THE EFFECT OF MAGNESIUM DEFICIENCY ON THE GLUCONEOGENIC ENZYMES WITH EMPHASIS ON THE EFFECTS OF FASTING AND ANOREXIA

by

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CHAPTER I. INTRODUCTION

Magnesium is the second most abundant intracellular cation (1). It is involved in enzyme activation (2), phosphorylation (2), and hormone secretion (3). It thus directly and indirectly influences the metabolism of protein, fat, carbohydrate, and minerals. Omission of magnesium from the diet has the potential for affecting all major metabolic pathways. Intermediary metabolism has not, however, been extensively studied in magnesium deficiency.

Gluconeogenesis has, in particular, been studied very little during magnesium deficiency. Gluconeogenesis refers to the process of glucose synthesis from non-carbohydrate precursors. This process is important for carbohydrate homeostasis allowing the animal to maintain blood glucose levels during periods of fasting or deficit of dietary carbohydrate (4). Gluconeogenesis is important in the pathogenesis of some diseases. For instance, in diabetes there is increased glucose synthesis which contributes to elevation of the blood glucose concentration (5).

The rate of gluconeogenesis is controlled, in part, by the activities of the four key gluconeogenic enzymes: glucose-6-phosphatase, fructose-1,6-bisphosphatase, phosphoenolpyruvate carboxykinase, and pyruvate carboxylase (6). There is substantial evidence that the activities of these enzymes are controlled by the endocrine system. In particular, insulin, glucagon, epinephrine and the glucocorticoids appear to be involved (4). Magnesium plays a role in the secretion of all four of these hormones. Specifically,

in magnesium deficiency the secretion of corticosterone is decreased (7). In vitro, the secretion of glucagon, insulin, and epinephrine are increased by decreasing the concentration of magnesium in the medium (8-10).

In magnesium deficiency the activities of the key gluconeogenic enzymes may be altered. It is difficult to predict the direction in which the activities of the enzymes may change under a given set of experimental conditions. If the insulin:glucagon ratio were decreased the activity of phosphoenolpyruvate carboxykinase, and possibly fructose bisphosphatase may be increased (11,12). If the circulating level of corticosterone were decreased, then the activities of these enzymes and that of glucose-6-phosphatase may be decreased. It must be recognized that magnesium may influence the activity of fructose bisphosphatase through a mechanism other than an effect on the endocrine system. Magnesium is a cofactor for fructose bisphosphatase (2). Therefore, in magnesium deficiency the activity of this enzyme may decrease.

The purpose of the experiments described herein was to initiate studies of gluconeogenesis in the magnesium deficient rat. The studies focused on the effects of magnesium deficiency on glucose-6-phosphatase, fructose bisphosphatase, and phosphoenolpyruvate carboxykinase. It was reasoned that if magnesium directly or indirectly affects the activities of the gluconeogenic enzymes, then the adaptive response of these enzymes to fasting may be altered in the deficient state. The first experiment was therefore carried out to test the following hypothesis:

1. The gluconeogenic enzymes in the liver of the magnesium deficient rat respond to fasting in the same manner as do the enzymes in the liver of the control rat.

Based on observations made in this first experiment the following hypothesis was tested in a second experiment:

2. Alterations in activities of the key gluconeogenic enzymes in the magnesium deficient rat are due to anorexia.

A third experiment designed to explore the role of insulin and glucagon in producing the changes in the gluconeogenic enzymes observed in magnesium deficiency was also carried out. The hormone assays were not completed at the time of this writing; however, the enzyme data did provide verification of the results of the previous experiments.

CHAPTER II. REVIEW OF LITERATURE

A. MAGNESIUM DEFICIENCY

Omission of magnesium from the diet of experimental animals and man produces a complex syndrome. Abnormalities have been observed in every organ system and in the metabolism of each major class of biological compounds. The features of the syndrome vary with species and may be modified by age, diet composition, and the endocrine system. In this section the symptomatology of magnesium deficiency will be described. Emphasis will be placed on studies carried out in the rat, although studies carried out in other species will be summarized where the results provide relevant information. Clinical symptoms, the metabolism of minerals, protein, fat, and carbohydrate, and the function of tne endocrine system in magnesium deficiency will first be discussed. Subsequently, the effects of age, diet, and the endocrine system on the course of magnesium deficiency will be reviewed.

The Symptoms of Magnesium Deficiency

Clinical symptoms: Kruse et al. (14) were the first investigators to describe the clinical, i.e., external, symptoms of magnesium deficiency in the rat. Rats of an unspecified strain, weighing initially 35 to 45 g, were fed a diet containing 18 ppm magnesium. After three to five days vasodilation was noted in areas of exposed skin. The intensity of the hyperemia increased until day 11 to 14

after which it subsided. The hyperemia was followed by pallor and slight cyanosis. The rats became hyperexcitable during the hyperemic period. This persisted, and on day 18 convulsions were noted. The convulsions were characterized by tonic-clonic spasms. Eighty-six percent of the animals died following the first convulsion. After four to six weeks of deficiency surviving rats developed skin lesions in the form of erythematous, desquamated areas. Loss of hair was noted around the eyes, ears, and under surfaces of the jaws.

These symptoms of magnesium deficiency have been observed in many studies. Most of this work has been carried out in relation to the effects of such variables as age and diet on the course of magnesium deficiency. These studies will be reviewed below.

Tissue magnesium levels: In numerous studies of magnesium deficiency the levels of magnesium in the blood, bone, and soft tissues have been determined. The following paragraphs will highlight the major findings.

Smith et al. (15) studied female Holtzman rats weighing initially 160 to 180 g. The animals were placed on diets containing either 10 or 400 ppm magnesium. The control rats were pair-fed to the deficient rats. After one, two, and four weeks the serum magnesium values of the control rats were 1.83, 1.85, and 2.10 mEq/l, respectively. In the magnesium deficient rats the serum magnesium values were 1.36, 0.94, and 0.70 mEq/l, respectively. Differences between control and experimental animals were significant at all three sampling times. After two to four weeks analysis of thigh muscle revealed a significant

decrease in the magnesium concentration (mg magnesium/kg fat free dry solids) of tissues obtained from the magnesium deficient rats relative to values obtained in controls.

Ko et al. (16) fed Sprague-Dawley rats, weighing initially 75 g, diets containing either 16 or 650 ppm magnesium for a period of six weeks. The diets were fed ad libitum. Muscle was obtained from three deficient and two control rats at weekly intervals. The results obtained from weeks two and five were pooled for statistical analysis. The mean concentrations of magnesium in the muscles of the control and deficient rats were 3.54 and 3.42 mm magnesium/100 g fat free dry solids, respectively. The difference was not statistically significant.

Martindale and Heaton (17) carried out extensive studies on the magnesium concentration of tissues of magnesium deficient rats. Male Sprague-Dawley rats, weighing initially 190 to 220 g, were fed diets containing either 3 or 400 ppm magnesium for up to 62 days. After 31 and 62 days serum magnesium levels in control rats were 2.80 and 2.55 mg/dl, respectively, and 0.89 and 1.19 mg/dl, respectively, in deficient rats. The differences were significant after both 31 and 62 days. Significant decreases in the total magnesium content and concentration (mg magnesium/g non-collagenous nitrogen) were observed in heart, liver, muscle, and kidney of rats receiving 3 ppm magnesium after both 31 and 62 days, relative to values observed in controls.

In female Wistar rats, weighing initially about 100 g, and fed diets containing either 3 or 750 ppm magnesium for two weeks, Heaton and Anderson (18) observed a plasma magnesium concentration of 2.31 mg/dl in control rats as compared to a concentration of 0.24 mg/dl in deficient rats. The difference was statistically significant. Analysis of kidneys showed no significant differences in tissue concentrations of magnesium between the two treatment groups.

In a study carried out by Britton and Stokstad (19) male weanling Sprague-Dawley rats were fed, ad libitum, diets containing 80, 100, 120, 160, or 1080 ppm magnesium for 28 days. Rats fed the lowest level of magnesium did not survive the experiment. In rats fed 100, and 120 ppm magnesium, serum magnesium levels were 7.5 and 7.1 µg/ml, respectively. In rats fed 160 and 1080 ppm magnesium the serum magnesium levels were 10.3 and 18.9 µg/ml, respectively. The difference between these two latter values was statistically significant. Both latter values were significantly greater than values obtained in rats fed the lower levels of magnesium. No significant differences among treatment groups were found in heart or kidney magnesium concentrations.

Elin et al. (20) fed male Holtzman rats, weighing initially 110 g, diets containing either 0.11 or 16.5 mm magnesium/kg diet for up to nine months. Rats maintained on the deficient diet were given a supplement of 4.6 mm magnesium once per week. At the end of the experiment plasma and bone ash magnesium concentrations were both significantly decreased in deficient rats relative to values observed

in controls. Liver and muscle magnesium concentrations were similar in control and deficient rats.

In general, bone and plasma magnesium levels are consistently decreased in magnesium deficiency. The effects of feeding a magnesium deficient diet on soft tissue concentrations of magnesium are quite variable.

Tissue levels of minerals other than magnesium: A number of researchers, in the course of studies on magnesium deficiency, have noted changes in levels of minerals other than magnesium in tissues of depleted rats. Calcium, phosphorus, sodium, and potassium have been most frequently measured. Only major findings will be reviewed here.

In magnesium deficiency there is marked calcification of soft tissues. Tufts and Greenberg (21) fed rats of an unspecified strain diets containing either 40 or 500 ppm magnesium for up to 100 days. Calcium concentrations of muscle, heart, and kidney increased two, eight, and twelve fold, respectively, in magnesium deficient rats relative to control values.

Martindale and Heaton (17) carried out extensive studies of tissue calcium concentrations. Female and male Sprague-Dawley rats, weighing 190 to 220 g, were fed diets containing 30 or 400 ppm magnesium for up to 62 days. At the end of the study plasma calcium concentrations were 11.52 and 13.11 mg/dl in control and deficient rats, respectively. The difference was statistically significant. Significant increases were also observed in calcium concentrations

of bone ash and of heart, liver, kidney, and skeletal muscle in magnesium deficient rats relative to control values.

Considerable research has been carried out on the histological characteristics of the soft tissues in magnesium deficiency. Battifora et al. (22) fed male Wistar rats diets containing 50 to 70 or 650 ppm magnesium for up to six weeks. Histological examination of kidneys obtained from deficient rats revealed apatite crystal deposition in the pars recta of the proximal convoluted tubule, the loops of Henle, and the collecting tubules. Deposits were both extracellular and intracellular. Schneeberger and Morrison (23) carried out similar studies and observed that the calcification produced by magnesium deficiency is unlike that produced by vitamin D, parathyroid hormone, or calcium gluconate.

Heggtveit et al. (24) carried out histological studies of the heart in magnesium deficiency. Female Sprague-Dawley rats, weighing an average of 70 g, were fed diets containing 10, 1500, or 2000 ppm magnesium for up to 36 days. After 14, 21, 29, and 36 days myocardial calcium deposition was seen as discrete basophilic granules within the cytoplasm of degenerating muscle fibers in the deficient rats. As time progressed the lesions became more severe with the calcium granules enlarging and coalescing.

Since phosphorus is frequently associated with calcium in biological systems, this mineral, too, has been measured in tissues of magnesium deficient rats. For instance, Itokawa and Fujiwara (25) carried out a study in which bone and soft tissue phosphorus levels

were assessed in male Wistar rats. The rats weighed 80 to 100 g initially, and were fed diets containing zero or 0.6 g $MgCl_2$ per 100 g diet for four weeks. Phosphorus concentrations, expressed on a dry weight basis, were decreased in serum and bone of magnesium deficient rats relative to values observed in tissues of controls. No changes, relative to control values, were observed in phosphorus concentrations of brain, heart, liver, spleen, kidney, or muscle of magnesium deficient rats.

