Single degree of freedom orthogonal contrasts were used to compare LO vs HI (linear) and to compare MED vs LO and HI (curvilinear). Least square means and standard errors are presented.

Blood variables were analyzed using a split plot design on time. The model used was:

$$Y_{ijklm} = \mu + A_i + C_j(A_i) + P_k + T_l + (A_i*T_l) + (A_i*P_k) + (P_k*T_l) + (P_k*T_l*A_i) + S_m + (S_m*T_l) + (S_m*A_i) + (S_m*P_k) + E_{ijklm}$$

Where A_i is the average effect of the age of the cow (primiparous vs. multiparous),

$C_j(A_i)$ is the average effect of cow within age,

P_k is the average effect of period,

T_l is the average effect of treatment,

S_m is the average effect of sampling time,

E_{ijkl} is the random residual, assumed normally and independently distributed.

Treatment was tested with $(P_k*T_l*A_i)$ as the error term; sampling time was tested by the residual. Single degree of freedom orthogonal contrasts were the same as for the previous model. Least square means and standard errors are presented.

The effects of infusion on milk yield and composition, and feed intake were tested using the model:

$$Y_{ijk} = \mu + A_i + C_j(A_i) + P_k + E_{ijk}$$

Where A_i is the average effect of the age of the cow

(primiparous vs. multiparous),

$C_j(A_i)$ is the average effect of cow within age,

P_k is the average effect of period (pre-infusion (I), infusion periods (II and IV), between-infusion (III), and post-infusion (V))

E_{ijkl} is the random residual, assumed normally and independently distributed.

Single degree of freedom orthogonal contrasts compared infusion periods (II and IV) vs non-infusion periods (I, III, and V). Means and the mean square error are presented.

RESULTS

Trial 1

One cow was removed from the trial due to pulmonary hypertension and cardiac insufficiency which began during Period I. This was presumably due to shedding of blood clots from the jugular catheter and was accompanied by erratic and depressed feed intake.

Infusing $MgCl_2$ increased plasma Mg concentration ($P<0.05$) and did not affect ($P>0.10$) DM intake, milk composition or milk secretion compared to saline infusion (Table 4). Infusing $MgCl_2$ tended to increase plasma TG ($P>0.12$) and did not affect ($P>0.50$) plasma Ca and NEFA. There was no significant ($P>0.10$) interaction of treatment sequence with

Table 4. Milk yield and composition, DM intake, and plasma variables for Trial 1.

	TREATMENT			
	Saline	$MgCl_2$	SE	P[1]
kg/d				
Intake	23.5	22.2	0.7	0.30
Milk	30.3	29.0	0.6	0.23
3.5% FCM	27.0	26.3	0.5	0.37
Fat	0.85	0.84	0.02	0.77
Protein	1.01	0.97	0.02	0.18
Concentration, g/100 ml				
Fat	2.82	2.91	0.06	0.35
Protein	3.38	3.35	0.01	0.35
Plasma variables, mg/dl				
Mg	1.90	3.26	0.30	0.05
Ca	9.05	8.64	0.38	0.50
TG[2]	7.72	8.74	0.43	0.12
NEFA[3]	203.6	208.8	8.3	0.68

[1] Probability that contrast is between treatments that do not differ.
[2] Triglyceride.
[3] Non-esterified fatty acid, μEq/l.

day for the production of milk and milk components, or concentration of milk components.

Trial 2

Infusing MED tended ($P<0.12$) to decrease milk energy output and milk fat concentration compared to the combined effect of infusing LO and HI (Table 5). Infusing HI decreased ($P<0.04$) the milk concentration of K. There were no other effects ($P>0.17$) due to treatment on milk yield and composition.

Intake of dietary constituents was not affected ($P>0.22$) by treatment (Table 6, Appendix B). Infusing MED increased ($P<0.05$) the digestibility of Na compared to the combined effect of infusing LO and HI. Infusing HI increased ($P<0.06$) serum Mg compared to infusing LO (Table 7). Infusing MED tended to increase ($P<0.10$) insulin compared to the combined effect of infusing LO and HI. There were no other effects ($P>0.32$) of treatment on serum variables. Infusing MED increased the urine concentration of Ca ($P<0.01$), Mg ($P<0.005$), and Na ($P<0.03$) compared to the combined effect of infusing LO and HI. Treatment had no effects ($P>0.37$) on the kidney filtration ratio of cations. Figure 1 shows the variation of serum Mg from 950 to 1830 h. There was no treatment X sampling time interaction ($P>0.95$). The increase in serum Mg observed around 14 h was probably associated with feeding. The decrease in serum Mg observed around 16 h was probably associated with the preparation for milking. There

Table 5. Milk yield and composition for Trial 2.

	TREATMENT[1]			
	LO	MED	HI	SE
Yield, kg/d				
Milk	37.2	37.4	36.8	0.6
3.5% FCM	42.4	42.0	42.3	0.73
Fat	1.62	1.60	1.63	0.03
Protein	1.19	1.20	1.18	0.02
Lactose	1.81	1.81	1.79	0.03
SNF	4.88	4.86	4.86	0.08
Energy[2]	776.8	769.6	784.1	4.4
Concentration, g/100 ml				
Fat[3]	4.38	4.30	4.44	0.05
Protein	3.21	3.21	3.22	0.01
Lactose	4.87	4.87	4.88	0.01
SNF	13.15	13.08	13.24	0.06
Minerals, mg/dl				
Ca	125.03	120.29	120.03	2.94
Mg	107.81	105.33	103.98	2.28
K[4]	124.0	117.0	105.0	5.0
Na	98.0	91.0	95.0	6.0

[1] LO = 0, MED = 6, and HI = 12 g Mg infused/d.
[2] Units are Kcal/kg of milk. Calculated from Tyrrel and Reid (1965). Probability that the contrast MED vs LO and HI is among treatments that do not differ ($P<0.12$).
[3] Probability that the contrast MED vs LO and HI is among treatments that do not differ ($P<0.11$).
[4] Probability of overall treatment effect ($P<0.09$). Probability that the contrast LO vs. HI is among treatments that do not differ ($P<0.04$).

Table 6. Intake and digestibility of dietary constituents for Trial 2.

	TREATMENT[1]			
	LO	MED	HI	SE
Intake, kg/d				
DM	21.9	22.1	22.1	0.1
Intake, g/d				
Mg	42.29	42.45	41.90	0.69
Digestibility				
DM	0.64	0.64	0.65	0.02
OM[2]	0.66	0.66	0.67	0.02
CP	0.68	0.69	0.69	0.02
ADF	0.48	0.46	0.49	0.03
Ca	-0.03	0.06	-0.02	0.05
Mg	.20	.25	.26	0.04
K	0.86	0.87	0.86	0.01
Na[3]	0.72	0.83	0.72	0.04

[1] LO = 0, MED = 6, and HI = 12 g Mg infused/d.
[2] Organic matter.
[3] Probability of overall treatment effect ($P<0.13$). Probability that the contrast MED vs LO and HI is among treatments that do not differ ($P<0.05$).

Table 7. Cations in serum and urine, serum hormones, and kidney filtration ratio for Trial 2.

	TREATMENT[1]			
	LO	MED	HI	SE
Serum, mg/dl				
Ca	9.81	10.02	9.50	0.06
Mg[2]	2.84	2.92	3.11	0.02
K	31.17	31.08	31.19	0.21
Na	555.04	573.72	576.83	4.99
Triglyceride	6.66	9.60	9.85	1.55
Creatinine	1.44	1.33	1.18	0.21
Insulin[3]	0.46	0.49	0.46	0.02
ST[4]	2.88	2.84	2.90	0.10
Urine, mg/dl				
Ca[5]	57.13	69.47	96.33	7.76
Mg[6]	521.96	1046.79	1316.94	99.67
K	8.19	7.65	7.44	0.72
Na[7]	2.40	0.86	1.45	0.30
Creatinine	172.47	150.26	108.82	59.54
Kidney filtration ratio[8]				
Ca	0.002	0.004	0.003	0.002
Mg	0.003	0.007	0.005	0.003
K	0.002	0.005	0.004	0.002
Na	0.005	0.014	0.011	0.005

[1] LO = 0, MED = 6, and HI = 12 g Mg infused/d.
[2] Probability that the contrast LO vs. HI is among treatments that do not differ (P<0.06).
[3] Units are ng/ml. Probability that the contrast MED vs LO and HI is among treatments that do not differ (P<0.10).
[4] Somatotropin. Units are ng/ml.
[5] Probability that the contrast LO vs. HI is among treatments that do not differ (P<0.01).
[6] Probability that the contrast LO vs. HI is among treatments that do not differ (P<0.001).
[7] Probability that the contrast LO vs. HI is among treatments that do not differ (P<0.07). Probability that the contrast MED vs LO and HI is among treatments that do not differ (P<0.03).
[8] (urine cation/serum cation) * (serum creatinine/urine creatinine).

Figure 1. Effects of Mg infusion on serum Mg in Trial 2. Pooled standard error is 0.10. Cows were fed at 1300 h and preparation for milking started at 1600 h.

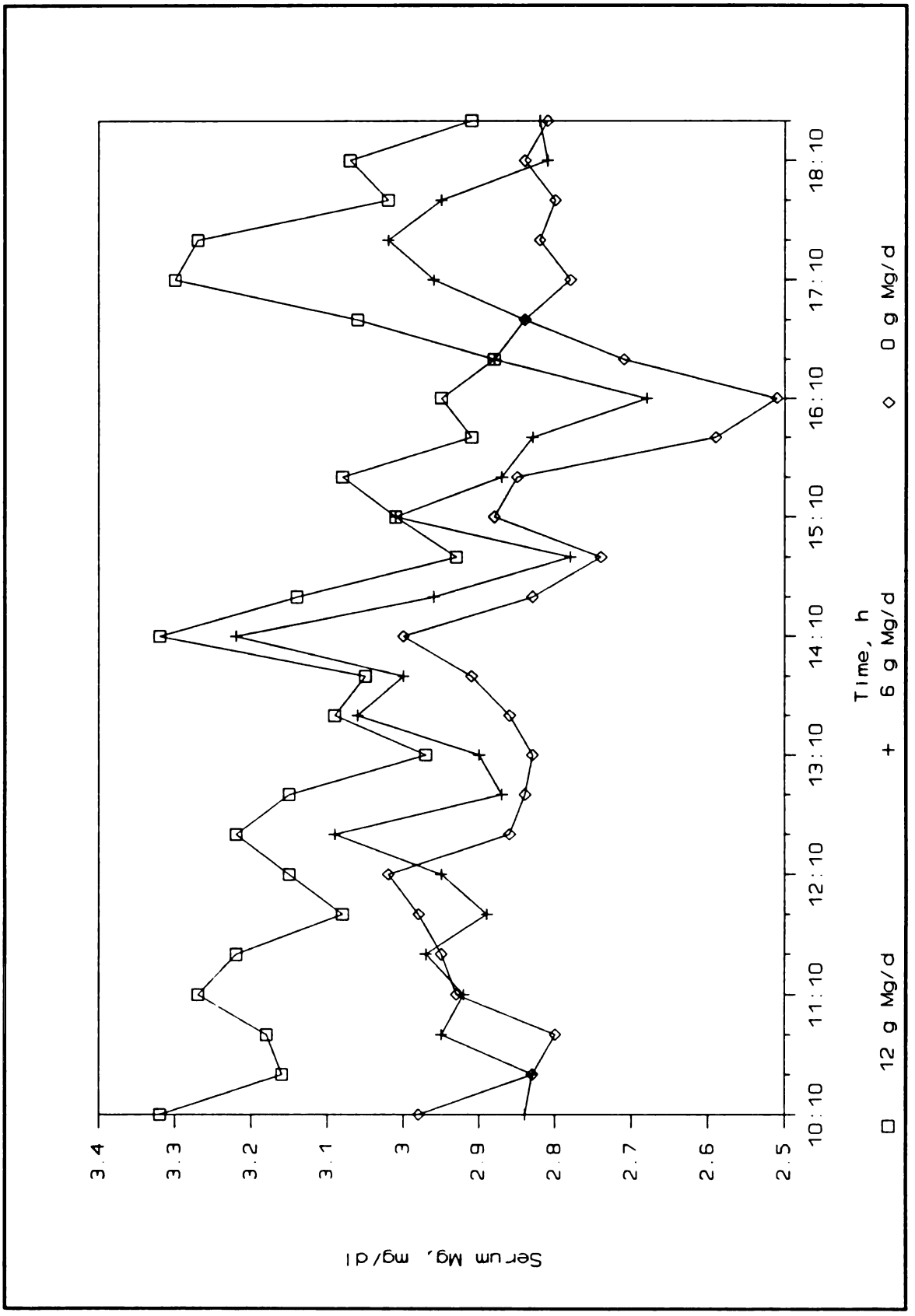
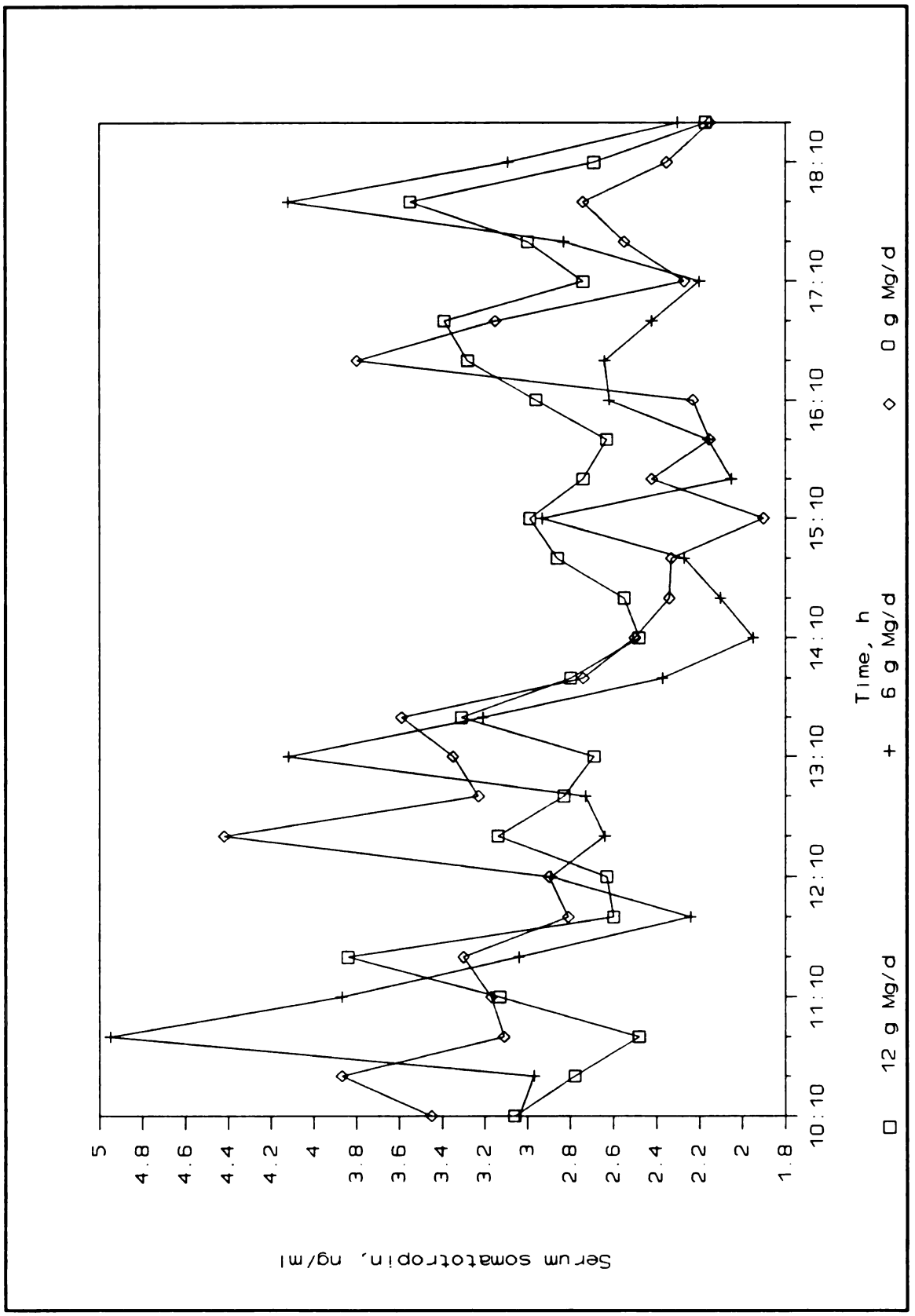