Brautbar et al. (26) fed weanling, male, Sprague-Dawley rats diets containing either 600 or 1000 ppm magnesium for up to 14 days. Phosphorus was determined in serum, bone, muscle, liver, and kidney. Although neither of the two diets were deficient in magnesium there was a marked interaction between magnesium and phosphorus. Phosphorus concentration in bone was significantly decreased in rats receiving the diet containing 600 ppm magnesium relative to that of rats receiving the higher level of dietary magnesium. Serum phosphorus levels were unaffected by dietary treatment. Muscle, liver, and kidney phosphorus levels were increased by feeding the diet containing 600 ppm magnesium relative to values obtained by feeding 1000 ppm magnesium. The statistical significance of the results was not stated.

Measurements of sodium and potassium levels of tissues of magnesium deficient rats have frequently been made. Whang and Welt (27) fed female Sprague-Dawley rats a diet deficient in sodium, potassium, magnesium, chlorine, and phosphorus. Solutions containing

sodium, potassium, chlorine and phosphorus, with and without the addition of magnesium, were administered daily by gavage. The experiment was carried out for 31 days. In the magnesium deficient group, relative to values observed in the control group, no changes were observed in the serum sodium or potassium concentrations. Muscle and erythrocyte sodium levels were increased while potassium levels were decreased in the deficient group relative to values obtained in controls.

As described above, Martindale and Heaton (17) carried out a 62 day study on the magnesium deficient rat. At the end of the study there was a significant elevation in the plasma sodium concentration with no change in the plasma potassium concentration in magnesium deficient rats, relative to values observed in controls. The potassium concentration of liver, heart, and thigh muscle was decreased, while the concentration of sodium was increased in these tissues in the magnesium deficient group, relative to values obtained in the control group.

To summarize: in magnesium deficiency there is calcification of soft tissues. The concentration of phosphorus decreases in bone and plasma. The deficient state is, furthermore, characterized by sodium accumulation and potassium loss. There has been, and continues to be, considerable research interest in defining the mechanisms responsible for these changes. This subject has been reviewed in a number of books and publications (28-30) and will not be reviewed here.

Protein, fat, and carbohydrate metabolism: Delayed growth was an early noted feature of magnesium deficiency (14). This prompted numerous later researchers to explore the effect of magnesium deficiency on protein metabolism. Studies carried out have emphasized effects on protein synthesis.

Schwartz et al. (31) studied male, weanling, Sprague-Dawley rats fed diets containing either 100, 500, or 1000 ppm magnesium with either 12 or 36 percent protein for five weeks. Carcass nitrogen (expressed as a percentage of fresh weight) was unaffected by the dietary magnesium level at either protein level. Percent dietary nitrogen retained and liver nitrogen were unaffected by the level of dietary magnesium, regardless of protein level. There was an increase in the plasma protein concentration as the level of dietary magnesium increased. This increase was significant only in the group of rats fed the 1000 ppm magnesium diet, relative to the value observed in the group of rats fed the lowest level of magnesium at the 36 percent protein level.

In subsequent work Schwartz et al. (32) studied the synthesis of plasma albumin by the magnesium deficient rat. Male, weanling, Sprague-Dawley rats were fed diets containing 80, 400, or 800 ppm magnesium for five weeks. One hour before sacrifice the rats were administered 10 μCi L-^{14}C-valine intraperitoneally. Incorporation of the isotope into liver albumin was determined as a measure of albumin synthesis. Albumin synthesis was successively and significantly increased by increasing the dietary magnesium level.

Zieve et al. (33) studied protein synthesis in various tissues of the rat. Male Holtzman rats were fed diets containing either 50 or 1300 ppm magnesium for 4.5 months. The rats were then injected with 10 μCi ^{3}H-leucine intraperitoneally. Fifteen minutes later the rats were sacrificed. Levels of radioactivity in protein of liver and kidney were depressed while those in spleen and thymus were increased in the magnesium deficient group relative to values obtained in the control group.

Protein synthesis in magnesium deficiency has recently been studied at the subcellular level. Magnesium ions are required for the attachment of mRNA and tRNA to the ribosomes and for the aggregation of the 30S and 50S subunits (2). Grace and O'Dell (34) studied polysome structure in livers of magnesium deficient guinea pigs. Weanling guinea pigs were fed diets containing 20 or 1200 ppm magnesium for 36 or 37 days. Polysomes were subsequently isolated from liver. Polysomes obtained from livers of magnesium deficient guinea pigs lacked the 131S ribosomal unit found in polysomes of control guinea pigs. Polysomes of the former group has a 64S particle not found in polysomes of the latter group.

Fat metabolism has been little studied in the magnesium deficient rat. Schwartz et al. (31) observed that as the dietary magnesium concentration was increased the fat content of the carcass increased. This was observed at both the 12 and 36 percent protein levels.

Rademeyer and Booyens (35) observed changes in plasma cholesterol levels of magnesium deficient rats. Young rats of an unspecified strain

were fed a diet with an unspecified but deficient level of magnesium for nine weeks. After 6 weeks the glucose in the diet of one half of the rats was replaced by an equal weight of maize meal high in magnesium. Blood was obtained three weeks later. Addition of maize meal to the diet of the magnesium deficient rats resulted in a significant reduction in the plasma cholesterol level relative to the value obtained in unsupplemented control rats.

Only studies of a preliminary nature have been carried out on the effects of magnesium deficiency on glucose metabolism. Suzuki and Fuwa (36) fed female rats of the Donryu strain diets containing either 25 or 75 percent casein. The magnesium levels in the control and deficient 25 percent protein diets were 530 and 46 ppm, respectively. The levels of magnesium in the high protein diets were 510 and 26 ppm, respectively. The diets were fed, ad libitum, for a period of 14 days. The rats were then sacrificed without preliminary fasting. The activity of glucose-6-phosphatase was measured in the kidney. With both the high and low protein diets, magnesium deficiency was accompanied by a reduction in the activity of renal glucose-6-phosphatase. In control rats raising the dietary protein level increased the renal glucose-6-phosphatase activity 140 percent; raising the dietary protein level in the magnesium deficient rats increased the activity of this enzyme 136 percent. The difference was not significant. Thus magnesium deficiency did not appear to affect the ability of renal glucose-6-phosphatase to respond to a high protein diet, but it did affect the absolute level of this enzyme.

Two studies on the role of magnesium in glucose production have been reported. In the first investigation, that carried out by Rutman et al. (37), kidney slices obtained from normal Wistar rats were incubated in a Krebs-Ringer buffer containing pyruvate in the presence and absence of 2 mM magnesium. The kidney slices were incubated for 60 minutes at 38 C. Glucose production, as measured by the release of glucose into the incubation medium, was not altered by the absence of magnesium from the medium.

Roobol and Allyne (38) studied the effect of magnesium on glucose production by liver slices. Male adult Sprague-Dawley rats, presumably fed an adequate diet, were fasted for 18 to 22 hours then sacrificed by cervical fracture. Liver slices were incubated in a Krebs bicarbonate buffer at 37 C for 90 minutes. The effect of magnesium on glucose production was determined by omitting this cation from the medium. Release of glucose into the medium was similar in samples incubated in the presence and absence of magnesium.

Kahil et al. (39) undertook a study of peripheral glucose metabolism in the magnesium deficient puppy. Hound puppies received either a magnesium deficient diet alone or a magnesium deficient diet supplemented with 16 mg magnesium/kg body weight/day. The latter, control, group was pair-fed to the former, magnesium deficient, group. The experiment was carried out for 35 days. Carbohydrate metabolism was studied before and after induction of magnesium deficiency. Glucose tolerance was measured by the intravenous administration of 500 mg glucose/kg body weight. The blood glucose

concentration was measured before and 20 minutes after glucose administration. Prior to induction of magnesium deficiency increases in blood glucose in control and test groups, after glucose administration, were 37.9 and 39.3 mg/dl, respectively. After one week on the experimental diets the blood glucose concentration rose 34.6 mg/dl in the control group and fell 6.1 mg/dl in the magnesium deficient group. Plasma insulin was determined in samples obtained during glucose tolerance testing. No significant differences were noted between control and experimental puppies in plasma insulin concentration. Plasma magnesium values were not measured at the time glucose tolerance testing was carried out. However, after two to four weeks of study, magnesium deficient puppies displayed significantly decreased plasma magnesium levels relative to values obtained in controls.

In addition to this study on intact animals, in vitro studies on the effect of magnesium deficiency on glucose uptake by the rat diaphragm were carried out. Rats weighing 115 to 125 g were fed either a low magnesium diet (magnesium level not stated) or the low magnesium diet supplemented with 0.27 mEq magnesium daily. The feeding trial was carried out for two weeks. After sacrifice whole diaphragms were incubated at 37 C in a Krebs-Ringer bicarbonate buffer in the presence of 2-deoxyglucose. After 30 minutes of incubation 2-deoxyglucose uptake was significantly increased by diaphragms of magnesium deficient rats in the presence or absence of 1 mU insulin/ml incubation fluid relative to values observed in control diaphragms. With 400 mU insulin there were no differences

between control and experimental rat diaphragms in 2-deoxyglucose uptake. The 2-deoxyglucose uptake by diaphragms of magnesium deficient rats could be decreased to control values by addition of physiological amounts of magnesium to the incubation medium. In additional studies 2-deoxyglucose uptake by rat diaphragm was studied after 0, 3, 6, 8, 10, and 14 days of magnesium deficiency. As the serum level of magnesium decreased, uptake of 2-deoxyglucose by the diaphragm increased. Glucose and inulin spaces of magnesium deficient and control rat diaphragms were measured after 30 and 90 minutes of incubation in a medium containing ^{14}C-inulin and ^{14}C-glucose. The volume of distribution of glucose exceeded that of inulin, but the results were similar in control and deficient rats.

No studies of glucose degradation, per se, have been carried out on magnesium deficient rats, but two research groups have reported changes in tissue levels of enzymes involved in glucose catabolism. Zieve et al. (40) undertook a study of blood and liver transketolase in magnesium deficiency. Female Holtzman rats were fed diets containing 602 or 32 ppm magnesium for various time periods. After 33 and 59 days the whole blood magnesium concentration was significantly reduced in magnesium deficient rats relative to values observed in controls. Blood transketolase activity was significantly reduced in magnesium deficiency after two months. After 33 and 59 days of magnesium deficiency, liver transketolase activity was significantly reduced in experimental rats, relative to values observed in control rats.

Cadell and Olson (41) studied the effects of magnesium deficiency on liver hexokinase, glucokinase, and pyruvate kinase. Female Wistar rats, weighing initially 45 to 60 g, were fed diets containing either 50 or 2000 ppm magnesium. The experiment was carried out for 60 days with enzyme activity determinations made when the rats weighed between 100 and 150 g. Of the three enzymes, only the activity of liver hexokinase was reduced by magnesium deficiency. Liver magnesium concentration was unchanged by experimental treatment.

Little is known regarding the effect of magnesium deficiency on glycogen metabolism. However, Rayssigieur (42) has obtained preliminary results which indicate that the concentration of glycogen in liver of magnesium deficient rats is decreased.

In summary, magnesium deficiency is characterized by a decrease in protein synthesis. Alterations in fat and carbohydrate metabolism have been observed but are little understood.

The endocrine system: Magnesium is involved in cellular secretory processes; it is, therefore, involved in hormone secretion (3). Abnormalities in calcium metabolism occurring in magnesium deficiency early suggested that parathyroid hormone (PTH) secretion may be increased (43). Buckle (44) studied the effect of blood magnesium concentration on PTH secretion by goat parathyroid glands. Parathyroid glands were perfused at a constant rate with dialyzed blood containing an unspecified level of magnesium or no magnesium. When blood containing magnesium was perfused, PTH secretion was decreased relative to that observed when unsupplemented blood was perfused.

These findings were supported by those obtained by Massry et al. (45).

The action of calcitonin (CT) opposes that of PTH, and this hormone, too, has been studied in relation to magnesium. Littledike and Arnaud (46) studied CT secretion by the C cells of pig thyroid. Seven female crossbred pigs were infused, through the femoral artery, with a solution containing 1.38 M $MgCl_2$ at varying rates. As the rate of magnesium infusion was increased from 1.38 to 27.6 μM/kg/minute there were successive increases in the levels of CT in peripheral blood.

Corradino and Park (47) studied the effect of magnesium deficiency on the function of the thyroid gland in the rat. In rats fed diets containing 0, 50, 280, 560, or 2240 ppm magnesium for five weeks, there were slight, but statistically significant, decreases in thyroid weight with each successive increase in dietary magnesium. The ^{131}I content of the thyroid gland, 24 hours after the injection of 10 μCi $K^{131}I$, decreased as the level of magnesium in the diet decreased.

Williams (48) reported studies on the effect of magnesium on the release of ^{131}I by the thyroid gland of the rat. Thyroid glands, obtained from apparently normal rats, were labeled, by an unspecified method, with ^{131}I. The glands were incubated in a buffered medium with and without addition of 20 mM magnesium. Addition of magnesium to the medium suppressed ^{131}I release by thyroid glands.

Ginn et al. (7) investigated the effect of magnesium deficiency on corticosterone secretion. Male Sprague-Dawley rats of an

unspecified age were fed diets containing 40 or 500 ppm magnesium for 43 days. Blood was collected from the left adrenal vein for 10 minutes for the purpose of measuring the corticosterone secretion rate. The rate of corticosterone secretion was significantly decreased in the magnesium deficient group relative to the value obtained in the control group. Evidence that decreased secretion of corticosterone is accompanied by a decreased circulating level of this hormone in magnesium deficiency was obtained by Richer et al. (49).

In the study carried out by Ginn et al. (7) the secretion rate of aldosterone was also measured. Aldosterone secretion was significantly increased in deficient rats relative to that observed in control rats. The level of aldosterone in plasma was subsequently found to be increased in magnesium deficient rats relative to that of adequately fed control animals by Soulunias and Schwartz (50).

The secretion of adrenalin by the adrenal medulla has also been studied in relation to magnesium. Ishikawa et al. (10) observed that perfusion of the rat adrenal gland with solutions containing increasing amounts of magnesium successively decreased adrenalin secretion. The secretion of this hormone has not been studied in the magnesium deficient animal.

The function of the endocrine pancreas appears to be affected by magnesium. Curry et al. (8) studied insulin secretion by the _in vitro_ perfused pancreas. At fixed calcium levels increasing the magnesium concentration of the perfusing medium from 0.6 to 1.2 to 2.4 mEq/l resulted in successively decreased insulin secretion.

Leclerq-Meyer et al. (9) studied the secretion of glucagon by pancreatic tissue incubated in a bicarbonate buffered medium. Omission of magnesium from the medium resulted in increased glucagon release by the tissue.

The central role of the pituitary in the function of the endocrine system has prompted studies of the effects of magnesium on hormone secretion by this gland. Gautvik and Tashjian (51) studied the secretion of growth hormone and prolactin by clonal cells of rat pituitary gland. Increasing the concentration of magnesium in the medium suppressed secretion of both hormones. Wakabayashi et al. (52) studied the effect of magnesium on the release of follicle stimulating hormone (FSH) and leutinizing hormone (LH) by rat pituitary. Whole pituitary glands obtained from adult male Sprague-Dawley rats were incubated in the presence and absence of 20 mM magnesium. The presence of magnesium decreased the release of both hormones.

Studies of pituitary function have not been carried out in the magnesium deficient rat. Reproduction is adversely affected in magnesium deficiency (53) and studies of pituitary hormone secretion may provide insight into the mechanism of this abnormality.

It is apparent from the above that magnesium exhibits a variety of effects on the endocrine system. The function of the endocrine system in magnesium deficiency has not been well researched. Calcium as well as magnesium is involved in cellular secretory processes (3). As we have seen calcium metabolism is abnormal in magnesium deficiency. The interaction of calcium and magnesium with respect to hormone

secretion in magnesium deficiency is an unexplored area of study.

Variables Affecting the Course of Magnesium Deficiency

Comparison of studies on magnesium deficiency carried out by various research groups frequently reveals discrepancies in the time of onset of symptoms and tissue mineral levels. These discrepancies have prompted research on such variables as age and diet on the course of magnesium deficiency. In addition, limited work has been carried out on the role of the endocrine system in this process. The studies described below could be used to determine the factors responsible for differences in results of various studies. Such an analysis will not be carried out here.

Age: Smith and Field (54) compared the effects of magnesium deficiency in young and adult hooded Lister rats. Young rats used in this study were 8 weeks old at the start of the experiment whereas adult rats were aged 9 to 12 months. The rats were fed a magnesium deficient diet (0 ppm added magnesium) for 18 days. Controls received a diet containing 500 ppm added magnesium. Hyperemia developed in young rats after 8 to 11 days; in adult rats it was observed 11 to 14 days after the beginning of the study. Convulsions were noted in young rats but not in old rats. The weights of the magnesium deficient adult rats remained almost constant throughout the experiment whereas the weights of the control rats increased. The young magnesium deficient rats gained weight throughout the experiment but at a slower rate than controls. In both young and adult deficient animals

the plasma magnesium concentrations were lower after 8 days relative to values observed in controls. The concentration of magnesium in the femur was significantly decreased in all deficient rats relative to values observed in controls. The decrease in bone magnesium was significantly greater in young deficient rats than in adult deficient rats. No significant differences were noted among the various treatment groups in the concentrations of magnesium in liver or muscle. Similar results were reported by Smith and Nisbet (55,56).

Hunt (57) investigated rats classified as young, mature, or old on the basis of body weight (100, 190, and 382 g, respectively). The animals all received a diet containing 66 ppm magnesium. Control rats received an additional 20 to 30 mg magnesium/100 g body weight daily in their drinking water: deficient rats received 1/60th of this amount. The experiment was carried out for 20 days. Hyperemia and edema of the extremities were noted in all magnesium deficient rats. The onset of these symptoms was day five in the young rats and day eight in the mature and old rats. Neuromuscular irritability was most severe in young rats and 25 percent of these animals died following convulsions. Convulsions were less frequent in mature rats and were not observed in old rats. Skin lesions were observed in all rats but they were most severe in mature rats. The weight gain of old magnesium deficient rats was similar to that of old control rats. Weight gains in young and mature magnesium deficient rats were less than those of their respective controls. Significant decreases in the plasma magnesium levels were observed in all deficient rats

relative to their respective controls. The decrease in the plasma magnesium level in old magnesium deficient rats was significantly greater than that observed in either young or mature rats. All deficient rats displayed a significantly lower bone magnesium concentration than did control rats. Bone magnesium concentration in deficient rats increased as the initial starting age increased.

In summary, the severity of the magnesium deficiency syndrome increases as the initial age of the rat decreases as indicated by the mortality rate, incidence of convulsions, and the bone magnesium concentration. The severity of magnesium deficiency appears to be greatest where growth is most rapid.

Diet: A variety of diets have been used in studies of magnesium deficiency and several research groups have attempted to define the effects of the mineral, protein, fat, and carbohydrate content of the diet on the course of magnesium deficiency.

Forbes (58) studied the relationship of dietary sodium and potassium to tissue levels of magnesium. Weanling, male, Sprague-Dawley rats were fed, ad libitum, diets containing 40, 220, 650, and 1255 ppm magnesium at each of the following levels of potassium: 0.09, 0.27, 0.62, and 1.40 percent. Diets containing 40, 215, and 1020 ppm magnesium were fed at sodium levels of 0.048, 0.497, and 0.927 percent. The experiment was carried out over a 28 day period. Normal levels of magnesium in serum were found only in rats receiving 650 or 1255 ppm magnesium at each level of potassium and in rats receiving 1020 ppm magnesium at each level of sodium. At each level of dietary

magnesium serum magnesium concentration was increased in rats receiving the lowest levels of sodium or potassium. In rats receiving 40 ppm magnesium concentrations of magnesium in heart and muscle were decreased at each level of dietary sodium or potassium relative to values in controls fed the highest level of magnesium. Neither dietary sodium nor potassium level affected soft tissue magnesium concentrations.

McAleese and Forbes (59) studied the effect of dietary calcium on serum and kidney magnesium concentrations. Weanling male rats, weighing initially 90 to 110 g, were fed diets containing 20, 80, 175, 285, 360, 420, and 510 ppm magnesium at each of three calcium levels: 0.2, 0.4, and 0.8 percent. The diets were fed ad libitum for 28 days. Serum magnesium concentration rose as dietary magnesium was increased from 20 to 360 ppm. Values obtained for rats receiving 360, 420, and 510 ppm magnesium were similar. At each level of dietary magnesium the serum magnesium concentration was greater in rats fed 0.2 percent calcium relative to the values obtained in rats fed the two higher levels of calcium at each dietary magnesium level. Kidney magnesium concentration was unaffected by dietary magnesium or calcium intake.

Forbes (60) studied the effect of phosphorus on magnesium deficiency in the rat. Male Sprague-Dawley rats, weighing initially 60 g, were fed diets containing either 142 or 420 ppm magnesium with either 0.19 or 0.50 percent phosphorus. The study was carried out for 28 days. The classical symptoms of magnesium deficiency were not

observed in rats receiving the magnesium deficient, low phosphorus diet. Erythema and edema of the extremities were noted in rats receiving the magnesium deficient diet with the higher level of phosphorus. The percentage of magnesium in femur ash was significantly lower in magnesium deficient rats fed 0.5 percent phosphorus than the value obtained in rats fed the magnesium deficient diet with the lower level of phosphorus. The percentage of magnesium in femur ash of rats receiving the higher level of magnesium was not affected by the addition of phosphorus to the diet.

Colby and Fry (61) were the first researchers to report the effects of dietary protein level on the features of magnesium deficiency. Sprague-Dawley rats, weighing initially 60 to 80 g, were fed, for 14 days, diets containing either deficient or adequate levels of magnesium (exact levels of magnesium not stated), with either 24 or 50 percent casein. At each level of dietary magnesium whole blood magnesium concentrations were decreased in rats fed 50 percent casein, relative to values observed in rats fed 24 percent casein. The statistical significance of the results was not stated.

Bunce ta al. (62) fed male Holtzman rats diets composed of either 12 or 36 percent casein with three levels of dietary magnesium: 100, 200, and 600 ppm. With either 200 or 600 ppm magnesium the average weight gain of the rats was higher with the higher protein level. Seventy-five percent of the rats fed 36 percent protein with 200 ppm magnesium developed hyperemia; no hyperemia developed in rats fed 12 percent casein with 200 ppm magnesium or in rats fed the

highest level of magnesium at both protein levels. Serum magnesium concentration rose with dietary magnesium level at each protein level. Dietary protein level had no consistent effect on serum magnesium concentration. Liver magnesium concentration rose with dietary magnesium concentration at each protein level. The differences observed between rats fed the lowest and highest levels of magnesium were statistically significant. At each level of dietary magnesium the bone magnesium concentration was higher in rats fed the higher level of protein. The statistical significance of the results were not stated.