Serum Mg, mg/dl
3.4
3.3
3.2
3.1
3
2.9
2.8
2.7
2.6
2.5
10:10
11:10
12:10
13:10
14:10
15:10
16:10
17:10
18:10
Time, h
□ 12 g Mg/d
+ 6 g Mg/d
◇ 0 g Mg/d

Figure 2. Effects of Mg infusion on serum somatotropin in Trial 2. Pooled standard error is 0.44. Cows were fed at 1300 h and preparation for milking started at 1600 h.


Serum somatotropin, ng/ml
5
4.8
4.6
4.4
4.2
4
3.8
3.6
3.4
3.2
3
2.8
2.6
2.4
2.2
2
1.8
10:10
11:10
12:10
13:10
14:10
15:10
16:10
17:10
18:10
Time, h
□ 12 g Mg/d
+ 6 g Mg/d
◇ 0 g Mg/d

was a treatment X sampling time interaction (P>0.09) for plasma ST (Figure 2, Appendix G).

Infusing MED decreased the fecal content of Ca (P<0.01), and Na (P<0.06) compared to the combined effect of infusing LO and HI (Table 8). Treatment had no effect on the fecal content of Mg (P>0.18). The concentration of milk components was greater (P<0.0001) during the two infusion periods, when cows were milked in their stanchion, compared to the non-infusion periods, when cows were milked in the parlor (Table 9). There were no effects of infusion period on milk yield (P>0.17, Table 9) or feed intake (P>0.60, data not shown).

DISCUSSION

Actual amount of infusate delivered to each cow varied. Average infusion per cow was 45.07 ± 1.30 l/infusion period for Trial 1, and 1010.4 ± 5.9 ml/infusion period for Trial 2. Animal activity can account for most of the variation of infusate delivered.

The concentration of milk fat for Trial 1 and 2 averaged 3.40 and 4.7 g/100 ml of milk pre-trial and 3.70 and 3.52 g/100 ml of milk post-trial when cows consumed an alfalfa based diet. Milk fat content was 2.87 (Trial 1) and 3.47 g/100 ml of milk (Trial 2) while cows were on the experimental, milk-fat depressing diet. Therefore, the experimental diet did depress the concentration of milk fat as

planned, although the recovery of milk fat concentration was not as marked in Trial 2.

The infusion periods (II and IV, Table 9) during Trial 2 significantly altered milk composition. This alteration of milk composition was likely due to milk sampling and not to an infusion effect. During the infusion periods, cows were milked in their stanchion using a portable bucket milking system, compared to milking in the parlor at any other time. Milk was sampled manually from the bucket during the infusion periods, and from an on line milk sampler when cows were milked in the parlor. However, since all cows were treated the same, the relative differences among treatments are still valid. Furthermore, milk yield and feed intake were not affected during the infusion periods.

One must bear in mind that neutral salts were infused in these trials. Therefore, the anion-cation balance of these cows was not affected. Tucker et al. (1988) reported that oral Cl created an unfavorable cation-anion balance for lactating cows. Apparently, this was due to alterations in the absorption of the relative amounts of Na, K and Cl from the gastro-intestinal tract. In Trial 1, solutions were isotonic, and in Trial 2 LO and MED treatments were isochloric while HI provided twice as much Cl. Based on the results of these trials, it appears that the infusion of Cl had no effect on lactational performance.

The Mg that was supplied in excess in Trial 2, was

primarily excreted via urine. There were no differences detected in kidney filtration ratio, digestibility and fecal content of Mg. Our results contradict those of Allsop and Rook (1979), who reported that intravenous Mg infusion to hypomagnesemic sheep increased the endogenous fecal loss of Mg. The concentration of Mg in milk was not altered by any excess Mg provided. This was expected since there is no significant correlation between serum Mg and milk Mg concentrations (Hardt et al., 1989).

Normal Mg concentrations in plasma of cattle range from 1.7 to 3.3 mg/dl (Reinhardt et al., 1988). The intravenous infusion of Mg to normomagnesemic cows resulted in significantly higher blood Mg levels that were within physiological range. However, there were no responses in lactational performance or alleviation of milk-fat depression. Although a direct comparison between intravenous and oral Mg supplementation was not made in these trials, the lack of response to infused Mg suggests that the improvement of lactational performance (Teh et al., 1985) and alleviation of milk-fat depression (Solórzano et al., 1989) observed when supplementing Mg salts orally is not mediated via an increased supply of Mg to the tissues. Emery and Thomas (1967) reported an increase in milk fat concentration due to parenteral administration of Mg. However, milk yield was decreased with no effect on milk fat yield. Wilson (1980) compared oral (10 g Mg/d) and subcutaneous (2 g Mg/d) administration of Mg to

grazing dairy cows with plasma Mg levels of 1 mg/dl. Although both treatments increased plasma Mg to 1.5 mg/dl, only the cows supplemented orally improved milk and milk fat yield. Supplementing up to 0.48% Mg as $MgPO_4$ did not alter plasma Mg levels but increased the yield of milk (O'Connor et al., 1988). The same workers showed that feeding 0.60% Mg increased plasma Mg levels, but decreased milk yield compared to feeding lower levels of Mg. This negative effect was probably due to palatability effects. The intravenous infusion of 12 g Mg/d in our studies is equivalent to adding 35 g of dietary Mg from $MgPO_4$, assuming a coefficient of absorption of 35% (O'Connor et al., 1988). The increased available Mg from our infusions falls between the estimated increase in available Mg obtained by feeding 0.42 and 0.8% $MgPO_4$ in the study by O'Connor et al. (1988). These data also support the idea that the effects of Mg salts on lactational performance are at the gastro-intestinal tract level and not by increasing the level of Mg supplied to the tissues.

Increased insulin levels were implicated in the development of milk-fat depression (McClymont and Vallance, 1962, Jenny et al., 1974). Because Mg salts alleviate milk-fat depression, it is possible that Mg has a post-absorptive effect on insulin. Omission of Mg from the medium perfusing rat pancreas inhibited the release of insulin (Curry et al., 1977). On the other hand, high levels of Mg can suppress insulin production (Durlach et al., 1990). In Trial 2, serum

Mg level had no effect on serum insulin concentration. However, oral MgO may be alleviating milk-fat depression by decreasing blood insulin concentrations in response to decreased rumen propionate production.

Somatotropin is a well recognized lipolytic agent. Higher levels of ST could mean a higher mobilization of fatty acids from adipose tissue, thus providing the mammary gland with extra substrate for milk fat synthesis. This could mean that ST could alleviate milk-fat depression or in cows not milk-fat depressed, increase milk fat yield. McGuffey et al. (1991) reported a 0.19% increase in milk fat concentration due to administration of 11.5 mg/d of exogenous recombinant ST compared to placebo administration. To our knowledge, only Emery et al. (1986) showed a positive relationship between Mg and ST in cattle. Our work shows that cows infused HI had a more constant ST concentration, but there was no overall treatment effect on ST concentrations.

An interesting observation, although non-significant ($P>0.12$ for Trial 1, $P>0.20$ for Trial 2), is the trend followed by blood TG concentration. Based on data from Tables 4 and 7, blood TG concentration increased as the blood Mg level increased. Rayssiguier and Gueux (1983) showed using hypomagnesemic rats that a Mg deficiency produces hypertriglyceridemia. These authors concluded that hypertriglyceridemia was the result of impaired TG clearance. Thus, elevated or low serum Mg levels cause

hypertriglyceridemia. In our work, impaired TG removal is unlikely. The major site of TG removal in the lactating cow is the mammary gland, and no differences were detected in milk fat concentration or yield among treatments. On the other hand, if the mammary gland was impaired in clearing TG, it could explain both the increased blood TG and the lack of alleviation of milk-fat depression by intravenous Mg supplementation.

In summary, short term intravenous infusions of varying levels of Mg as $MgCl_2 \cdot 6\ H_2O$ significantly increased blood Mg levels within the physiological range, but failed to improve the lactational performance or alleviate milk-fat depression in early or mid lactation Holstein cows fed high-grain diets. Our data suggest that the positive effects of orally administered MgO on lactational performance and the alleviation of milk-fat depression are not mediated via an increased Mg supply to the tissues.

Varying the levels of serum Mg failed to modulate serum insulin concentrations. Serum level of Mg did modulate serum ST concentrations. However, more research is needed to characterize the effects of serum Mg level on the concentration of serum ST.

Table 8. Fecal content of nutrients (g/100 g fecal DM) in Trial 2.

	TREAMENT[1]			
	LO	MED	HI	SE
DM	18.62	19.10	18.58	0.45
Lignin	4.37	4.32	4.41	0.19
OM[2]	91.15	91.83	91.39	0.22
CP	15.04	15.02	15.07	0.38
ADF	16.68	16.64	16.48	0.53
Ca[3]	1.94	1.77	1.95	0.04
Mg	0.44	0.42	0.43	0.01
K	0.31	0.29	0.33	0.03
Na[4]	0.13	0.08	0.14	0.02

[1] LO = 0, MED = 6, and HI = 12 g Mg infused/d.
[2] Organic matter. Probability that the contrast MED vs. LO and HI is among treatments that do not differ (P<0.09).
[3] Probability of overall treatment effect (P<0.02). Probability that the contrast MED vs. LO and HI is among treatments that do not differ (P<0.01).
[4] Probability that the contrast MED vs. LO and HI is among treatments that do not differ (P<0.06).

Table 9. Effect of infusion on milk yield and components during Trial 2.

	PERIOD[1]					
	I	II	III	IV	V	MSE
N, cows	12	12	12	12	12	
Yield, kg/d						
Milk[2]	37.3	37.4	37.5	36.7	34.2	3.5
Concentration, g/100 ml						
Fat[3]	3.54	4.54	3.50	4.21	3.36	0.25
Protein[3]	3.19	3.13	3.35	3.29	3.33	0.04
Lactose[3]	4.84	4.88	4.83	4.86	4.76	0.05
SNF[3]	12.27	13.25	12.38	13.07	12.14	0.25

[1] Period I = pre-infusion; II = 1st infusion period; III = between infusion periods; IV = 2nd infusion period; and V = post infusion period.

[2] Probability that the contrast II and IV vs I, III, and V is among the periods that do not differ (P>0.17).