A similar study was carried out by Schwartz et al. (31). Male Sprague-Dawley rats were fed, one week post-weaning, diets containing 12 or 36 percent casein at each of three levels of magnesium: 100, 500, and 1000 ppm. The diets were fed ad libitum for five weeks. The concentration of magnesium in plasma rose with the level of dietary magnesium at each protein level. At the lowest level of magnesium the plasma magnesium concentration was slightly depressed in rats fed the higher level of protein. At each of the other two levels of dietary magnesium the plasma magnesium concentration was significantly increased in animals fed 36 percent protein relative to values observed in rats fed 12 percent protein. The level of magnesium in the liver was unaffected by dietary treatment.

There has been only one well controlled study on the effects of dietary carbohydrate on magnesium metabolism. Scholz and Featherston (63) carried out three experiments designed to test the effects of

dietary glucose, sucrose, lactose, and starch on magnesium utilization in the chick.

In the first study day-old, white cockerel chickw were fed, for four weeks, diets containing 50 percent glucose, sucrose, or starch, or 25 percent glucose with 25 percent lactose at each of four dietary magnesium levels: 250, 300, 350, and 400 ppm. The lowest mortality rate in chicks fed 250 ppm magnesium was observed in birds fed the lactose diet. Mortality was highest during the fourth to the sixth day of the experiment in chicks fed glucose, sucrose, or starch and during the second week of the experiment in chicks fed the lactose diet. Growth was similar in chicks receiving the various carbohydrate sources at the three lower levels of magnesium. With 400 ppm magnesium growth was greatest with the sucrose diet and lowest with the starch diet. Dietary carbohydrate source had no consistent effect on femur magnesium concentration. In chicks fed each dietary carbohydrate source an increased dietary magnesium level was associated with an increased plasma magnesium concentration. At the two lowest levels of dietary magnesium the dietary carbohydrate source had no effect on the plasma magnesium concentration, but with 350 and 400 ppm magnesium lactose feeding produced the highest plasma magnesium level. In a second experiment this increased magnesium level was shown to be due, in part, to hemoconcentration.

In the third experiment carried out by these workers femur and plasma magnesium levels were determined weekly, for four weeks in chicks fed glucose or lactose with 250, 300, 350, or 400 ppm dietary

magnesium. Higher plasma magnesium levels and bone magnesium concentrations were found by week two in birds fed lactose at each level of dietary magnesium.

Only a single study had been carried out on the effects of dietary fat on magnesium metabolism. Redemeyer and Booyens (35) fed albino rats of an unspecified strain a diet containing a salt mixture prepared without the addition of magnesium. The diet contained 20 percent sunflowerseed oil and was fed for three weeks. At this time the sunflowerseed oil was replaced by butter, and this altered diet was fed to one-half of the rats for an additional three weeks. The substitution of butter for sunflowerseed oil had no effect on the serum magnesium levels of experimental rats.

In a second experiment four groups of rats were fed a diet containing 2550 to 3000 ppm magnesium. The control group received a diet of unspecified composition. The three additional groups received diets containing 25 percent butter, 25 percent "dripping," or 25 percent sunflowerseed oil. The diets were fed for four weeks. Blood samples were obtained weekly by heart puncture. The serum magnesium level declined progressively in rats receiving the diets containing dripping or butter, relative to values observed in the serum of control rats. Feeding the sunflowerseed oil diet had no effect on the serum magnesium concentration.

These studies, taken together, indicate that manipulations of dietary sodium, potassium, calcium, protein or carbohydrate have the potential for altering the course of magnesium deficiency. Protein

is the dietary component which has the most striking effect. The effect of protein bears a similarity to the effect of age. In both the young rat and the rat given a high level of dietary protein growth is rapid (31). This suggests that a high growth rate, by increasing the need for magnesium, increases the severity of a dietary imposed deficiency.

The endocrine system: A number of factors have led to the study of endocrine effects on magnesium metabolism. These factors include species differences in the features of magnesium deficiency, observations on human patients with endocrine disorders, and similarities between the effect of magnesium deficiency and various hormones on the metabolism of calcium, sodium, and potassium. Research in this area has not been extensive and has often been anecdotal. The major findings will be reviewed here.

In a study carried out by Palmieri et al. (64) male rats of the Holtzman strain were fed a low calcium diet for 24 hours. At this time groups of animals were given subcutaneous injections of either 60 MRC mU porcine calcitonin, 5 mg cortisone twice a day for four days, or 30 USP units of parathyroid extract (PTE) every 12 hours for four days. Control rats received an injection of 0.1 M sodium acetate buffer. Administration of thyrocalcitonin produced a reduction in plasma magnesium concentration while administration of cortisone or PTE produced an increase relative to values observed in controls.

Farnell (65) studied the effects of cortisone administration on the course of magnesium deficiency in the rat. Female Sprague-Dawley

rats, weighing 76 to 92 g, were fed a diet prepared without the addition of magnesium. One-half of the rats received daily injections of 2.5 mg cortisone acetate. Control rats received injections of 0.9 percent saline. The experiment was carried out for 28 days. Control rats developed hyperemia and convulsions by day 10 of the experiment. No hyperemia was observed in rats receiving cortisone and convulsions did not develop until day 15. Growth of rats receiving cortisone was depressed relative to that of control rats.

In a subsequent study Farnell (66) carried out experiments using weanling male mice of the CD-1 Swiss strain. The mice were fed either a diet prepared without magnesium or the same diet supplemented with 42 mg MgO and 250 mg $MgSO_4$/100 g. The experiment was carried out for 28 days. Convulsions developed in all mice fed the deficient diet beginning day six. The total number of convulsions was reduced in cortisone treated mice relative to values observed in deficient mice receiving the vehicle. No convulsions were noted in control mice.

In a similar study histological examination of various tissues revealed necrotic and inflammatory lesions in skeletal muscle, heart, and liver of magnesium deficient mice. Lesions were more severe in tissues of magnesium deficient mice receiving cortisone than in tissues of mice receiving the vehicle.

The finding of decreased convulsions but increased tissue lesions in magnesium deficient mice treated with cortisone suggests that this hormone may cause a redistribution of magnesium in the animal resulting in protection of the nervous system at the expense of the

soft tissues. Analysis of various tissues for magnesium in cortisone treated mice may provide some insight into this problem.

Rayssiguier (67) studied the effects of adrenalin administration on the plasma magnesium levels. Fasted pregnant ewes, weighing 40 to 55 kg, received either an infusion of 40 µg adrenalin/kg/hr or physiological saline for five hours. Plasma magnesium was determined at half-hour or hourly intervals. The administration of adrenalin caused a significant decline in the plasma magnesium concentration in experimental ewes relative to values observed in control ewes.

Forbes (68) carried out a study on the effect of thyroxine administration on magnesium deficiency in the rat. Sprague-Dawley rats were fed diets containing 80, 170, 280, 490, and 970 ppm magnesium with and without the addition of 5 ppm thyroxine for four weeks. In general, as the dietary magnesium level was increased the weight gain of the rats increased. This was observed both in rats receiving thyroxine and in those receiving no hormone. The severity of the clinical symptoms of magnesium deficiency was similar in control and thyroxine treated groups. In both experimental and control rats each successive increase in dietary magnesium was accompanied by an increase in serum magnesium concentration. At each level of dietary magnesium thyroxine treated rats displayed increased serum magnesium levels relative to values observed in controls.

Jones et al. (69) measured the plasma magnesium concentration in eight hypothyroid and eight hyperthyroid patients. The cause of altered thyroid activity in each patient was not stated. The mean

plasma magnesium concentration in the hypothyroid group was 1.51 mEq/1; that in the hyperthyroid group was 2.05 mEq/1. The statistical significance of the results was not stated.

Humphray and Heaton (70) studied the effect of thyrotoxicosis or hypothyroidism on magnesium metabolism in the rat. Male Wistar rats, weighing about 100 g, were fed diets containing 3800 ppm magnesium with and without addition of 1.5 ppm 6-propyl-2-thiouracil (PTU) or 10 ppm thyroxine. The experiment was carried out for one week. Addition of thyroxine to the diet elevated the plasma magnesium concentration while the addition of PTU decreased it relative to values obtained by feeding the control diet. Magnesium concentrations in thigh muscle and bone were significantly increased in thyrotoxic rats relative to values observed in controls. Addition of PTU to the diet had no effect on tissue levels of magnesium.

In summary, thyrotoxicosis increases plasma and tissue levels of magnesium. It is not clear whether this is due to increased absorption and retention of the mineral or to redistribution of magnesium in the body.

Two studies have been carried out on the effects of pancreatic hormones on magnesium metabolism. Madsen et al. (71) utilized four Holstein bull calves in a study on the effect of glucagon on the plasma magnesium level. Four calves, weighing an average of 102 Kg were infused with either 0.9 percent saline or 20 ppm glucagon in saline. Blood samples were obtained before, during, and after hormone treatment. Glucagon administration had no effect on plasma magnesium concentration during or after treatment.

Mellerup (72) investigated the effects of insulin on magnesium metabolism. Female Wistar rats, weighing initially 100 g, were fasted for 20 to 24 hours. Subsequently 0.1 or 1.0 mU insulin and 5 μCi ^{28}Mg/rat were administered intraperitoneally. Control rats received an injection of the isotope alone. Fifteen minutes, one or two hours later the rats were sacrificed and blood and tissues were obtained for analysis. Serum magnesium levels were significantly increased after two hours in rats administered either dose of insulin relative to values obtained in controls. Levels of radioactivity were decreased in bone and increased in muscle and liver in rats receiving insulin relative to control values.

These studies of the effects of the endocrine system on magnesium metabolism have been preliminary in nature. They indicate the need for studies on the distribution of magnesium in tissues of hormone treated rats. It is tempting to speculate that hormones may mediate some of the effects of magnesium deficiency. Studies carried out on the effects of PTH on the magnesium deficient rat indicate that this hormone may mediate some of the alterations in calcium metabolism observed in this state (43). Speculation with respect to the role of other hormones in mediating the effects of magnesium deficiency appears unwarranted at present. Clearly, this is an area which deserved further investigation.

B. GLUCONEOGENESIS: THE GLUCONEOGENIC ENZYMES

Gluconeogenesis, or the process of glucose synthesis from non-carbohydrate precursors, is carried out primarily in the liver and kidney. The sequence of enzyme reactions leading to glucose production is the reverse of glycolysis except at three points: the glucokinase reaction, the phosphofructokinase reaction, and the pyruvate kinase reaction. These reactions are reversed by four enzymes: glucose-6-phosphatase (G6Pase, E.C. 3.1.3.9), fructose 1,6-bisphosphatase (fructose diphosphatase, FDPase, E.C. 3.1.3.11), phosphoenolpyruvate carboxykinase (PEPCK, E.C. 4.1.1.32) and pyruvate carboxylase (PC, E.C. 6.4.1.1). These enzymes are the so-called "key" gluconeogenic enzymes. This terminology is based on the fact that all of these enzymes catalyze thermodynamically irreversible reactions, and that they are all present at low levels in the cell relative to other enzymes in the gluconeogenic sequence. Thus, it is assumed that these enzymes play a role in the control of glucose production (6).