[3] Probability that the contrast II and IV vs I, III, and V is among the periods that do not differ (P>0.0001).

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CHAPTER IV

EFFECTS OF SERUM Mg LEVEL ON STIMULATED LIPOLYSIS IN THE EARLY LACTATION HOLSTEIN COW

L. C. Solórzano, R. S. Emery, and J. S. Liesman

Department of Animal Science

Michigan State University

East Lansing 48824

Key words: magnesium, dairy cow, lipolysis, free fatty acid

INTRODUCTION

Lipolytic hormones or agents bind to receptors located in the plasma membrane of adipocytes and activate the enzyme adenylate cyclase (AC). Cyclic AMP (cAMP) is formed from ATP by the action of AC with Mg as a cofactor. The cAMP activates a protein kinase which phosphorylates and activates fatty acid mobilizing lipase (FAML) with Mg as a cofactor. This enzyme converts triglycerides (TG) to diglycerides and is considered rate limiting in lipolysis (Vance, 1988). Almost all reactions involving ATP hydrolysis occur in the presence of Mg ions (Ingraham, 1988). In lactating cows, glyceride synthesis seems a better control of fatty acid (FA) release from adipose tissue than FAML (Emery, 1973).

There is an accumulation of Mg in rat adipocytes when lipolysis is stimulated by catecholamines or adrenocorticotropin (Elliot and Rizack, 1974). There is a 470% increase in Mg content in the fatty tissue of cold treated rats provided with insufficient dietary Mg (Meyer et al., 1982). Stimulating lipolysis in sheep by fasting results in increased blood non-esterified fatty acids (NEFA) and decreased blood Mg (Rayssiguier and Larvor, 1976). Similar results were shown in dogs following ethanol withdrawal (Flink et al., 1979). Declines in plasma Mg were observed in calves and ewes fed low Mg diets and exposed to low ambient temperatures (Meyer et al., 1982, Terashima et al., 1984). An

increase in serum NEFA and a concomitant decrease in serum Mg were found after acute myocardial infarction in humans (Cohen et al., 1984). Possible explanations for the decrease in blood Mg are sequestration by adipocytes when lipolysis is stimulated (Elliot and Rizack, 1974), chelation by NEFA followed by possible sequestration by cell membranes (Flink et al., 1979), or a combination of both.

Objectives of this study were to determine: 1) the effect of different levels of serum Mg on serum NEFA concentration when lipolysis was stimulated in early lactation Holsteins fed a high-grain diet, 2) if Mg is redistributed to perirectal, subcutaneous adipose tissue when lipolysis is stimulated in early lactation Holsteins fed a high-grain diet, and 3) the effects of different Mg supply to perirectal, subcutaneous adipose tissue (SAT) on the activity of FAML, lipoprotein lipase (LPL), and glyceride synthesis after lipolysis is stimulated in early lactation Holsteins fed a high-grain diet.

MATERIALS AND METHODS

Animals and treatments

Six multiparous and six uniparous Holstein cows averaging 34 d in lactation were assigned to a 2 x 3 cross over design (Gill, 1978). Treatments were the aseptic, intravenous infusion of LO) 287.7 g NaCl/l, MED) 416.7 g $MgCl_2 \cdot 6 H_2O$/l, and HI) 833 g $MgCl_2 \cdot 6 H_2O$/l. We expected that LO and MED were

isochloric, but because of a mistake in calculations LO provided an extra 3.5 g Cl/d. Our goal was to infuse via jugular vein 120 ml/d using an Autosyringe pump (Model AS*2BH, Hooksett, NH) that dispensed .3125 ml every 1/16 h. Infusion periods lasted 10 d each, with 10 d between the two infusion periods. Jugular catheters were installed on d 1 of each infusion period, and on d 8 of each infusion period in the contralateral jugular vein for blood sampling. The last day of each infusion period, lipolysis was stimulated by injecting intravenously 6 mg of epinephrine (EPI, The Butler Co., Columbus, OH). Blood sampling at 10 min intervals started 20 min pre-EPI challenge and continued for 60 min post-EPI challenge. Immediately after the last blood sample was taken, a small, perirectal area of the cows was anesthetized using 10 ml of Lidocaine · HCl (AmVet, Fort Collins, CO) subcutaneously. A small incision was made, and 3 to 5 g of SAT were obtained. The SAT sample was rinsed with sterile water, and placed in dry ice within 10 min of the last blood sample (i.e., within 70 min of the EPI challenge). Infusions were maintained throughout this trial and were stopped at 1800 h, 3 to 4 h after the last biopsy was performed. Rectal temperature was monitored twice daily, and 15 million units penicillin (Aquacillin, Vedco, Inc., Overland Park, KS) applied twice daily only if temperature exceeded 39.4 °C. Details of animal handling, care, and lactational performance were provided elsewhere (Chapter III).

Analysis of serum and adipose tissue

Blood samples were treated as described in Chapter III. Serum and SAT were analyzed for Ca, Mg, K, and Na by atomic absorption spectroscopy (Perkin Elmer Model 5000, Cornwalk, CT). Dry matter (DM) was determined in SAT by drying at 55 °C for 24 h. Non-esterified fatty acids were analyzed according to McCutcheon and Bauman (1986). Triglycerides (TG) were analyzed using a modification (Appendix A) of the method by Patton (1989).

Analysis of enzymes in adipose tissue

Fatty acid mobilizing lipase

Activity was determined using a modification (Appendix C) of the method by Frederickson et al. (1981) as the release of ^{3}H oleic acid from 1.5 Mm triolein in a lecithin emulsion. Incubations lasted 10 min at 37 °C. Extraction of the labeled NEFA was according to Liesman et al. (1988). Activity was corrected by subtracting counts extracted at the same time point when no enzyme was present. Protein concentration in the homogenate was determined using a modification (Appendix F) of the method by Markwell et al. (1978). Triglyceride concentration in the homogenate was determined as described above.

Glyceride synthetase

Activity was determined using a modification (Appendix D) of the method by Benson and Emery (1971) as the incorporation

of ^{14}C palmitate into glycerides for 30 min at 37 °C. Activity was corrected by subtracting the counts extracted when no enzyme was present at time 0. Protein concentration was determined as above.

Lipoprotein lipase

Activity was determined by a modification (Appendix E) of the procedure by Liesman et al. (1988) as the release of ^{3}H oleic acid from 7.5 Mm triolein in a bovine serum albumin and Triton X-100 emulsion for 60 min at 37 °C. Activity was corrected by labeled NEFA released in the presence of heat denatured serum from a lactating cow containing 3.33 M NaCl at the same time point. Protein was determined as described above.

Statistical analysis

Serum NEFA, TG, Ca, Mg, K, and Na were analyzed by analysis of variance (SAS, 1985) as a split plot design on time with repeated measurements (Gill, 1978). The model used was:

$$Y_{ijklm} = \mu + A_i + C_j(A_i) + P_k + T_l + (A_i*T_l) + (A_i*P_k) + (P_k*T_l) + (P_k*T_l*A_i) + S_m + (S_m*T_l) + (S_m*A_i) + (S_m*P_k) + E_{ijklm}$$

Where A_i is the average effect of the age of the cow (primiparous vs. multiparous),

$C_j(A_i)$ is the average effect of cow within age,

P_k is the average effect of period,

T_l is the average effect of treatment,

S_m is the average effect of sampling time,

E_{ijkl} is the random residual, assumed normally and independently distributed.

When an interaction term was not significant ($P>0.20$), the term was added to the appropriate error term and the variable reanalyzed with the reduced model. Treatment was tested with ($P_k*T_l*A_i$) as the error term; sampling time was tested by the residual. Single degree of freedom orthogonal contrasts compared LO vs. HI (linear) and MED vs. LO and HI (curvilinear). Least square means and standard errors are presented.

Mineral concentration and DM content in adipose tissue, baseline levels of serum variables, activity of enzymes, and TG content of FAML homogenate were analyzed using the model:

$$Y_{ijkl} = \mu + A_i + C_j(A_i) + P_k + T_l + (A_i*P_k) + E_{ijkl}$$

Where A_i is the average effect of the age of the cow (primiparous vs. multiparous),

$C_j(A_i)$ is the average effect of cow within age,

P_k is the average effect of period,

T_l is the average effect of treatment,

E_{ijkl} is the random residual, assumed normally and independently distributed.

Single degree of freedom orthogonal contrasts were as described above. Least square means and standard errors are presented.

RESULTS

Treatment affected baseline levels of serum NEFA, Ca, Mg, and K (Table 1). Infusing HI compared to LO increased serum NEFA ($P<0.04$), Mg ($P<0.08$) and decreased serum Ca ($P<0.06$). Infusing MED increased serum K ($P<0.03$) compared to the combined effect of infusing LO and HI.

Infusing HI caused a smaller decrease ($P<0.11$) in serum K in response to the EPI challenge compared to the effect of infusing LO. Infusing MED caused a greater decrease ($P<0.02$) in serum K in response to the EPI challenge compared to the combined effect of infusing LO and HI (Table 2). There was no overall treatment effect ($P>0.70$) on serum TG. The concentration of serum TG pre-EPI injection was 6.73, 10.45, and 5.91 (± 1.82) mg/dl for LO, MED, and HI, respectively. Twenty minutes after the EPI injection TG concentrations were increased ($P<0.0001$) to 21.3, 18.9, and 21.3 (± 1.82) for LO, MED, and HI, respectively. Treatment did not have a significant ($P>0.18$) effect on any of the other serum variables in response to the EPI challenge.

Treatment did not affect the concentration of cations in SAT ($P>0.24$, Table 3) or the activity of enzymes in homogenates of SAT ($P>0.28$, Tables 4 and 5). There were no differences ($P>0.47$) in the activity of FAML expressed as nmoles FA released/mg endogenous TG/h, which were 137.7, 220.2, and 213.5 (± 68.1) for LO, MED, and HI, respectively.

Endogenous TG provided (mg/assay tube) did not differ among treatments (P>0.28) and were 0.03, 0.06, and 0.08 (± 0.03) for LO, MED, and HI, respectively. The overall response to challenge with EPI was increased serum NEFA concentration (P<0.0001, Figure 1) and decreased serum Mg concentration (P<0.0001, Figure 2). No treatment differences on serum NEFA (P>0.18, Figure 3) and serum Mg (P>0.61, Figure 4) could be detected in response to the EPI challenge.

DISCUSSION

The availability of qualified personnel to perform the biopsies was a limitation in this trial. We were able to work with 2 cows at one time. Therefore, over 3 h elapsed between the start of the EPI challenge of the first pair of cows and the last pair of cows (Table 6). This asynchronous handling of the animals can explain the effects observed in the baseline levels of serum variables. Synchronized measurements of serum variables for over 8.5 h for all 12 cows were reported elsewhere (Chapter III). Feeding at 1300 h affected serum Mg levels (Chapter III). Thus, it is more appropriate to report serum data as the response to the EPI challenge.

The regulation of FA mobilization from the adipose tissue of lactating dairy cows is important to economics and health. The cow will mobilize FA from endogenous stores in an attempt to meet the energy needs for high levels of milk production. This can result in an increase of health problems such as

Table 1. Pre-epinephrine challenge levels of serum variables.

	Treatment[1]			
	LO	MED	HI	SE
NEFA, μEq/l[2]	176.3	223.5	238.7	17.2
Ca, mg/dl[3]	10.13	9.97	9.73	0.12
Mg,mg/dl[4]	2.77	2.76	2.97	0.07
K, mg/dl[5]	27.87	29.62	27.24	0.61
Na, mg/dl	451.4	439.7	438.0	14.2

[1] LO = 0, MED = 6, and HI = 12 g Mg infused/d.
[2] Probability of overall treatment effect (P<0.09). Probability that the contrast LO vs. HI is among treatments that do not differ (P<0.04).
[3] Probability of overall treatment effect (P<0.15). Probability that the contrast LO vs. HI is among treatments that do not differ (P<0.06).
[4] Probability of overall treatment effect (P<0.12). Probability that the contrast LO vs. HI is among treatments that do not differ (P<0.08).
[5] Probability of overall treatment effect (P<0.07). Probability that the contrast MED vs LO and HI is among treatments that do not differ (P<0.03).

Table 2. Overall change of serum variables in response to the epinephrine challenge

	Treatment[1]			
	LO	MED	HI	SE
NEFA, μEq/l	156.2	174.9	203.6	14.0
Ca, mg/dl	0.25	0.10	0.11	0.05
Mg, mg/dl	-0.12	-0.08	-0.08	0.02
K, mg/dl[2]	-3.28	-4.43	-2.15	0.28
Na, mg/dl	-15.85	4.32	0.10	3.90

[1] LO = 0, MED = 6, and HI = 12 g Mg infused/d.
[2] Probability of overall treatment effect (P<0.81). Probability that the contrast LO vs. HI is among treatments that do not differ (P<0.11). Probability that the contrast MED vs. LO and HI is among treatments that do not differ (P<0.02).

Table 3. Concentration (μg/g DM) of minerals in adipose tissue.

	Treatment[1]			
	LO	MED	HI	SE
Ca	29.1	22.8	52.9	11.8
Mg	349.8	177.0	108.9	100.6
K	1.1	0.8	2.2	0.6
Na	1.4	1.0	2.9	0.9

[1] LO = 0, MED = 6, and HI = 12 g Mg infused/d.

Table 4. Activity of enzymes in fresh adipose tissue[1].

	Treatment[2]			
	LO	MED	HI	SE
	nmoles FA released or incorporated/g fresh tissue/h			
FAML[3]	258.6	272.2	226.0	65.0
GS[3]	78.4	65.6	89.7	30.0
LPL[3]	827.6	890.1	406.2	212.9

[1] Dry matter content of adipose tissue did not differ among treatments (P>0.50) and was 55.8, 62.2, and 51.4% (± 9.6) for LO, MED, and HI, respectively.
[2] LO = 0, MED = 6, and HI = 12 g Mg infused/d.
[3] FAML = fatty acid mobilizing lipase; GS = glyceride synthetase; and LPL = lipoprotein lipase.

Table 5. Activity of enzymes in adipose tissue.

	Treatment[1]			
	LO	MED	HI	SE
	nmoles FA released or incorporated/ mg soluble protein/h			
FAML[2]	24.7	19.9	26.0	6.5
GS[2]	4.9	6.6	8.2	2.0
LPL[2]	61.2	74.5	35.6	15.7

[1] LO = 0, MED = 6, and HI = 12 g Mg infused/d.
[2] FAML = fatty acid mobilizing lipase; GS = glyceride synthetase; and LPL = lipoprotein lipase.

Table 6. Starting times (h) of bleeding and biopsy for each cow within period.

COW	TRT[1]	PAIR	BLEEDING	BIOPSY
PERIOD I				
2034	MED	2	1054	1207
2086	HI	1	1032	1135
2167	MED	2	1051	1205
2344	LO	3	1158	1305
2370	HI	3	1155	1312
2373	LO	1	1036	1142
2413	MED	6	1335	1441
2419	LO	5	1253	1403
2420	LO	5	1256	1412
2425	HI	4	1218	1337
2432	HI	6	1337	1448
2436	MED	4	1220	1343
PERIOD II				
2034	LO	2	1050	1205
2086	MED	1	1033	1150
2167	HI	2	1055	1209
2344	MED	3	1115	1225
2370	LO	3	1110	1222
2373	HI	1	1030	1140
2413	LO	4	1215	1323
2419	HI	6	1250	1400
2420	MED	6	1255	1410
2425	MED	5	1235	1345
2432	LO	4	1210	1316
2436	HI	5	1230	1342

[1] Treatments LO = 0, MED = 6, and HI = 12 g Mg infused/d.

Figure 1. Overall response of serum non-esterified fatty acids to epinephrine challenge. Pooled standard error is 31.6.

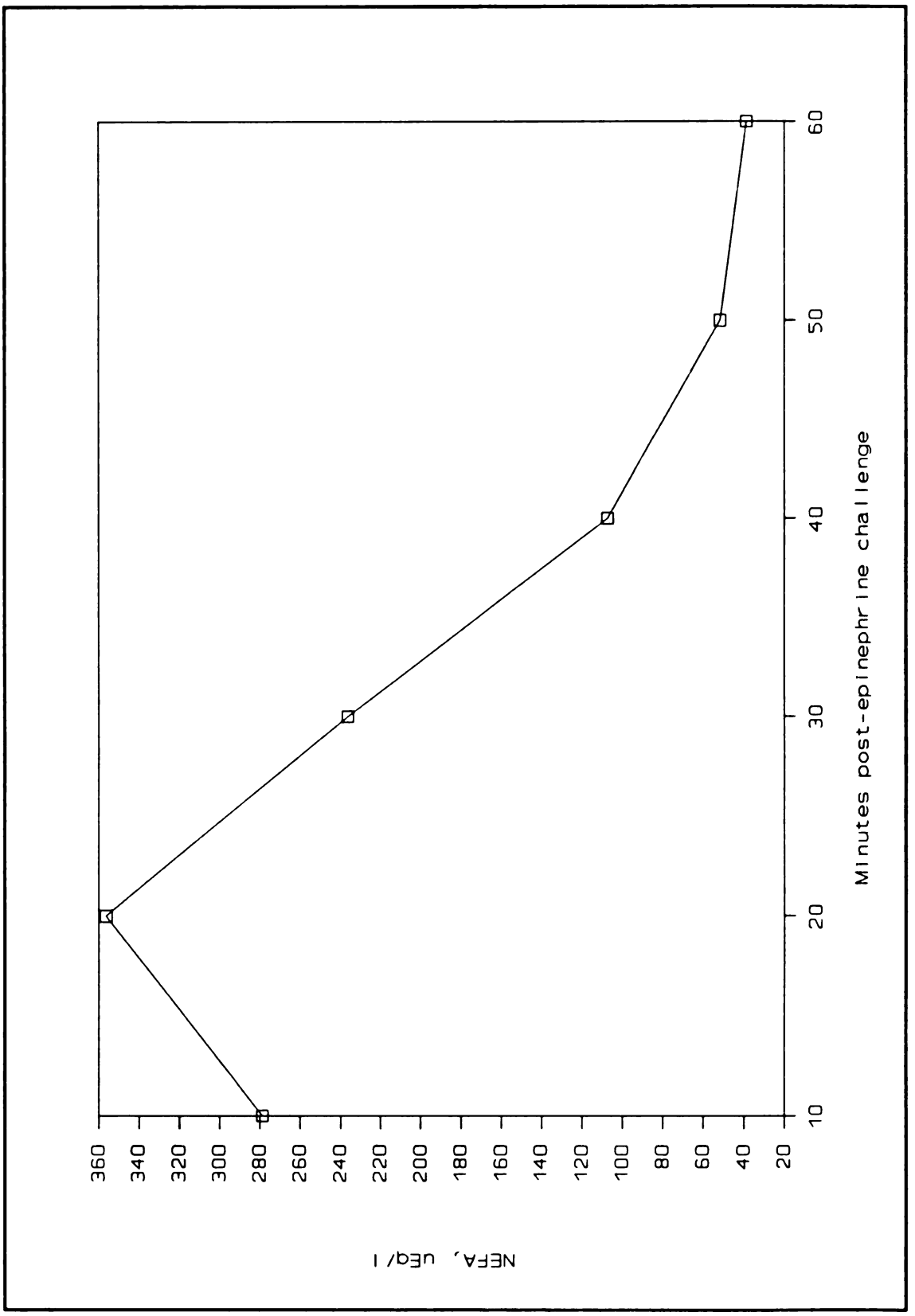

NEFA, uEq/l
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
10
20
30
40
50
60
Minutes post-epinephrine challenge

Figure 2. Overall response of serum Mg to epinephrine challenge. Pooled standard error is 0.04.

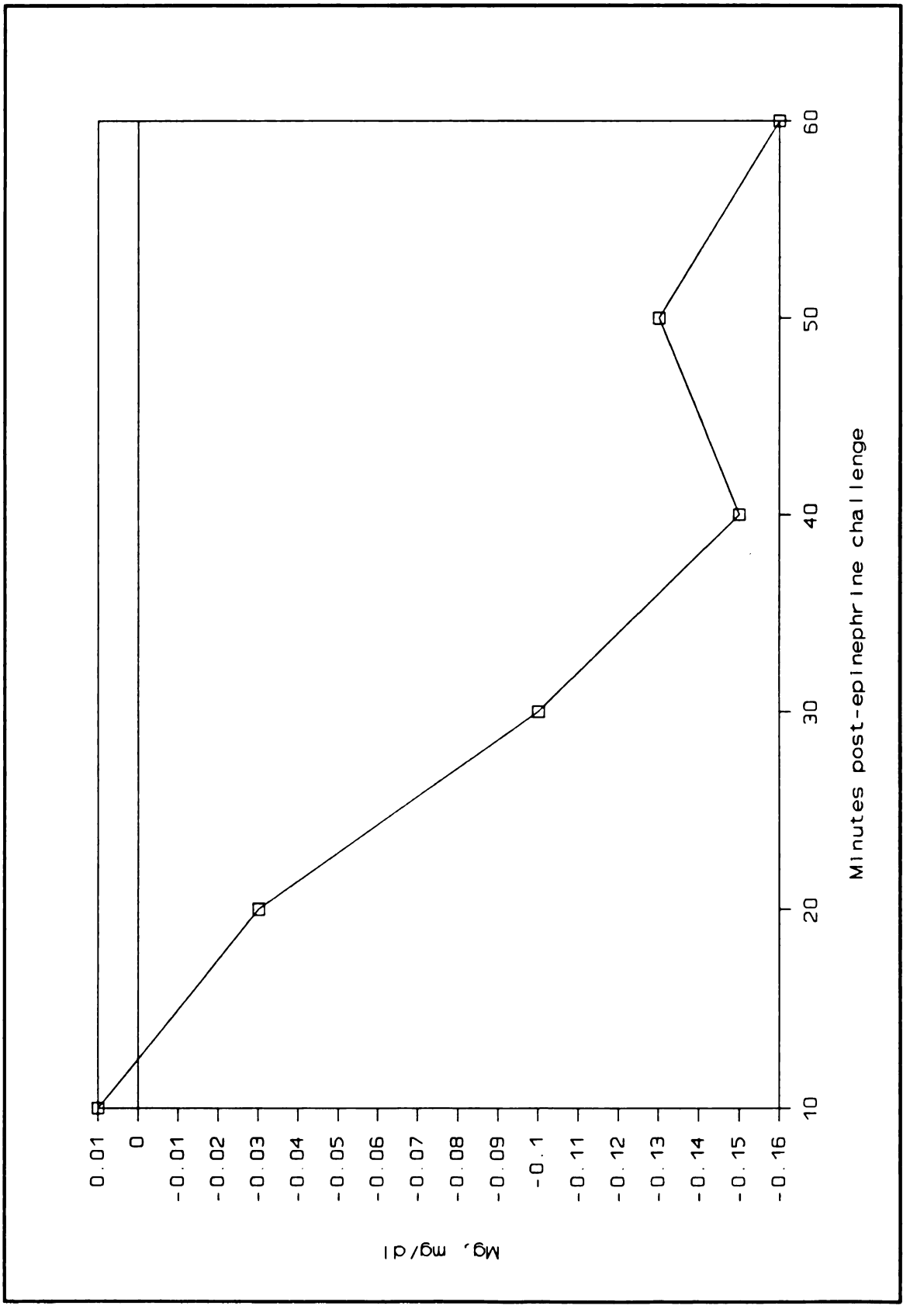

0.01
0
-0.01
-0.02
-0.03
-0.04
-0.05
-0.06
-0.07
-0.08
-0.09
-0.1
-0.11
-0.12
-0.13
-0.14
-0.15
-0.16
Mg, mg/dl
10
20
30
40
50
60
Minutes post-epinephrine challenge

Figure 3. Treatment response of serum non-esterifed fatty acids to epinephrine challenge. Pooled standard error is 17.9.