In the following sections the chemistry of the gluconeogenic enzymes and their role in the physiology of the animal will be discussed. Due to the nature of the work carried out here, the discussion will focus on G6Pase, FDPase, and PEPCK as they occur in the liver of the rat.

The Chemistry of the Key Gluconeogenic Enzymes

Glucose-6-phosphatase: Glucose-6-phosphatase is a microsomal enzyme found primarily in the liver and kidney (73). It is multifunctional exhibiting a wide variety of synthetic and hydrolytic activities (74-78). The glucose-6-phosphatase (Reaction 1), mannose-6-phosphatase (Reaction 5), inorganic pyrophosphatase (Reaction 3), pyrophosphate-glucose-phosphotransferase (Reaction 4), and nucleoside triphosphate-glucose-phosphotransferase (Reaction 5) reactions are as follows:

$$\text{Glucose-6-P} + H_2O \rightarrow \text{glucose} + P_i \quad (1)$$

$$\text{Mannose-6-P} + H_2O \rightarrow \text{mannose} + P_i \quad (2)$$

$$PP_i + H_2O \rightarrow 2P_i \quad (3)$$

$$PP_i + \text{glucose} \rightarrow \text{glucose-6-P} + P_i \quad (4)$$

$$\text{Nucleoside 5'-triphosphate} + \text{glucose} \rightarrow \text{Glucose-6-P} + \text{nucleoside 5'-diphosphate} \quad (5)$$

That these activities all reside in a single enzyme species has been concluded from kinetic studies (73-76), parallel activation in fractions obtained from ammonium sulfate treatment, parallel inactivation by molybdate and heat (75) and the response of the rat liver enzymes to fasting and a variety of hormonal treatments (77,78).

For the hydrolysis of glucose-6-phosphate, the enzyme exhibits a Michealis constant for glucose-6-phosphate of 1.75×10^{-3} M (74). For this reaction the enzyme is inhibited by glucose (74,75). *In vitro* the enzyme is activated by bile acids (79).

The enzyme has been considerably purified (80), but to date it has not been solubilized. This has limited its study and, hence, neither its molecular weight nor amino acid composition are known.

Glucose-6-phosphatase is inhibited by the metal binding agents sodium azide, sodium cyanide, sodium oxalate, 1,10-phenanthroline, diethyl thiocarbamate, and 8-hydroxyquinoline suggesting that it is a metalloenzyme. The inhibition by 1,10-phenanthroline indicates that magnesium is not the metal involved (81).

Feuer and Goldberg (82) reported studies of the effects of several metal ions on the activity of G6Pase. Both the G6Pase and the inorganic pyrophosphatase activities were increased by Fe^{2+} and Ca^{2+} at concentrations between 10^{-4} and 10^{-3} M. Higher concentrations of the ions caused inhibition of both activities. Magnesium ion and K^{+} also enhanced these enzyme activities but to a lesser extent than Ca^{2+} and Fe^{2+}.

Johnson and coworkers (83) carried out detailed studies on the effects of Mg^{2+} on the various hydrolytic and synthetic activities of G6Pase. Glucose-6-phosphatase activity was increased by less than 5 percent by concentrations of Mg^{2+} greater than 10^{-3}. The pyrophosphate-glucose-phosphotransferase activity was inhibited by concentrations of magnesium up to 13 mM. Mannose-6-phosphatase activity was unaffected by the presence of the magnesium ion. The authors pointed out that these results are strong evidence against the belief, held by the authors of several textbooks (84-86), that G6Pase is activated by magnesium.

Fructose 1,6-bisphosphatase: Fructose bisphosphatase was first positively identified as a liver cytoplasmic enzyme by Gomori in 1943 (87). It is also present in the cytoplasm of the kidney and muscle (88). The enzyme cleaves fructose 1,6-diphosphate to fructose-6-phosphate and inorganic phosphate. It is also capable of cleaving sedoheptulose-diphosphate to sedoheptulose-7-phosphate and inorganic phosphate (89). The enzyme has been extensively purified (90) and subjected to detailed studies.

Rat liver FDPase is a tetrameric enzyme of molecular weight 140,000 to 143,000. Its amino acid composition is known (90,91). The enzyme exhibits a K_m for fructose diphosphate of between 1 to $3x10^{-6}M$ (91,92). It is inhibited by high substrate concentrations and by AMP. The inhibition by AMP is specific and noncompetitive. Three to four binding sites for AMP exist on each molecule of the enzyme (92). Divalent zinc, Fe^{2+}, Fe^{3+}, (93) and Ca^{2+} (90) inhibit the enzyme.

Detailed studies on the effect of EDTA on the enzyme and the relation of its action to the pH optimum have been carried out. The pH optimum of the enzyme is in the alkaline range (pH 8 to 9) in the absence of EDTA. In the presence of 0.1 M EDTA and 2 to 5 mM Mg^{2+} the activity of FDPase is increased and the pH optimum is shifted to about 7. Histidine and citrate have similar effects on the pH optimum and activity of the enzyme (90,94).

McGilvery (95) studied the effect of pH on the activity of FDPase and on the requirement by the enzyme for magnesium. As the pH was raised from 6.0 to 8.5 the peak of the activity versus

magnesium curve was shifted to the left. This was taken as evidence of competition between H^+ and Mg^{2+} for a chelating site on the enzyme. Optical rotary dispersion studies confirmed the presence of a magnesium-enzyme complex.

Phosphoenolpyruvate carboxykinase: Phosphoenolpyruvate carboxykinase catalyzes the following reversible reaction:

$$\text{Oxaloacetate + GTP (ITP)} \rightleftarrows \text{phosphoenolpyruvate + GTP (ITP)}$$

The enzyme has been purified from the liver of the chick, rat, lamb, and guinea pig (88,96,97). The intracellular distribution of PEPCK varies with species. In the rat, mouse and hamster most of the activity is in the cytosol; in the pig, guinea pig, and rabbit most of the activity is localized in the mitochondrion (80).

Rat liver PEPCK has a molecular weight of 74,500 and an isoelectric point of 5.04 (97). The pH optimum of the enzyme lies in the range 7.3 to 8.1. It has an apparent K_m for oxaloacetate of 13×10^{-3}M and an apparent K_m for GTP of 6×10^{-5}M.

Magnesium and manganese ions appear to have a synergistic effect on the activity of the enzyme. Foster et al. (98) carried out an experiment in which the total concentration of $MgCl_2$ and $MnCl_2$ was held constant at 6 mM, while the proportion of each ion was varied. Activity increased with increasing concentration of $MgCl_2$ until at 5.8 to 5.95 mM $MgCl_2$ and 0.05 to 0.2 mM $MnCl_2$ optimum activity was reached. The high ratio of Mg^{2+} to Mn^{2+} for optimum activity prompted the authors to advance the hypothesis that Mn^{2+} activates the enzyme while

Mg^{2+} is required to complex ITP. This hypothesis was further supported by the finding of an optimal magnesium to ITP ratio of 1:1.

Magnesium and the gluconeogenic enzymes: As indicated above magnesium is not required for the activation of G6Pase. It activates FDPase and functions in the PEPCK reaction by chelating ITP. This suggests that if magnesium is lost from the cell in magnesium deficiency the activity of G6Pase will be unaffected while the activity of FDPase may be decreased. Manganese may substitute for magnesium in the PEPCK reaction (98); therefore in magnesium deficiency a decrease in the activity of PEPCK may not occur.

A problem may arise in interpreting the results of enzyme assays, however. The assay for PEPCK and FDPase require that magnesium be added to the incubation medium. If synthesis of the enzyme molecules proceeds normally in magnesium deficiency then the addition of magnesium to the enzyme system may mask the effects of a cellular deficit of the cation.

Variables Affecting the Activities of the Key Gluconeogenic Enzymes

The activities of the key gluconeogenic enzymes in the liver may be altered by a variety of experimental manipulations. The effects of fasting, diet, the glucocorticoids, insulin, glucagon, and epinephrine will be reviewed here. The observations which have been made will be reviewed first. This will be followed by a discussion of the mechanisms involved.

Fasting: In 1954, Weber and Cantero (99) reported the first study carried out on the effects of fasting on the activity of G6Pase. Male Wistar rats, weighing 210 g, were fed either a commercial laboratory ration or fasted for 48 hours. The activity of G6Pase, expressed on a milligram liver basis, was, in the livers of the fasted rats, 160 percent of the level found in the livers of the fed rats.

Using Sprague-Dawley rats fasted for 18 to 24 hours, Langdon and Weakley (100) observed that the specific activity of G6Pase rose during fasting but the total enzyme activity of the liver was not significantly increased. The failure of the total liver enzyme activity to increase with fasting was attributed to a decrease in liver weight. Similar results were reported by Ashmore et al. (101).

Wimhurst and Manchester (102) carried out time course studies on the activity of G6Pase. Male Wistar rats weighing 150 to 250 g were fed an unspecified diet or fasted for up to three days. Enzyme activity was measured in liver after one, two, and three days. Glucose-6-phosphatase activity (μm product formed/min/g liver) rose from 6.42 in fed rats to 16.58 in rats fasted for one day and rose further to 21.15 by the third day. Activity expressed on a DNA basis rose in similar fashion during this time period.

These authors also presented data on the response of FDPase and PEPCK to fasting. Fructose bisphosphatase activity (μm product formed/min/g liver) rose steadily from 7.00 in fed rats to 12.80 after three days of fasting. When the activity was expressed on a unit

DNA basis, little change with fasting was noted. Phosphoenolpyruvate carboxykinase responded to fasting as did G6Pase, i.e., the activity expressed on a gram liver or unit DNA basis rose rapidly over the first day of fasting and more gradually over the next two days.

No other reports on the effects of fasting on the activity of FDPase have appeared in the literature. Young and coworkers (103) have, however, reported their findings on PEPCK. Male Sprague-Dawley rats were fed either a commercial laboratory ration or fasted for up to 96 hours. The activity of PEPCK (μm product formed/min/mg protein) doubled to a level of 115 over the first 24 hours of fasting, but fell to 91 over the next 72 hours.

In summary, the activities of G6Pase, FDPase, and PEPCK increase in fasting. Such increases may reflect the need for a greater capacity for glucose production in time of glucose need. Whether or not this increased capacity results in increased glucose production will depend on the ability of the organism to provide an adequate substrate supply.

Diet: A variety of dietary manipulations appear to alter the level of G6Pase in the liver of the rat. Freedland and Harper have made a substantial contribution to our understanding of this subject; therefore, the following discussion will emphasize their work.

In the first study reported by these workers (104) the activity of liver G6Pase in response to eight different diets was measured. The diets used were a control (25 percent casein, 65 percent dextrin, 5 percent fat), three high fat diets (Fat I: 25 percent casein,

70 percent fat; Fat II: 95 percent fat; Fat III: 55 percent fat, 40 percent casein), a high protein diet (90 percent casein, 5 percent fat), a protein free diet (90 percent dextrin, 5 percent fat), a fructose diet (25 percent casein, 65 percent fructose, 5 percent fat), and a galactose diet (25 percent casein, 65 percent galactose, 5 percent fat). Male Sprague-Dawley rats were fed these diets for four days. The activity of G6Pase was expressed on a gram liver, unit body weight, and a unit liver protein basis.

Substitution of casein, fructose, or galactose for dextrin resulted in significant increases in the activity of G6Pase. Substitution of fat for all or part of the dextrin also increased liver G6Pase activity. These results were obtained regardless of how enzyme values were expressed. Feeding the protein free diet also resulted in an increased liver G6Pase activity relative to values obtained in control rats. This result was, however, attributed to rejection of the diet by the rats which resulted in a state of fasting.

In a subsequent study (105) the response of G6Pase activity to protein, fat, or sucrose over a longer time period was reported. The control, high fat, and high protein diets were the same as the control, Fat I, and high protein diets of the previous study. In addition, a high sucrose (25 percent casein, 65 percent sucrose, 5 percent fat) diet was used. With the high fat and high protein diets, the activity of G6Pase was measured at three day intervals up to 25 days after the diets were first fed. After six days the activity

of G6Pase (expressed on a body weight basis) in the rats fed the high protein diet was 145 percent of the control value and 139 percent of the control value in rats fed the high fat diet. Glucose-6-phosphatase activity declined thereafter to levels of 114 percent and 115 percent of the control levels in rats fed the high protein and high fat diets, respectively, at 25 days.

A separate experiment was carried out in which the sucrose diet was compared with the control diet. Glucose-6-phosphatase activity was measured in liver after one, two, 21, and 31 days. Glucose-6-phosphatase activity in liver of sucrose-fed rats was significantly greater than control values at all times.

The authors theorized, on the basis of these experiments, that the initial rise in G6Pase activity seen in rats fed high fat and high protein diets reflects an immediate need for glucose by the rat. The fall in G6Pase activity which occurs as the rats are fed the diets for longer time periods reflects a secondary adaptation. This secondary adaptation was thought to have its basis in an increase in the ability to use protein and fat directly as energy sources by organs of the body other than the liver. The authors did not comment on the failure of the rats to exhibit a secondary adaptation to sucrose.

Two questions posed by these investigators were studied in further experiments (106). These questions were: 1) what is the minimal level of glucose in the diet required to prevent the initial rise in G6Pase activity when a high protein or a high fat diet is fed, and 2) what is the minimal level of dietary sucrose or galactose required to induce the enzyme.

To answer the first question dextrin was fed to rats at levels ranging from 5 to 65 percent of the diet. The diets also contained 5 percent fat and 5 percent minerals and vitamins. The remainder of the diets consisted of casein. Three diets were used to study the effect of fat: a control diet containing 65 percent dextrin, a diet containing 30 percent dextrin, and one containing 40 percent dextrin. Twenty-five percent casein, 5 percent minerals and vitamins, and fat to 100 percent completed the diets. Rats were fed the diets for one, two or four days. In brief, the results showed that a minimum of 30 percent dextrin in the high protein diet and 40 percent dextrin in the high fat diet was sufficient to prevent the induction of the enzyme.

To explore the second question sucrose and galactose diets containing 25 percent casein, 5 percent fat, 5 percent minerals and vitamins, 3 to 45 percent sucrose or 15 to 60 percent galactose, and dextrin to 100 percent were fed. Only 6 percent sucrose but 45 percent galactose were required to increase enzyme activity. The authors related the differing effects of the two sugars to differences in their metabolism.

This last experiment suggested that glucose is a specific suppressor of G6Pase. To study this possibility Harper (38) fasted male Wistar rats for 48 hours. The animals were then refed glucose alone, casein alone, or fat (butter) alone. Enzyme activity was measured 24 hours later and compared with values found in fed and 48 hour fasted rats. Fasting alone caused a doubling of G6Pase activity

(expressed on a gram liver basis). Refeeding with glucose depressed enzyme activity while refeeding with casein or fat maintained the activity of the enzyme at fasting levels.

The response of G6Pase to a fructose diet was verified by Fitch and Chaikoff (39). Male Long-Evans rats were fed either a 60 percent fructose or a 60 percent glucose diet for seven days. The specific activity of the enzyme was 32 after feeding the glucose diet, and it was 42 after feeding the fructose diet.

In contrast to what is known of G6Pase, little is known regarding the effects of diet on FDPase and PEPCK. Freedland and Harper (40) were the first investigators to report the effect of diet on FDPase. Control male Sprague-Dawley rats were fed diets containing 65 percent dextrin, 25 percent casein, and 5 percent fat. Experimental rats were fed diets in which protein, fructose, or fat were substituted for all of the dextrin. The diets were fed for four days. Fructose bisphosphatase activity (expressed on a gram liver basis) was significantly increased by all three dietary components tested. The long term effects of feeding these diets were not assessed in this study. The short term effect of a diet high in fructose on liver FDPase was confirmed by Kvam and Parks (41). No studies of the effects of glucose on this enzyme have been reported.

Peret and Chanez (111) reported the effect of dietary protein on liver PEPCK. Make Wistar rats were fed diets containing either 10.6 or 59.6 percent casein for 23 days. The activity of liver PEPCK (μm oxalacetate converted/min/100 g body weight) was 3.70 in rats fed

the low protein diet and 15.24 in the rats fed the high protein diet. The difference between these values was statistically significant.

That PEPCK, like G6Pase, is suppressed by glucose may be concluded from a study carried out by Foster et al. (112). Sprague-Dawley rats were fasted for 24 hours. Glucose (5g/kg body weight) was then administered by stomach tube. After four hours hepatic PEPCK activity was decreased to 72 percent of the value found in fasted rats receiving no glucose.

The effects of dietary sucrose and fat on the activity of liver PEPCK have not been assessed. The time course of changes in the activity of the enzyme with protein, fat, or sucrose feeding have, likewise, not been investigated.

The glucocorticoid hormones: In a short communication Weber et al. (113) first reported the effects of cortisone on G6Pase. Male Wistar rats weighing 100 g were injected with 25 mg cortisone acetate daily for five days. Control rats received daily injections of the vehicle. On day six the rats were sacrificed and G6Pase was assayed in the liver. Enzyme activity in the experimental rats was increased 49, 62, and 95 percent when expressed on the basis of wet weight, nitrogen, and cell, respectively.

These results were presented in a second report (114) and, in addition, studies on the effects of cortisone on the subcellular distribution of G6Pase were described. In the control rats, 50 percent of enzyme activity was found in the microsomal fraction with the remaining activity divided equally between the mitochondrial and

nuclear fractions. Significant increases in the activity of the enzyme resulted from cortisone administration with the greatest increases occurring in the microsomal and mitochondrial fractions. Contamination of the nuclear and mitochondral fractions by the microsomal fraction was not ruled out in this study.

The effects of cortisone on liver G6Pase were confirmed by Ashmore and coworkers (101,115) who also noted that total liver enzyme activity was increased by the hormone.

Weber et al. (6) reported the response of hepatic G6Pase to varying doses of cortisone. Either zero, 2.5, 5, 10, or 25 mg cortisone was administered to rats daily for five days. Activity, expressed as percent change on a cell or body weight basis, was significantly increased by administration of 2.5 mg cortisone and reached a maximum with administration of 10 mg cortisone.

Ashmore et al. (101) studied the effect of adrenalectomy on liver G6Pase activity. Male Wistar rats, weighing 200 to 300 g, were adrenalectomized. Unoperated controls were included in the experimental design. The adrenalectomized rats were studied one to six days after surgery. Adrenalectomy was accompanied by no change in activity of G6Pase when expressed on a unit liver basis. However, adrenalectomy caused a significant decrease in enzyme activity when expressed on a total organ basis.

Similarly, Weber and Cantero (116) noted no change in the concentration of G6Pase in livers of fed adrenalectomized rats relative to values obtained in unoperated controls. However, activity, expressed

on a cell basis, was significantly decreased by the removal of the adrenal glands.

Less attention has been focused on effects of the glucocorticoids on liver FDPase activity. Weber et al. (6) reported the effects of increasing doses of cortisone administered to rats daily for five days. A significant increase was seen in liver FDPase activity in rats receiving 2.5 mg cortisone per day relative to values observed in untreated controls. Maximal effects were seen with the administration of 10 mg cortisone/day.

Weber et al. (117) also reported the effects of adrenalectomy on FDPase. Male Wistar rats, weighing 110 to 160 g, were adrenalectomized and studied seven days later. Unoperated rats served as controls. There was no change in hepatic FDPase activity in adrenalectomized rats relative to values observed in control rats regardless of whether activity was expressed on a cell or body weight basis. Adrenalectomized rats receiving daily injections of cortisone were also studied. Hormone administration significantly increased FDPase activity when expressed on a body weight basis. The effect of cortisone on adrenalectomized rats was confirmed by Parks and coworkers (110,118).

As with FDPase scant attention has been paid to the effects of the glucocorticoids on hepatic PEPCK activity. Foster et al. (112) compared the activity of hepatic PEPCK in normal rats receiving a single dose of cortisone to that of controls receiving the vehicle. In experimental rats a detectable rise in PEPCK activity was seen

two hours after hormone administration relative to control values. Enzyme activity rose to a maximum five to six hours after hormone administration then declined over the next six hours. In a later experiment (11) increasing doses of hydrocortisone were administered daily for five days to adrenalectomized rats. Administration of 5 and 10 mg hydrocortisone/day was accompanied by increasing levels of PEPCK activity. Further increases in hormone dose were not accompanied by further increases in enzyme activity.

Studies were also carried out on adrenalectomized rats. Slight, nonsignificant, decreases in PEPCK activity were observed in adrenalectomized rats relative to values observed in unoperated controls.

In summary, the administration of cortisone to the rat results in marked increases in the activities of G6Pase, FDPase, and PEPCK. Adrenalectomy tends to have the opposite effect although the significance of the results depends on how enzyme values are expressed. These results suggest that the adrenal gland may play a critical role in determining the capacity of the liver for glucose synthesis.

Insulin: The effect of diabetes on G6Pase was first investigated by Langdon and Weakley in 1955 (100). Rats were made diabetic by injection of 50 mg alloxan/kg body weight. After induction of diabetes, as indicated by the blood glucose level, the activity of G6Pase was measured in the liver. The specific activity of the enzyme in the microsomal fraction was more than doubled in diabetic rats relative to values in untreated controls.

Ashmore et al. (101) induced diabetes in adult male Wistar rats by the injection of 40 mg alloxan/kg body weight. Liver G6Pase activity was assessed after 15 days. Enzyme activity in these rats was compared with that measured in the livers of normal rats receiving 5 U of protamine-zinc insulin at 12 hour intervals for the duration of the experiment. Glucose-6-phosphatase activity, expressed on a gram liver basis, was significantly elevated by the diabetic condition. Administration of insulin to normal rats depressed enzyme activity to a level below that of untreated controls.

Wimhurst and Manchester (102) measured, simultaneously, the levels of G6Pase, FDPase and PEPCK in the livers of male Wistar rats made diabetic by the injection of either alloxan or streptozotocin. Normal rats served as controls. All enzyme activities were expressed on a unit DNA basis. A three-fold increase in liver G6Pase was observed in rats made diabetic by either alloxan or streptozotocin relative to values observed in controls. No increase in FDPase was observed in alloxan-diabetic rats, but a 60 percent increase in activity was seen in streptozotocin-treated rats. The activity of PEPCK was increased 3.5 fold over control values in alloxan-diabetic rats and 3 fold over control values in streptozotocin-diabetic rats.

The simultaneous effects of diabetes and insulin administration on PEPCK were studied by Shrago et al. (11). Male Sprague-Dawley rats, maintained on a commercial laboratory ration, were made diabetic by the injection of alloxan. The rats were studied one to two weeks after alloxan administration. The activity of liver PEPCK was measured

and compared with that of normal rats, normal rats receiving insulin, and diabetic rats receiving insulin. The diabetic rats, relative to the untreated normal rats, exhibited a six fold increase in PEPCK activity expressed on a protein basis and a 10 fold increase when expressed on a body weight basis. Administration of insulin to normal rats depressed enzyme activity slightly relative to values observed in untreated controls, and hormone administration to diabetic rats depressed enzyme activity significantly relative to values found in normal or diabetic rats.