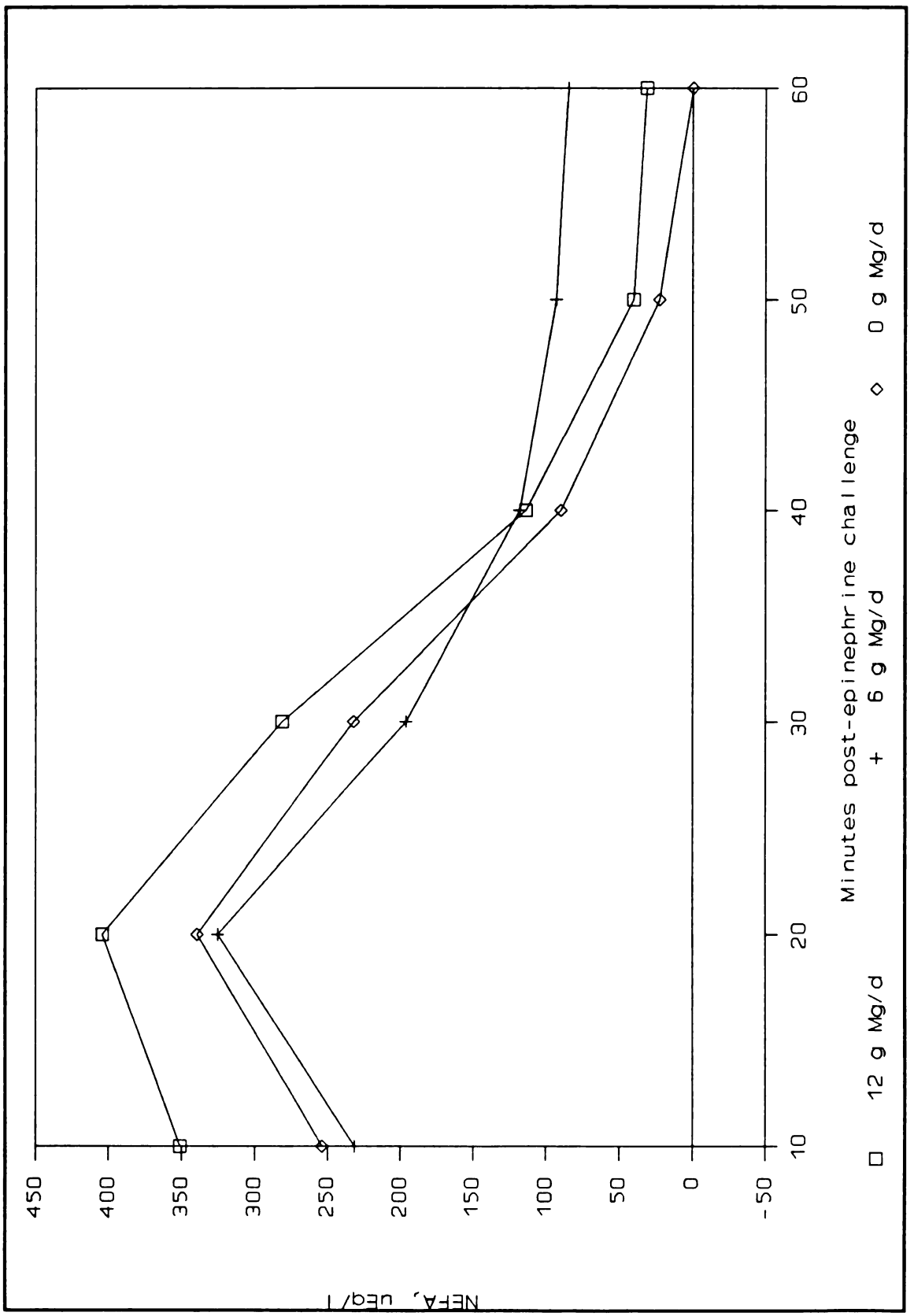
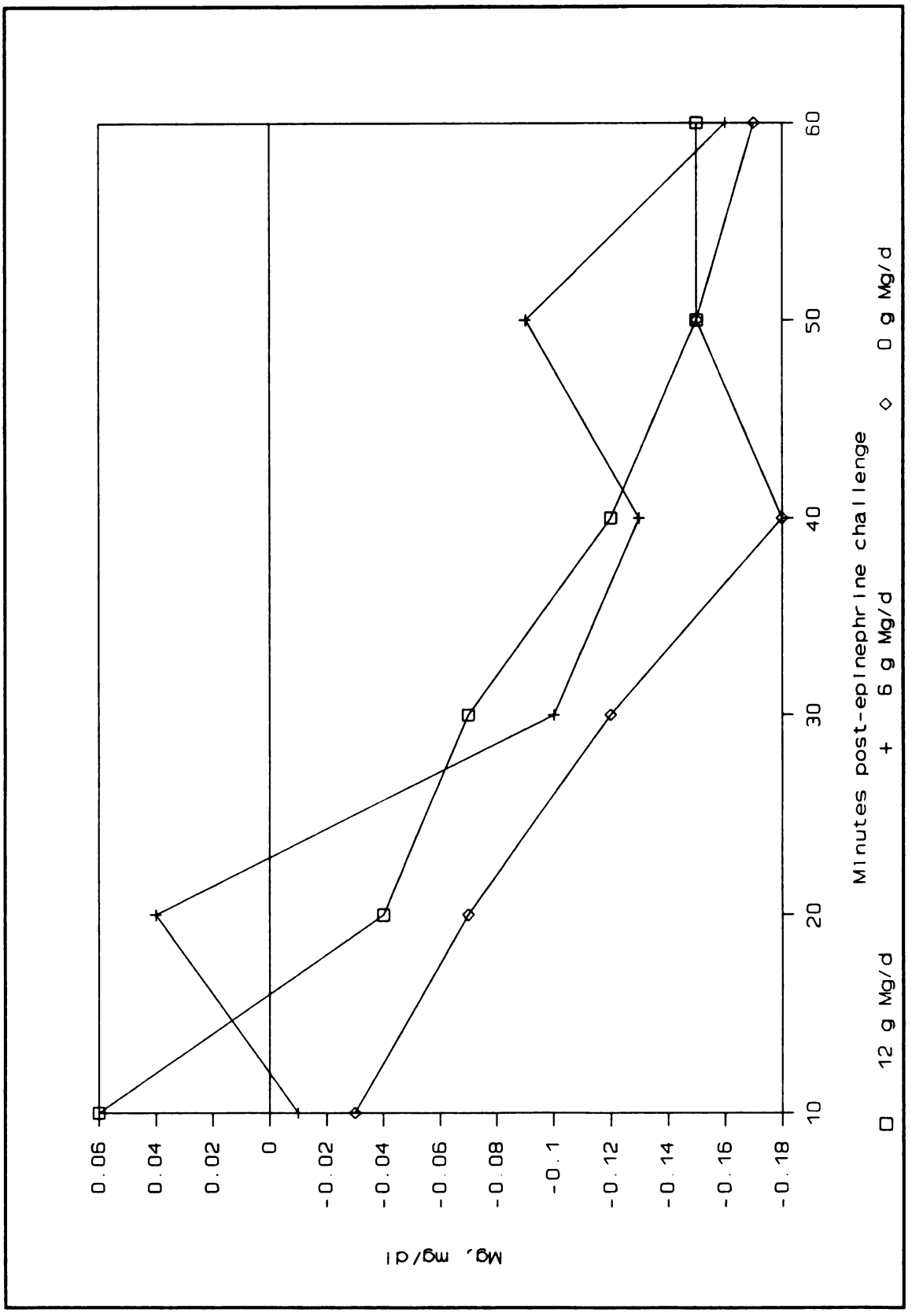

NEFA, ueq/l
450
400
350
300
250
200
150
100
50
0
-50
10
20
30
40
50
60
Minutes post-epinephrine challenge
□ 12 g Mg/d
+ 6 g Mg/d
◇ 0 g Mg/d

Figure 4. Treatment response of serum Mg to epinephrine challenge. Pooled standard error is 0.02.


0.06
0.04
0.02
0
-0.02
-0.04
-0.06
-0.08
-0.1
-0.12
-0.14
-0.16
-0.18
Mg, mg/dl
10
20
30
40
50
60
Minutes post-epinephrine challenge
□ 12 g Mg/d
+ 6 g Mg/d
◇ 0 g Mg/d

ketosis, fatty liver and alterations in the reproduction cycle. Also, lipid metabolism can influence Mg metabolism and contribute to the development of hypomagnesemia.

As expected, the stimulation of lipolysis via EPI injection in normomagnesemic lactating cows increased serum NEFA and a concomitant decrease in serum Mg was observed. This is in agreement with the results obtained in sheep (Rayssiguier and Larvor, 1976), dogs (Flink et al., 1979), and humans (Cohen et al., 1984). When the effects of the EPI challenge were separated according to treatment, neither the overall response in serum NEFA and Mg nor the treatment response overtime were significantly different. However, the trends followed by serum NEFA during the first 40 min post-EPI injection were the type of trends that one would expect. An interesting observation is that serum NEFA levels for LO and HI approached baseline levels at 60 min post-EPI injection, while the decline in serum NEFA for MED was more persistent. Thus, it appears that upon lipolysis stimulation the rate of FA mobilization is rapid but of short duration in those cows with high or low serum Mg levels, while cows with medium level of serum Mg have a slower but more sustained release of FA. These effects could be explained by differences in the activities of FAML and (glyceride synthetase + LPL) caused by varying levels of Mg supply to SAT. Also, increased FA recycling within the adipocyte is possible. Another possible explanation is that the level of serum Mg modulates the

effects of EPI. The feeding of high Mg diets to rats decreases the secretion of catecholamines (Porta et al., 1990).

Elliot and Rizack (1974) documented an increase in total Mg content in whole fat cells following EPI, norepinephrine, or adrenocorticotropic hormone stimulation in vitro. Meyer et al. (1982) showed that the Mg content of fat tissue from cold treated rats was increased 470% relative to controls. Cohen et al. (1984) reported that the reduced serum Mg level in acute myocardial infarction did not reflect a total body Mg deficiency, since normal serum Mg levels were established within 3 d after infarction. Our results show that cows infused LO had the largest decrease in serum Mg following the stimulation of lipolysis. These cows also had the highest concentration of Mg in SAT. The increase in Mg concentration in SAT when cows were infused LO was 191 and 341% relative to infusing MED and HI, respectively. Even though this increase in the concentration of Mg in SAT was not statistically significant, we would like to suggest that the drop in serum Mg following the stimulation of lipolysis is due to a redistribution of Mg to SAT. This redistribution of serum Mg into SAT may be involved in the pathogenesis of the development of hypomagnesemia.

The deposition of fat should depend upon FAML as well as synthesis of glycerides and LPL activities in adipose tissue (Emery, 1973). In lactating cows, glyceride synthesis seems

a better control of FA release from adipose tissue than FAML (Emery, 1973). In cows that are milk-fat depressed, lipolysis of adipose TG is increased, as observed in glycerol release while the release of FA decreases (Sidhu and Emery, 1973). This can be the result of increased recycling of FA into glycerides within the adipocyte. Benson et al. (1972) reported that FA esterification in adipose tissue is elevated by feeding high-grain diets. In our trial, FAML activity expressed in a per mg soluble protein basis was similar among treatments. However, the activity of glyceride synthetase increased non-significantly as the level of Mg infused increased. Mammary gland LPL increases 94 fold at the initiation of lactation while adipose tissue LPL decreases (Shirley et al., 1973). When milk-fat depression is caused by high-grain feeding mammary LPL decreases and adipose LPL increases (Emery, 1973). Emery et al. (1965) suggested that Mg increases the uptake of TG from blood into mammary gland tissues. This increased uptake could be due to increased LPL activity in the mammary gland. In our work, intravenous Mg infusion had no significant effect on the activity of LPL in SAT. However, the LPL activity when cows were infused HI was only 58 and 48% of the activity determined for cows infused LO and MED, respectively. The activity of LPL in SAT shows no relationship to either baseline or post-EPI injection serum TG concentrations. This is not surprising since the major site of TG removal in the lactating cow is the mammary gland.

Rayssiguier et al. (1991) reported no effect of Mg deficiency in the activity of LPL in rat epididymal adipose tissue.

A problem faced by researchers conducting enzyme work in tissue slices or homogenates is that there is no adequate denominator to express enzyme activity. An alternative was offered by Gagliostro and Chilliard (1991), but as they pointed out, it has it's drawbacks. Since the adipocyte is the main site of TG storage, FAML data were also expressed in a per mg endogenous TG basis. Data expressed this way show that cows infused MED and HI had non-significantly higher lipolytic rates compared to cows infused LO.

To our knowledge, this is the first time that EPI injection is reported to increase serum TG. Three possibilities can explain the effect of EPI on serum TG: 1) the assay used to determine TG determines glycerol levels, and any free glycerol present, may bias the results, 2) the activity of LPL is decreased resulting in serum TG accumulation, or 3) there is an increased production of TG by the liver or the intestine. We determined that our modified assay for TG removed up to 9 mg/dl free glycerol, which is well above physiological levels (Appendix A). The second possibility does not appear any more likely. We did not determine LPL activity before EPI injection. Even if we had determined the activity of adipose LPL, mammary LPL is probably affected the most, since adipose LPL is secondary in the uptake of TG in the lactating cow. Furthermore, Emery et

al. (1965) reported an increased uptake of TG by the mammary gland of cows fed MgO. Therefore, the third possibility appears more likely.

In summary, serum NEFA concentrations tended to increase ($P<0.18$) in response to the stimulation of lipolysis as the level of Mg infused increased (Figure 3). Serum Mg levels decreased upon stimulation of lipolysis. The decrease in serum Mg was greater for cows infused LO. We hypothesized that serum Mg was redistributed to perirectal SAT. The infusion of Mg had no significant effects on enzyme activities of perirectal SAT.

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CHAPTER V

UNIFYING REMARKS

UNIFYING REMARKS

Different aspects of the metabolism of Mg in the lactating Holstein cow were investigated. But what do they mean to the cow, the producer and the dairy industry?

Supplementing Mg above current NRC (1989) recommendations is no magic cure for various problems associated with the production of milk. There is a place in the feeding of the dairy cow for supplemental Mg salts. As new knowledge emerges, the conditions needed for the inclusion of Mg salts in the diet of cows will be better characterized. Supplementation of Mg to alfalfa based diets usually provides no benefit in terms of improved lactational performance. The kind of Mg supplement can be an explanation for this lack of beneficial effect. An alternate explanation is conditions encountered within the lumen of the gastro-intestinal (GI) tract. These conditions in the GI tract are largely dictated by the source of forage provided to the cow. We have not determined if the effect of Mg supplements is dependent on the forage fed. In order to address this question, a large scale and costly trial would be required. Assuming 2 forages and 3 levels of Mg within forage, we have 6 possible treatments. Assuming 10 cows/treatment in order to detect a difference of 2 kg milk/d, makes this trial a dream that will not come true. However, for the dairy farmer in Michigan it is important to know that Mg supplementation above NRC (1989) recommendations will not improve the lactational performance of dairy cows fed

alfalfa based diets. An exception is in early lactation when low fiber diets are fed and feed intake is erratic. In Michigan, the trend is for farmers to feed more alfalfa based diets and to include less corn silage in the diet. Many feed companies provide excess Mg in their products, and this is an unjustified added cost to the producer.

We determined that the improvement of lactational performance and alleviation of milk-fat depression by Mg is not mediated via increases in the supply of Mg to the tissues. A question that remains unanswered is: does Mg have a pre- or post-absorptive effect on the alleviation of milk-fat depression? In order to answer this, a direct comparison between oral and infused Mg must be conducted. Many questions have to be answered before this type of work can be conducted. For example, what product and level should be provided orally? Differences among Mg products are well known, but we do not have the right kind of information to answer the question above. If milk-fat depression is not alleviated in the above comparison, then can we conclude that Mg does or does not alleviate milk-fat depression one way or the other? NO! We can not. As long as we have to deal with a Mg supplement that is affected by solubility in the rumen, rumen pH, rumen content of Na and K, regulation of Mg absorption in the forestomach of the ruminant animal by unknown factors, etc. we will not be prepared to compare oral vs. intravenous Mg supplementation. However, from two different angles, data from Chapters II and

III suggest the same idea: the effect of Mg on improvements in lactational performance and alleviation of milk-fat depression is pre-absorptive.