Two studies have been carried out on the interaction of insulin with the glucocorticoids. In the first study, that carried out by Ashmore et al. (101), male Wistar rats were made diabetic by the administration of alloxan. Similarly treated rats were adrenalectomized. The activity of G6Pase was measured in the livers of these rats and the values were compared with those obtained in the livers of normal intact rats; normal, adrenalectomized rats; normal, intact rats receiving insulin; and diabetic-adrenalectomized rats receiving hydrocortisone. The diabetic condition increased liver G6Pase activity expressed on a gram liver basis. This response was prevented by adrenalectomy and restored by the administration of adrenal cortical hormone. Adrenalectomy, alone, decreased the activity of liver G6Pase, and administration of insulin enhanced this effect.

In a second study Shrago et al. (11) studied intact rats made diabetic by the administration of alloxan, adrenalectomized-diabetic rats, and normal rats. The activity of PEPCK, expressed on a protein

basis, was significantly increased by diabetes and this effect was partially, but not completely, inhibited by adrenalectomy.

It is clear that insulin suppresses the activities of the gluconeogenic enzymes in the liver of the rat while the diabetic condition enhances their activities. The changes in the activities of the key gluconeogenic enzymes in diabetes provides a biochemical basis for the increased glucose production seen in this state.

Glucagon: There is considerably less data available on the effects of glucagon on the activities of the gluconeogenic enzymes. Shrago et al. (11) initiated studies on glucagon in 1963. These workers injected 150 to 250 g rats with 0.05 mg of glucagon 3 hours before sacrifice. Control rats received an injection of 1 percent NaCl. The results showed that the activity of hepatic PEPCK (expressed on a body weight basis) doubled in the glucagon treated rats relative to the value obtained in the controls.

Wicks et al. (119) obtained similar results. Make rats were maintained on a protein free diet, fed ad libitum for five days. The rats were injected with 150 µg glucagon and 2 mg theophylline/100 g body weight. Controls were injected with a solution of 0.15 M NaCl. A six fold increase was observed in the activity of liver PEPCK, expressed on a protein basis, in rats receiving glucagon and theophylline relative to the value obtained in controls.

Taunton et al. (12,120) studied the effects of glucagon and insulin, administered intravenously, on the activity of hepatic FDPase. Male rats fed a standard laboratory chow were anesthetized

with 5 mg pentobarbital administered intraperitoneally. The abdomen was exposed and a catheter was inserted into the inferior vena cava. At zero time a liver sample was taken for enzyme analysis. Three hundred micrograms glucagon/kg body weight was then administered and at four and fifteen minutes liver samples were again taken for enzyme determination. At zero time the activity of FDPase (expressed as nm substrate metabolized/min/mg protein) was 46.2. Activity increased to 145.6 at four minutes and fell to 112.9 at 15 minutes. In a separate experiment glucagon was administered five minutes after the injection of 0.15 U insulin/kg body weight. In these animals there was no increase in FDPase activity.

O'Neill and Langslow (121) investigated the effect of glucagon administration on hepatic G6Pase in the chick. Two hundred micrograms glucagon/chick was administered and G6Pase activity was determined in liver 60 minutes later. Glucagon administration had no effect on G6Pase activity when expressed on a gram liver basis. Activity was slightly, but not significantly, depressed when expressed on a whole liver or protein basis.

Greengard (122) studied the effect of glucagon on induction of hepatic G6Pase in fetal and neonatal rats. Fetuses obtained from 20 day pregnant rats were injected, in situ, with 50 µg glucagon. Controls received an injection of 0.9 percent saline. Glucose-6-phosphatase was measured five hours after hormone administration. Glucagon doubled the activity of fetal liver G6Pase relative to control values. Similar experiments were carried out on two day old

pups. At this age the rats receiving glucagon exhibited no increase in hepatic G6Pase activity relative to control values.

Two groups of investigators have measured the effect of glucagon on the activities of PEPCK, FDPase and G6Pase in the same liver preparation. Eisenstein and Strack (123) injected male Holtzman rats, intraperitoneally, with 40 µg glucagon three hours prior to sacrifice. Control rats received an injection of 0.9 percent NaCl. Liver PEPCK activity (expressed on a protein basis) was significantly increased in glucagon treated rats relative to control values. Activities of G6Pase and FDPase were unchanged in experimental relative to control rats.

A different approach to this problem was taken by Eaton et al. (124). These workers carried out a study in which rats, initially fasted for 16 hours, were fed a liquid diet consisting of 40 percent dextrose in 0.5 percent saline for four days. Controls were pair-fed, on a caloric basis, a commercial laboratory chow. After an overnight fast serum glucagon in control rats was 90 pg/ml. In dextrose fed rats serum glucagon was 15 pg/ml. In dextrose fed rats liver PEPCK activity was significantly reduced relative to values obtained in controls. The activities of G6Pase and FDPase were slightly, but not significantly increased in dextrose fed rats.

The above studies, taken together, indicate that glucagon administered as a single dose to young or adult rats increases hepatic PEPCK activity, but has little effect on hepatic G6Pase. The studies involving measurement of hepatic FDPase have yielded conflicting

results. These differences may be due to such factors as the time the liver was sampled and amount of hormone administered. Although no studies of the effect of chronic administration of glucagon on the gluconeogenic enzymes have been reported, the results obtained by Eaton et al. (124) suggest that under conditions of elevated glucagon secretion only the activity of hepatic PEPCK is altered.

Epinephrine: Data obtained from studies carried out on the perfused rat liver have led to the conclusion that epinephrine stimulates gluconeogenesis. For instance, Exton et al. (125) perfused livers obtained from 18 to 22 hour fasted rats with a Krebs-Henseleit bicarbonate buffer with and without addition of $1x10^{-5}$M epinephrine. With no hormone in the perfusion medium, glucose was produced at the rate of 132 µm/100 g body weight/hour. Addition of epinephrine increased glucose production to 421 µm/100 g body weight/hour. Similar results were obtained by Fain et al. (126).

Only one study has been carried out on the effects of the catecholamines on the activity of the gluconeogenic enzymes. Reshef and Hanson (127) injected adult male Wistar rats with 0.4 mg epinephrine/100 g body weight. The rats were sacrificed five and 14 hours later. Untreated rats served as zero time controls. At zero time hepatic PEPCK activity was 2.1 units/g tissue. At five and 14 hours activity rose to 3.7 and 6.0 units/g tissue, respectively. Fructose biphosphatase and G6Pase activities were not measured in this experiment.

The Mechanism of Enzyme Induction

Studies of enzyme induction in the fasted rat have focused on the role of various hormones in this process. Weber and Cantero (116) measured G6Pase activity in four groups of male Wistar rats: normal-fed, normal-fasted, adrenalectomized-fed, and adrenalectomized-fasted. Fasting was carried out for 24 hours. When enzyme activity was expressed on a cell basis, G6Pase activity was increased by fasting alone. This increase was not prevented by adrenalectomy. When enzyme activity was expressed on a concentration basis, adrenalectomy partially prevented the increased G6Pase activity observed in fasting. These results were confirmed by Froesch et al. (115).

That the glucocorticoids are not responsible for the induction of G6Pase caused by fasting may be concluded from these studies. If enzyme induction were prevented by adrenalectomy, there would be a decrease in G6Pase activity expressed on a cell basis. Decreases in enzyme activity expressed on a concentration basis probably reflect changes in liver composition which attend fasting.

There is some evidence that insulin is likewise not involved in the fasting-induced increase in G6Pase activity. Ashmore et al. (101) measured liver G6Pase activity in normal and diabetic male Wistar rats fasted for an unspecified length of time. Glucose-6-phosphatase activity was significantly increased in fasted rats, regardless of endocrine status, relative to the value found in fed controls.

Like G6Pase, the induction of PEPCK does not appear to be mediated by the adrenal cortical hormones. Shrago et al. (11) studied

adrenalectomized and intact male Sprague-Dawley rats which were subjected to a 48 hour fast. The activity of liver G6Pase was measured and compared with that of normal fed rats. The rise in specific activity of the enzyme induced by fasting was the same in adrenalectomized and intact rats.

The mechanism by which various dietary components increase levels of the gluconeogenic enzymes has received scant attention. Kvam and Parks (110) measured G6Pase and FDPase activities in livers of intact and adrenalectomized male Holtzman rats fed a 60 percent sucrose diet for two days. Additional groups of intact and adrenalectomized rats were fed a diet described as a normal laboratory ration. Adrenalectomy prevented the increases in both FDPase and G6Pase (expressed on a 100 g body weight basis) induced by the diet high in sucrose.

The mechanism of glucocorticoid induced increases in the levels of the gluconeogenic enzymes has been explored in several studies. Nordlie et al. (78) tested the <u>in vitro</u> effect of cortisone on G6Pase. Levels of 1.25 μM, 1.25×10^{-5}M, 1.25×10^{-4}M and 6.25×10^{-4}M cortisone were tested. The lowest level of the hormone stimulated enzyme activity about 10 percent. Similar results were found with enzyme preparations obtained from control, adrenalectomized, or cortisone treated adrenalectomized rats. The authors concluded that the hormone did not stimulate G6Pase induction by a direct interaction with the enzyme.

These same authors studied the activation of G6Pase by deoxycholate, *in vitro*, in relation to the hormone status of the animal. Control or adrenalectomized Sprague-Dawley rats were injected with 25 mg cortisone acetate or the vehicle, daily, for five days. The rats were sacrificed and G6Pase was assayed in the presence and absence of 0.2 percent (w/v) deoxycholate. The specific activity of G6Pase was depressed slightly by adrenalectomy and elevated significantly by administration of cortisone when assays were carried out in the absence of deoxycholate. However, no significant changes occurred in either adrenalectomized or cortisone treated rats when the assays were carried out in the presence of the detergent. When G6Pase activity was expressed on the basis of total liver activity, significant increases in response to cortisone were found in both the presence and absence of deoxycholate.

In view of the possibility that lipids or phospholipids were involved in the cortisone response mechanism, these substances were measured in the liver microsomal fraction. Significant decreases in both total lipids and phospholipids were found in livers of cortisone treated rats relative to values observed in controls. The authors hypothesized that cortisone may regulate the synthesis of unknown enzymes which, in turn, function to alter the nature or amount of a lipid component associated with the catalytic mechanism of G6Pase.

There is some evidence indicating that induction of the gluconeogenic enzymes by the glucocorticoids involves protein synthesis.

Parks (118) investigated the effect of the methionine antagonist, ethionine, on the hydrocortisone induced increases in G6Pase and FDPase. Six groups of adrenalectomized rats were studied: control, ethionine control, ethionine-methionine control, hydrocortisone, and hydrocortisone-ethionine-methionine. Hydrocortisone groups were administered 5 mg of the hormone every 12 hours for 48 hours. Groups receiving ethionine and methionine received 0.3 and 0.6 μm, respectively, twice daily for the duration of the experiment. The three control groups had similar levels of G6Pase and FDPase expressed on a concentration basis. Hydrocortisone, and hydrocortisone-ethionine-methionine rats had significantly increased levels of both enzymes relative to control values. Ethionine, given alone, prevented the induction of enzyme activity in hydrocortisone treated rats.