We were able to manipulate the temporal pattern of serum somatotropin (ST) concentrations by infusing increasing levels of Mg. Can we do the same via oral Mg? One can anticipate more problems, and it is very possible it can not be achieved. Again, the effects of the Mg supplement are variable, the absorption varies, the solubility varies. Most of the differences among Mg sources can be traced back to the processing of the raw material. Therefore, we have no tight control over the regulation of serum Mg level (Chapter II). Even when Mg was infused intravenously it was difficult to maintain stable levels of serum Mg (Chapter III). This last observation suggests that serum Mg homeostasis is achieved by means other than Mg absorption from the gastro-intestinal tract, in agreement with Reinhardt et al. (1988). Also, the activity of the cows, such as eating and milking can account for some of the fluctuation in serum Mg levels. The most immediate need appears to be for an "IDEAL" product that can be trusted in terms of Mg availability and absorption. Once this "IDEAL" product is obtained some interesting questions could be answered. If we can manipulate endogenous ST levels nutritionally, what will be the response to exogenous ST? If serum Mg level regulates lipolysis, then what will be the effect of exogenous ST on the lactational performance of dairy

cows? Another question that has not been studied is, can exogenous ST alleviate milk-fat depression?

Our results suggest that in the short term, serum Mg levels can regulate ($P>0.18$) serum non-esterified fatty acid (NEFA) concentrations in response to the epinephrine challenge. This supports the data obtained in vitro when expressed in a per mg endogenous TG basis. A question that remains to be solved is: are the effects observed in NEFA and in vitro enzyme work due to Mg per se or to Mg modulating the mode of action of epinephrine? Questions to be addressed in the future would include can we regulate serum NEFA concentration in the long term via serum Mg levels? Can fatty liver and ketosis be alleviated by manipulating lipolysis with serum Mg? In Chapter II, although we fed increasing levels of Mg, body weight or plasma Mg were not significantly affected during the first 12 weeks (negative energy balance) of lactation. However, the cows fed the high level of Mg had lower body weight and condition score. Once again, the need for the "IDEAL" Mg product is evident.

The observation that serum TG increased with increasing levels of Mg infused is interesting. Why is this happening? Is decreased uptake of TG the cause? As discussed in Chapter III, this is probably not the case. Furthermore, Emery et al. (1965) reported an increased uptake of TG from blood into the mammary gland. Then, it must mean increased NEFA esterification into TG or increased TG export as very low

density lipoproteins in the liver or intestine. If this increased TG production is taking place in the liver, then could it be involved in the alleviation of ketosis and fatty liver?

New information was gained regarding the development of hypomagnesemia (HM). We hypothesized that upon stimulation of lipolysis there is a redistribution of serum Mg to subcutaneous adipose tissue in the lactating dairy cow. This observation coupled to the following facts can help explain the pathogenesis of HM: it occurs during grazing in early spring when lipolysis is stimulated by low energy content in the pasture, cold and wet weather, and exercise. Furthermore, HM is not due to a decreased intake of Mg, although increased Mg intake can alleviate this problem.

We have reported selected aspects of Mg metabolism in the lactating dairy cow. Hopefully, the information reported herein will prove useful in improving milk production and maintenance of the cow's well being, helping the farmer provide diets balanced adequately, with the end result of a healthier dairy industry.

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APPENDICES

APPENDIX A

Triglyceride determination in bovine blood

References:

Sigma. Procedure 405 for the determination of blood triglycerides. Sigma Chemical Co., St. Louis, MO.

Patton, R. A. 1989. The effect of dietary fiber and body condition on the milk production , dry matter intake and blood metabolites of peripartum dairy cows. Ph. D. Thesis, Michigan State University.

Reagents

1. 3:2 hexane:isopropanol (v/v): 600 ml hexane, 400 ml isopropanol.
2. 7:2 hexane:isopropanol (v/v): 778 ml hexane, 222 ml isopropanol.
3. Isopropanol: 100%.
4. Hexane: 100%
5. 7% sodium sulfate (w/v): 70 g Na_2SO_4 to 1 l with dH_2O.
6. Triglyceride purifier (405-3, Sigma, St. Louis, MO).
7. 5% KOH: 50 g KOH, 600 ml Dh_2O, 400 ml isopropanol.
8. Sodium periodate solution: 2.5 g $NaIO_4$ (S-1147, Sigma, St. Louis MO), in 1 l of 2 N acetic acid (115 ml glacial acetic acid + 885 ml Dh_2O). Stable for 3 mo at 0-5° C.
9. Color reagent: 333.3 ml of 2 M ammonia acetate (144.16 g ammonia acetate in 1 l of Dh_2O), 666.6 ml isopropanol, and 2.5 ml acetyl acetone. Store in amber bottle. Needs to

age at least 2 h prior to using. Stable for 1 mo at 0-5° C.

10. Triglyceride standard: 300 mg triolein in 100 ml isopropanol (405-10, Sigma, St. Louis, MO).

Standard curve for bovine blood

ml Triolein	ug glycerol (TG)
Blank	0
0.02	60
0.06	180
0.1	300
Control sample	To be determined

Equipment

1. Incubation glass tubes (16 x 125 mm), 19 ml (2 sets)
2. 10 ml screw cap tubes (1 set).
3. Metabolic shaker.
4. Water bath (60° C and room temperature).
5. Nitrogen evaporator and heated sand bath.
6. Centrifuge.
7. Spectrophometer.

Procedure

Extraction:

1. Add 1 ml of plasma or serum (if animal in positive energy balance, 0.5 ml is enough) or appropriate amount of standard to 7 ml of the 3:2 hexane:isopropanol solution in a 19 ml glass incubation tube.
2. Add 3.5 ml of the 7% sodium sulfate solution, vortex and centrifuge at 1000 rpm for 5 min. Aqueous phase will settle to the bottom of tube.
3. Transfer upper phase into a 10 ml screw cap tube containing 1.6 g of the triglyceride purifier.
4. Shake the tube sideways for 10 min in a metabolic shaker.
5. Centrifuge for 5 min at 1000 rpm and room temperature.
6. Transfer liquid phase into another prelabeled 19 ml glass incubation tube (sample tube). Wash the tube containing the purifier (step 3) with 5 ml of the 7:2 hexane:isopropanol solution, vortex for 15 sec and centrifuge for 5 min at 1000 rpm and room temperature.
7. Transfer the 7:2 hexane:isopropanol wash into the sample tube (step 6). Evaporate sample to dryness under a continuous nitrogen flow in a heated sand bath.
8. Dissolve the dry sample in 1 ml isopropanol. Since heating may be required in some samples, time will be saved if all samples are heated at 60° C for 1 min.
9. Add 0.5 ml of the KOH solution and vortex to saponify. The procedure can be stopped here overnight if desired.
10. Complete saponification by incubating samples at 60° C for 5 min. Cool samples to room temperature by placing

them in a bath with tap water for 2-3 min.

Colorimetric assay:

11. Add 0.5 ml of the sodium periodate solution to each tube. Vortex sample immediately after each addition.
12. After 10 min, add 3 ml color reagent to each tube, and vortex after each addition.
13. Incubate covered tubes at 60° C for 0.5 h.
14. Remove tubes and cool to room temperature in tap water bath.
15. If samples are hemolyzed, extract samples with 2 ml hexane to remove interfering pigments. If samples are not hemolyzed, skip this step and proceed to step 16.
16. Read absorbance at 410 nm within 20 of removing tubes from 60° C water bath. Calibrate spectrophotometer with Dh_2O. If hexane wash was necessary, bottom layer should be read.

Calculations

Use linear regression to determine ug glycerol/assay tube (ug glycerol/assay tube=ug triglyceride (TG)/assay tube).

(ug TG)/(ml plasma assayed * 10) = mg/dl TG

Note: TG concentrations can be converted to mmol/l by multiplying mg/dl values by 0.0113.

Note: The lipid extraction procedure and triglyceride purifier steps should remove most of the free glycerol, therefore no corrections are needed.

Free glycerol determination in bovine blood

This is a modification of the TG procedure. The modifications are as follows:

Skip steps 9 and 10, go directly to step 11. Immediately after step 11, add the 0.5 ml of KOH (this is necessary to make the Ph basic. Otherwise the sample is cloudy and the reading yields artificially high values).

APPENDIX B

Table 1. Intake of dietary constituents for Trial 2.

	TREATMENT[1]			
	LO	MED	HI	SE
Intake, kg/d				
OM[2]	20.8	20.9	21.0	0.1
Lignin	0.31	0.32	0.31	0.01
CP	3.71	3.72	3.73	0.03
ADF	2.50	2.52	2.52	0.01
Intake, g/d				
Ca	138.47	143.35	133.50	4.41
K	178.22	179.99	177.74	2.18
Na	36.55	36.43	35.29	1.04

[1] LO = 0, MED = 6, and HI = 12 g Mg infused/d.
[2] Organic matter.
[3] Probability of overall treatment effect (P<0.13). Probability that the contrast MED vs LO and HI is among treatments that do not differ (P<0.05).

FATTY ACID MOBILIZING LIPASE

References:

Frederickson, G. et al. 1981. Methods Enzymol. 71:636-646.

Liesman, J. S. 1991. Personal files.

Purpose: to determine the activity of fatty acid mobilizing lipase (FAML) in homogenates of bovine adipose tissue (AT).

Reagents:

CAMP solution: 0.0133 mM cyclic-3,5 AMP (Sigma A-6885), 0.13 mM ATP (Sigma A-3377), 4.4 mM $MgCl_2$, 150 mM NaCl.

8% Bovine serum albumin (BSA): 8% BSA heat-shock treated (Sigma A-3803), in 150 mM NaCl, 0.05 M dibasic phosphate, pH 7.4 at room temperature.

Lecithin:

Tripalmitate:

^{3}H-tripalmitate:

HMC solution: 1 heptane (273 ml/l) : 1.41 methanol (385 ml/l) : 1.25 chloroform (342 ml/l). All solvents distilled.

Alkaline buffer: 1.266% (41 mM) $K_2B_4O_7 \cdot H_2O$, 2.756% (200 mM) K_2CO_3.

NaCl: 150 Mm.

Sucrose solution: 0.25 M sucrose, 1 X 10^{-3} M EDTA, pH 7.4 at room temperature.

Scintillation fluid: triton base fluid for aqueous samples.

Procedure:

1. Substrate premix: in a 20 ml plastic scintillation vial, pipet 0.4 ml ^{3}H-triolein

(~300,000 cpm), 1 ml cold triolein, and 0.1 ml lecithin. Evaporate solvents under N_2, then add 9 ml of 8% BSA solution. Caution: do not add Triton X-100, as it may inhibit the enzyme. Step by step procedure is described in Appendix E.

2. Quench tubes: prepare 2 tubes as described in the table below, except that the premix is replaced by 8% BSA solution. Otherwise, treat tubes as sample tubes. Add 10 μl of the TG premix or FA to the scintillation vial just prior to counting.

3. Fatty acid recovery tubes: prepare 4 tubes as described in the table below, except that the TG premix is replaced by FA premix. Otherwise, treat tubes as sample tubes.

4. Tissue homogenate: weigh out AT and add the sucrose solution in the proportion of 1 : 3 (w:v) AT:sucrose solution. Homogenize twice for 10 sec each time with polytron at 3/4 full speed in 15 ml (Corex) glass tube. Centrifuge in Sorvall RC2B at 6,500 rpm (5,000 X g, SS-34 rotor) and 5 °C for 30 min. Pour supernatant and save on ice for not more than 2 h before assayed.

5. Start by adding homogenate (or sucrose solution for blanks) and vortex. Incubate at 37 °C for 10 min with shaking (35 oscillations/min).

6. To stop assay, place the tubes on ice and add 1.65 ml HMC solution and vortex. Then, add 0.45 ml buffer and vortex for 10 s. Centrifuge in Sorvall RC3 at 3,000 rpm and 20 °C for 15 min.

7. Allow tubes to sit at room temperature for 1 h to equilibrate. Then, pipet 0.5 ml of supernatant into 20 ml scintillation vial with 10 ml aqueous scintillation fluid. Used cpm from 10 μl of premix quenched with from quench tubes. Count quench tubes using channel ratios to obtain a constant ratio.

ASSAY SETUP
(All volumes in µl)

TUBE	SUBSTRATE	CAMP	NaCl	HOMOGEN.
Blank	80	60	60	60, 30, 0
Sample	80	60	0	0, 30, 60

Calculations:

Specific Activity (SA) of TG = 260 nmoles/CPM in 10 µl TG premix.

FA recovery factor (RF) = CPM in 10 µl FA premix * 8/CPM in recovery sample.

FAML activity (nmoles FA/time) = (sample CPM - blank CPM) * SA * RF * 3 FA/TG.

Assay validation:
Refer to Figure 1.
1. Linearity with time.
2. Linearity with enzyme concentration.

Problems encountered during validation, steps taken to solve them that did not work, and other useful tips:

1. Homogenization:
   - Ice cold 0.15 M KCl did not work.
   - 50 ml plastic tubes: can not cool fast enough. Overheating is caused and enzyme killed.
   - Addition of PMSF (5 µl/ml) and pepstatin (3 µl/ml) did not help.
   - Centrifugation up to 48,000 rpm will not remove more TG from homogenate.
   - Do not over-homogenize (3 X 10 sec), enzyme is easily killed.

2. Substrate:
   - Use BSA indicated above ONLY. Other BSAs are contaminated with a lipase/hydrolase. We were able to validate an assay almost perfectly, just to find out we had validated this contaminant.
   - Tested adding 7.5 or 4.5 Mm TG, but 1.5 Mm was best.
   - Re-purified labeled TG in florosil column to reduce blanks, but did not help.
   - Use of Tris-buffer in BSA solution, is

inadequate.
- Enzyme may prefer endogenous substrate.

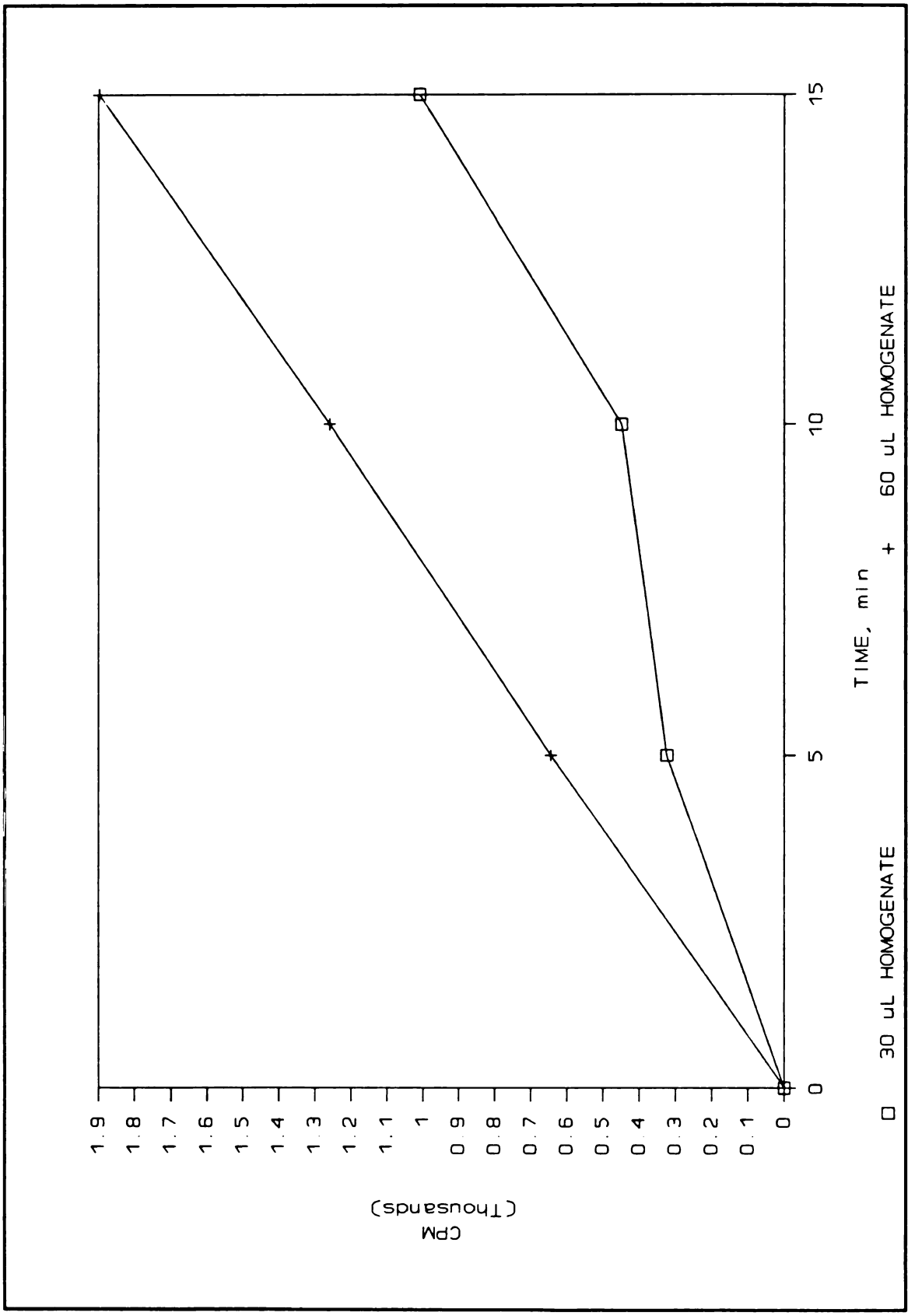
3. Blanks:
   - Hard to obtain stable blanks. Run many blanks (at least 6) and choose the lowest and most repetitive blanks. You may end up with negative activities after subtracting blanks.
   - Blanks are stabilized somewhat if the concentration of Mg in sample tubes is also provided in blank tubes.
4. Linearity:
   - Could not get past 15 min (tested up to 90 min).
   - Results at 15 min not consistent, chose 10 min.
   - If no linearity with enzyme concentration is obtained, suspect a contaminant. Usually linearity with time can be achieved.

5. Time:
   - Allow yourself PLENTY of time to validate this assay. For future reference, Dr. Emery, Jim Liesman and me spent since January until the last week of June validating this assay. The assays for LPL and GS were validated in 3 and 2 days, respectively.

6. Tissue handling:
   - Cut and handle frozen tissue. Cut with a clean (remove all paint) hack-saw. If sample is frozen, is easy to homogenize.
   - Mincing the tissue with a razor blade HELPS a lot in homogenization.

7. Scintillation counter:
   - We counted for 10 min. Accuracy could be increased by counting for longer times.

Figure 1. Validation of assay for hormone sensitive lipase.


CPM
(Thousands)
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
TIME, min
□ 30 uL HOMOGENATE
+ 60 uL HOMOGENATE

APPENDIX D

GLYCERIDE SYNTHETASE

Reference:

Benson, J. D. and R. S. Emery. 1971. J. Dairy Sci. 54:1034-1040.

Liesman, J. S. 1991. Personal files.

Purpose: to determine the activity of glyceride synthesis (GS) in homogenates of bovine adipose tissue (AT).

Reagents:

0.1 M Na phosphate buffer (pH 7): 3.4 g monobasic Na phosphate anhydrous (FW 120) and 10.23 g dibasic Na phosphate anhydrous (FW 142). Dilute to 1 l with dH_2O and check pH.

0.1 N NaOH: 1 g NaOH (FW 40) in 250 ml of dH_2O.

20 Mm FA solution: to 28 mg Na palmitate (Sigma P-9767, FW 278.4) and 10 μCi palmitic acid add 2 ml 0.1 N NaOH. Bring volume to 5 ml with Na phosphate buffer. Store at -20 °C.

2.7% Bovine serum albumin (BSA): 1.35 g BSA heat-schock treated (Sigma A-3803) to 50 ml with phosphate buffer.

150 Mm KCL: 5.6 g Kcl (FW 74.6) to 0.5 l with dH_2O.

Scintillation fluid: triton base fluid for aqueous samples.

COFACTOR SOLUTION
Total volume: 25 ml

Reagent	Assay mM	Sol. mM	FW	mg	Sigma #
ATP	4.7	23.5	551.2	324	A3377
CoA	0.1	0.5	767.5	9.6	C3019
Dl-glycero-phosphate	18.8	94	324.1	762	G21388
NaF	29.4	147	42	154	-
Dithiothreitol	1.9	9.5	154.2	37	D06322
$MgCl_2$	2.6	13	203.3	66	-

Remember to adjust for other molecules in reagents. Bring to 25 ml with phosphate buffer.

Heptane : isopropanol (1:1): make 1 l with distilled solvents.

10 mM NaOH: bring 100 ml 0.1 M NaOH to 1 l with Dh_2O.

Procedure:

1. FA-BSA solution: thaw FA solution then sonicate in sonicator bath for 1 min and vortex. Calculate amount of FA solution needed for the assay with the formula:

ml FA solution = # assay tubes * 0.01.

Pipet into a test tube and add BSA solution equal to 9 times the FA solution. Vortex and allow to equilibrate for 30 min. To determine specific activity, count 10 µl of FA-BSA solution in 10 ml scintillation fluid.

2. Assay tubes: into 16 X 125 mm glass tubes pipet 0.2 ml of cofactor solution and 0.1 ml FA-BSA solution.

3. Tissue homogenates: weigh frozen AT and add cold Kcl in the proportion of 1:3 (w/v) AT:Kcl. Homogenize with polytron at 3/4 full speed twice for 10 sec each time in 15 ml (Corex) glass tubes. Centrifuge in Sorvall RC2B at 5,000 rpm (3,000 X g, SS-34 rotor) and 5 °C for 10 min. Pour supernatant and save on ice for not more than 2 h before assayed.

4. Assay (1 ml): start assay by adding 0.7 ml of adipose tissue homogenate or KCL for blanks and vortexing. Incubate at 37 °C in water bath with shaking (35 oscillations/min) for 30 min.

5. Stop assay: place tubes on ice and add 4 ml heptane:isopropanol solution. Vortex and add 3 ml of the 10 Mm NaOH and vortex for 10 sec. Centrifuge and transfer supernatant to clean tubes and wash supernatant with 3 ml 10 Mm NaOH. Repeat the wash, then count 1 ml of the supernatant with 10 ml of aqueous scintillation fluid.

NOTE:

This assay is also validated (Figure 2) for an assay volume of 200 µl. Keep the proportions of all reagents the same. After the first 10 mM NaOH wash, add 1.2 ml pure heptane, this will help in pipeting off the 1 ml for counting. Repeatability decreases. It may be useful to increase radioactivity at least 5 times to increase specific activity.

Calculations:

Specific activity (SA) (µmoles/DPM) = 0.02 µmoles / CPM in 10 µl FA-BSA solution.

GS activity (μmoles FA incorporated into glycerides / time) = (sample CPM - blank CPM) * SA / time (h).

Assay validation:

Refer to Figure 1.

1. Linearity with time.
2. Linearity with enzyme concentration.

Figure 1. Assay validation for glyceride synthetase.

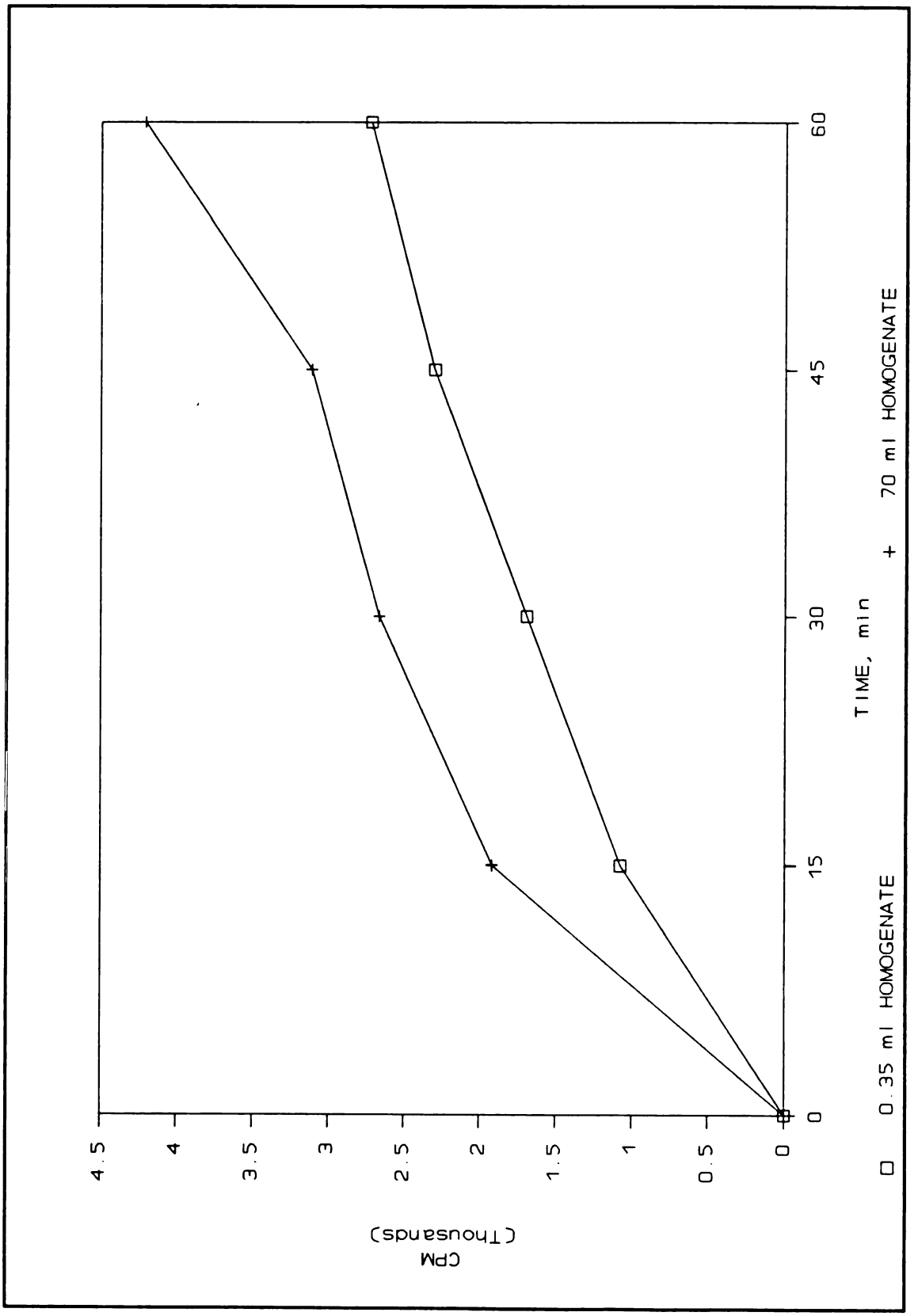
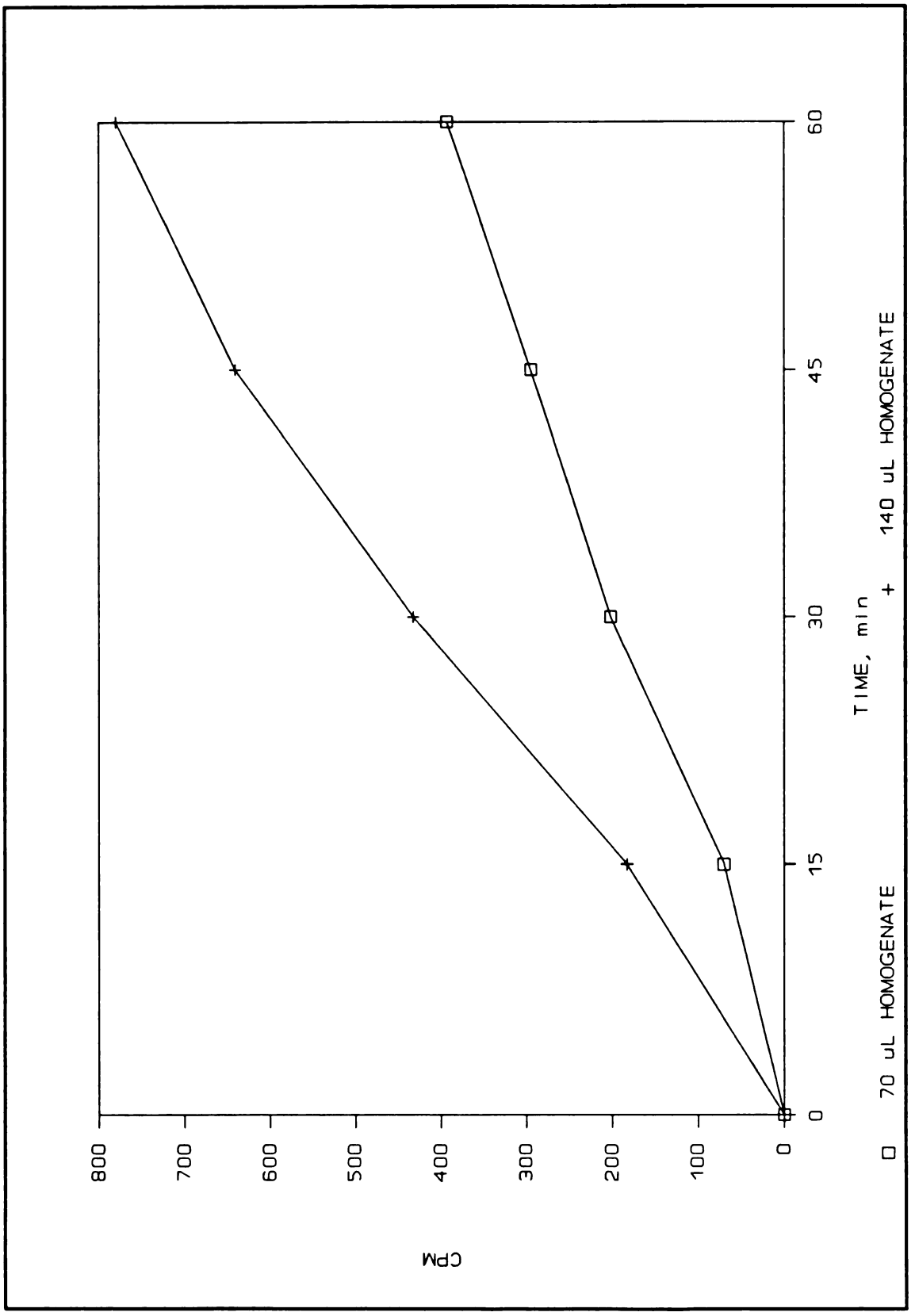

CPM
(Thousands)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
15
30
45
60
TIME, min
□ 0.35 ml HOMOGENATE
+ 70 ml HOMOGENATE

Figure 2. Micro-assay validation for glyceride synthetase.


CPM
800
700
600
500
400
300
200
100
0
0
15
30
45
60
TIME, min
□ 70 uL HOMOGENATE
+ 140 uL HOMOGENATE

APPENDIX E

LIPOPROTEIN LIPASE

References:

Liesman, J. S. 1991. Personal files.

Liesman, J. S. et al. 1988. Lipids 23:

Belfrage and Vaughn. 1969. J. Lipid Res. 10:341.

Nilsson-Ehle, et al. 1972. Clin. Chim. Acta 42:383.

Purpose: to determine the activity of lipoprotein lipase (LPL) in homogenates of bovine adipose tissue (AT).

Reagents:

Triolein: 10 g triolein (Sigma T-7140 or T-7502) diluted to 75.18 ml with toluene to yield 0.133 g/ml or 150 Mm. Store at 4-6 °C with tight solvent proof stopper.

Glycerol tri(9,10 (n)- ^{3}H) oleate: Amersham TRA 191. Should be >99% TG. 5 mCi/25 ml hexane.

^{14}C palmitic acid: Amersham. 5 Mci/0.25 ml toluene.

8% Bovine serum albumin (BSA, Sigma A-3803): BSA heat shock treated, 0.24 M tris, 0.15 M NaCl, Ph 8.6 at room temperature.

1% Triton X-100.

Heat denatured (HD) serum: serum from lactating dairy cow. Denature by heating at 60 °C for 30 min. Centrifuge at 48,000 X g for 20 min. Store at 5 ^{oc}.

Heat denatured serum + NaCl: as above, add 3.33 M NaCl.

HMC solution: 1 heptane (273 ml/l) : 1.41 methanol (385 ml/l) : 1.25 chloroform (342 ml/l). All solvents distilled.

Alkaline buffer: 1.266% (41 Mm) $K_2B_4O_7 \cdot H_2O$, 2.756% (200 Mm) K_2CO_3.

Scintillation fluid: triton base fluid for aqueous

samples.

Procedure:

Premix solutions:

a) Evaporate solvents under N_2 from 20 ml plastic scintillation vials containing:
   TG premix: 0.2 ml labelled trioleate and 1 ml triolein
   FA premix: 0.04 labelled oleate and 1 ml triolein
b) To each vial add:
   2.5 ml HD serum
   8.1 ml 8% BSA
   0.9 ml 1% Triton X-100
c) With the vials in ice sonicate each premix solution 3 X 1 min with pauses for cooling after each min. Make sure sonication tip is clean before immersing in premix containing a different isotope.

Procedure:

1. Quench tubes: prepare 2 tubes as described in the table below, except that the premix is replaced by 8% BSA solution. Otherwise, treat tubes as sample tubes. Add 10 µl of the TG premix or FA to the scintillation vial just prior to counting.

2. Fatty acid recovery tubes: prepare 4 tubes as described in the table below, except that the TG premix is replaced by FA premix. Otherwise, treat tubes as sample tubes.

ASSAY SETUP
volumes in µl

HOMOG.	Kcl	HD SERUM	HD SERUM + NaCl	PREMIX
30, 60	30, 0	0	60	80
30, 60	30, 0	60	0	80

3. Pipet all the solutions, except for the homogenate. Incubate for 20 min at 37 ° in a water bath. Place the tubes on ice, add the homogenate and incubate for 1 h at 37 ° in a water bath with shaking (35 oscillations/min).

4. To stop assay place tubes on ice and follow steps 5 and 6 for FAML.

Calculations:

FA recovery factor (RF) = (cpm in 10 μl FA premix X 10) / (cpm FA recovery)

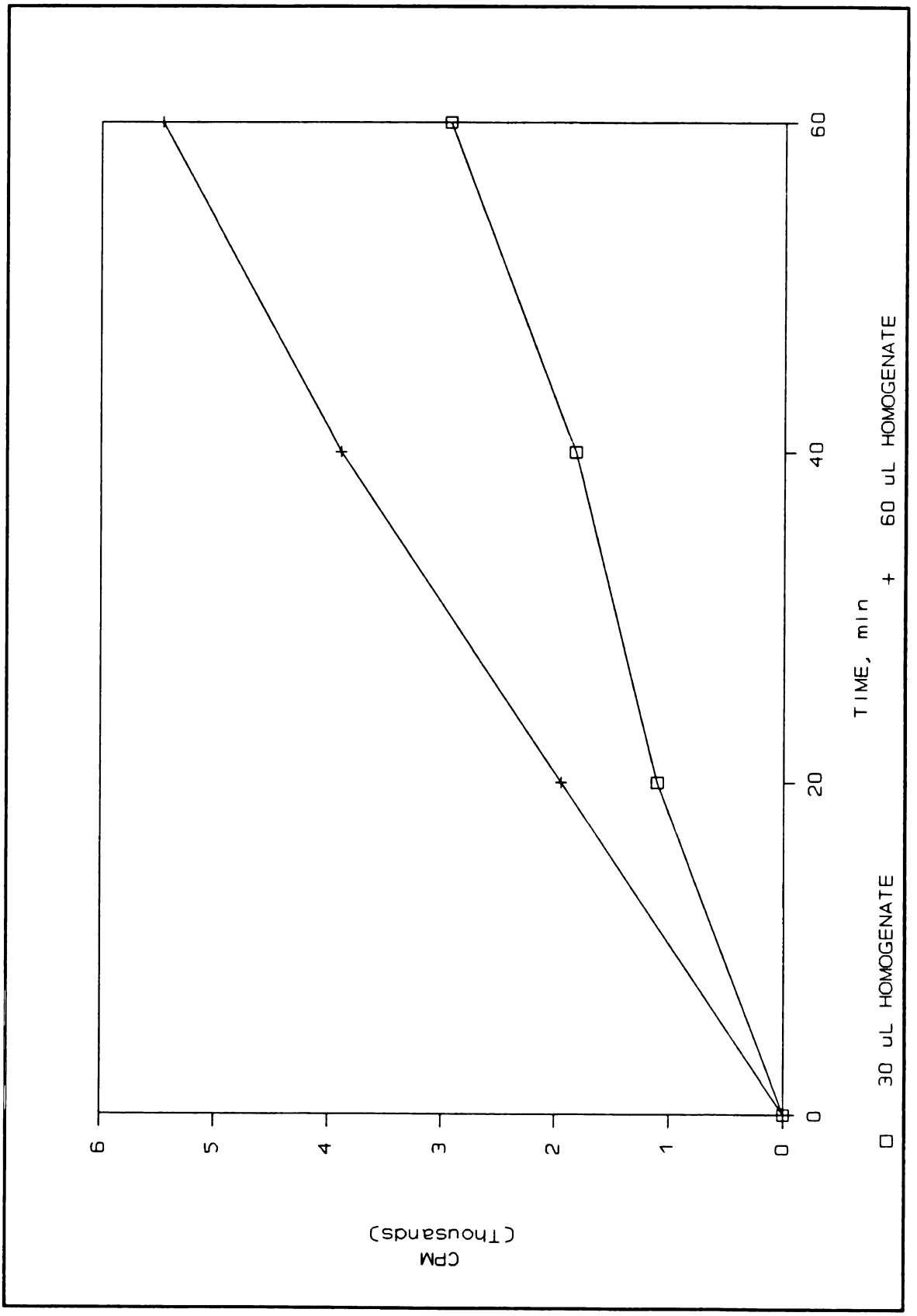
mMoles FA/cpm = [(RF) * (392.04 nmoles FA / cpm in 10 μl FA premix)] / (cpm in 10 μl TG premix)

nmoles FA/tube = [(cpm/tube) - (cpm/enzyme blank)] X (Nmoles FA/cpm)

Validation:

Refer to Figure 1.
1. Test for linearity with time.
2. Test for linearity with enzyme concentration.

Figure 1. Assay validation for lipoprotein lipase.


CPM
(Thousands)
0
1
2
3
4
5
6
0
20
40
60
TIME, min
□ 30 uL HOMOGENATE
+ 60 uL HOMOGENATE

APPENDIX F

MODIFICATION TO MODIFIED LOWRY PROTEIN DETERMINATION

References:

Markwell et al. 1978. Anal. Biochem. 87:206-210.
Liesman, J. S. 1989. Personal files.

Reagents:

A. 2% Na_2CO_3, 0.4% NaOH, 0.16% sodium tartrate, 1% sodium dodecyl sulfate.
B. 4% $CuSO_4 \cdot 5\ H_2O$.
C. 100 solution A : 1 solution B.
D. Folin-Ciocalteu phenol reagent, diluted in half with Dh_2O the day of use.

Procedure:

1. To 1 ml sample or standard (0-100 μg protein) add 3 ml solution C. Vortex and incubate 10 min at room temperature.

2. Add 0.3 ml of solution D, vortex and incubate at 60 °C in a water bath.

3. Cool in water bath at room temperature for 2-3 min, and read absorbance at 660 nm.