Phosphoenolpyruvate carboxykinase is affected similarly by ethionine. Shrago et al. (11) administered 0.25 percent dietary ethionine to adrenalectomized rats treated with daily injections of 10 mg cortisone for five days. Untreated adrenalectomized rats and cortisone treated adrenalectomized rats not receiving ethionine served as controls. A significant increase in the specific activity of PEPCK was observed 48 hours after cortisone administration relative to values found in untreated adrenalectomized controls. This response was inhibited by dietary ethionine.

Additional studies have resulted in the conclusion that the cortisone induced increases in hepatic gluconeogenic enzymes involves synthesis of RNA. Weber and Singhal (128) treated young

male Wistar rats with either puromycin or actinomycin D. Each inhibitor was studied in a separate experiment. In each experiment additional groups of rats were untreated (controls), treated with cortisone for five days, or treated with cortisone plus actinomycin or puromycin. Cortisone alone induced increases in liver FDPase and G6Pase. This response was completely prevented by actinomycin and partially prevented by puromycin.

Weber et al. (13) have advanced the hypothesis that the levels of the key gluconeogenic enzymes are regulated by the action of the cortical hormone as an inducer and insulin as a suppressor of a single genome unit. This was based on the observation that these enzymes are all induced by the glucocorticoids and that this process is prevented by any interference with protein synthesis and by insulin.

This hypothesis has been tested through studies designed to assess the simultaneous response of the gluconeogenic enzymes to various experimental manipulations. Freedland et al. (129) fed male Sprague-Dawley rats diets described as being high in glucose, fructose, or protein. One group of glucose fed rats was further treated with 5 mg cortisone/kg body weight. Each of these four groups of rats were further divided into two subgroups; one subgroup was left untreated and the other subgroup was treated with 4 U protamine-zinc insulin. The administration of insulin prevented the increase in liver G6Pase and FDPase in rats treated with cortisol. Insulin prevented the induction of G6Pase by a high protein diet, but it had no effect on the induction of FDPase. In the case of the high fructose diet,

insulin had no effect on either G6Pase or FDPase. In rats receiving the glucose diet the activity of G6Pase was decreased by the administration of insulin, but the activity of FDPase was not.

In the study of the response of G6Pase, FDPase, and PEPCK to streptozotocin or alloxan diabetes reviewed above (102), it was noted that the enzymes differed in the extent to which they were induced by the two drugs. Statistical analysis further indicated a lack of correlation between the rise in FDPase and either G6Pase or PEPCK.

These studies do not support, but they do not entirely disprove the "genome theory." Rather they indicate that this theory is perhaps an oversimplification of a complex process.

Several studies have been carried out on the relationship between cyclic 3'5'-adenosine monophosphate (cAMP) and the hormonal control of gluconeogenesis and the gluconeogenic enzymes. Exton et al. (130) perfused livers obtained from alloxan diabetic rats with a medium containing ^{14}C-lactate. In a similar fashion livers obtained from alloxan diabetic-adrenalectomized and normal rats were perfused. Removal of the adrenal glands significantly reduced glucose synthesis. Treatment of the adrenalectomized-diabetic rats with 1 mg cortisol one hour prior to the study restored glucose synthesis by the perfused liver. Addition of the hormone to the perfusion medium similarly increased glucose production. This response was prevented when either actinomycin D or cycloheximide was added to the medium. The levels of AMP and ADP in the livers of the diabetic rats were depressed by adrenalectomy and restored by in vitro or in vivo cortisone administration. Cyclic AMP was increased to a similar degree in diabetic,

adrenalectomized-diabetic, and cortisone treated adrenalectomized-diabetic rats relative to values found in controls. In the perfused liver the activity of PEPCK was increased significantly by diabetes, depressed by adrenalectomy, and restored by cortisol. It was concluded that in diabetes, hepatic gluconeogenesis is dependent upon the secretion of the glucocorticoids and that the steroids act by inducing PEPCK. This effect is not mediated by cAMP.

Studies carried out using the perfused liver preparation have provided evidence that the mechanism whereby glucagon enhances gluconeogenesis involves cAMP. For instance, Exton and Park (131) reported that both glucagon and cAMP stimulated glucose production from lactate in livers obtained from 18 to 22 hour fasted rats. In a later study (132) glucagon added to the perfusion medium produced a detectable increase in liver cAMP within 30 seconds and a maximal increase by three minutes. Similar results were reported by Pilkis et al. (133) in an isolated hepatocyte preparation.

Studies have been carried out by several groups of researchers on the effects of cAMP on the activities of the gluconeogenic enzymes. Wicks et al. (119) maintained male rats on a protein free diet, fed ad libitum, for five days, then fasted the rats overnight. At the start of the experiment the experimental rats received an injection of 6 mg dibutyrl cAMP/100 g body weight. Control rats received no treatment. The animals were sacrificed after four hours. Liver PEPCK activity in untreated controls was 11 units/mg protein; in treated rats enzyme activity was 58 units/mg protein.

Taunton et al. (12,120) obtained evidence that cAMP is involved in alterations in FDPase activity induced by glucagon. Fed male rats were injected with 0.3 mg glucagon intravenously. Liver samples were obtained before and at selected intervals after hormone injection. Livers were assayed for FDPase and cAMP. Within 30 seconds after hormone injection FDPase activity increased relative to control values. Enzyme activity rose to a maximum after five minutes and then declined slowly over the next 35 minutes. Detectable increases in liver cAMP concentration in experimental rats relative to control values were also noted after 30 seconds. Maximal levels were noted at one minute. The level of the nucleotide then declined over the next 39 minutes.

Further evidence that cAMP is involved in the regulation of hepatic FDPase activity was obtained by Riou et al. (134). A purified preparation of hepatic FDPase was obtained from rats fed an unspecified diet. The enzyme was incubated with a homogenous preparation of the catalytic subunit of cAMP-dependent protein kinase from bovine liver in the presence of ^{32}P-ATP. The incorporation of labeled phosphorus into the enzyme and the activity of the enzyme before and after ^{32}P incorporation were measured. In the presence of the catalytic subunit 4 moles of ^{32}P/mole of enzyme were incorporated in two hours. The phosphorylation of the enzyme was associated with a 40 percent increase in activity.

One study has been carried out in which the activities of the four key gluconeogenic enzymes were measured in the same liver

preparations. Kacew and Singhal (135) injected adult male Wistar rats with 10 mg cAMP plus 10 mg theophylline/100 grams body weight in two divided doses at zero and 2½ hours. The rats were sacrificed at five hours. Control rats receiving no treatment were also sacrificed at five hours. The activities of PC, PEPCK, FDPase and G6Pase (expressed on a milligram protein basis) rose 155, 165, 169, and 169 percent, respectively, in hormone treated rats relative to values obtained in controls.

In summary, glucagon increases the level of cAMP in the liver and the presence of cAMP is associated with increases in enzyme activity. There are some discrepancies when the effects of injected glucagon and injected cAMP are compared. For instance, cAMP injection apparently increases the activity of rat hepatic G6Pase whereas injected glucagon does not. These discrepancies may be due to differences in the concentration of cAMP at the cellular site. No studies have been reported in which an attempt was made to block the increases in FDPase and PEPCK induced by glucagon with inhibitors of protein synthesis. Thus it is not clear as to whether the alterations induced in these enzymes are due to de novo enzyme synthesis or to activation of preexisting enzyme molecules.

As noted above the catecholamines increase gluconeogenesis in the isolated perfused liver and increase PEPCK activity. Tolbert and Fain (136) observed that stimulation of gluconeogenesis due to epinephrine in the perfused liver was additive to that of glucagon or dibutyrl-cAMP. This was interpreted as evidence that epinephrine

acts through a cAMP-independent mechanism.

Potter et al. (137) obtained evidence that, in vivo, the effects of epinephrine may be mediated by glucagon. Unanesthetized fed and fasted, male New Zealand rabbits were infused sequentially with 0.05, 0.5, and 5 μg epinephrine per minute for 15 minutes. Samples of arterial blood were obtained at five minute intervals and assayed for glucose and glucagon. In both fed and fasted animals infusion of increasing levels of epinephrine led to successively higher plasma levels of glucose and glucagon.

Hormones, the Gluconeogenic Enzymes, and the Blood Glucose Level

In the fasting state glucose must be continually supplied to tissues which cannot utilize other substrates, e.g., fatty acids, for energy production. Such tissues include the nervous system, the red blood cells, and the kidney medulla. In short-term fasting this glucose is supplied primarily by the liver. In this organ the onset of fasting is accompanied by glycogenolysis. As fasting continues glucose is produced through gluconeogenesis. As we have seen the activities of the gluconeogenic enzymes are under hormonal control. It is reasonable to assume that there is a relationship between circulating hormone levels, the activities of the hepatic gluconeogenic enzymes and the blood glucose concentration. Unfortunately little work has been carried out on this subject. The few studies which have been reported will be reviewed here.

Evidence that glucagon acts on PEPCK so as to increase the blood glucose level was obtained by Eaton et al. (124). As noted above,

these workers observed increased hepatic PEPCK activity and increased blood glucagon levels chow fed rats relative to values obtained in dextrose fed rats. The basal serum glucose level in the former group was 116 mg/100 ml and in the latter group 113 mg/100 ml. When glucagon secretion was stimulated by the injection of 2 mg arginine/g body weight the blood glucagon level rose to 690 pg/ml in the chow fed rats, but rose to only 250 pg/ml in the dextrose fed rats. Blood glucose concentration, in the former group rose to 134 mg/100 ml. In the latter group it rose to 125 mg/100 ml. Ninety minutes after arginine administration the blood glucose level in chow fed rats fell to basal levels. In the dextrose fed rats the blood glucose level fell to 90 mg/100 ml.

Only one study has been reported in which the effects of glucagon administration on both FDPase and blood glucose have been related. Taunton et al. (12) administered glucagon intravenously to male rats fed, ad libitum, a commercial laboratory chow. The animals were sacrificed four and 15 minutes post injection. Hepatic FDPase activity increased at both four and 15 minutes. The authors stated that the blood glucose level was also raised at these times but no data was given.

In a single study Ashmore et al. (101) measured, over the course of 48 hours, the blood glucose concentration, the incorporation of pyruvate-2-^{14}C into glucose by liver slices, and the activity of liver G6Pase. The study was carried out on diabetic-adrenalectomized rats receiving adrenal cortical hormone (5 mg/12 hours). The

administration of cortical hormone was accompanied by an increase in the conversion of pyruvate to glucose within two hours. This was not paralleled by an increase in blood glucose. However, G6Pase activity did not increase until 12 hours after the initiation of the experiment.

It should be noted that in three of the above investigations the metabolic state of the rat, i.e., whether fed or fasted, was not stated. In these experiments glycogenolysis may have been occurring. This could mask any relationship between hormone factors, the gluconeogenic enzymes, and the blood glucose level. It is hoped that this factor will be controlled in future studies.

C. OVERVIEW

The development of magnesium deficiency in the rat is accompanied by hyperemia, poor growth, and convulsions. Soft tissue levels of magnesium are preserved but the levels of this cation in the plasma and bone are decreased. There are modifications in the metabolism of protein, fat, carbohydrate and minerals. There is also evidence that the function of the endocrine system is altered. The course of magnesium deficiency is influenced by age, diet, and some hormones.

A number of metabolic processes have not been well studied in magnesium deficiency. Gluconeogenesis is one such process. Gluconeogenesis refers to the process of glucose synthesis. The activities of the key gluconeogenic enzymes are thought to be one factor controlling the rate of glucose production by the liver. The

metabolic state of the animal, and the protein, fat, and carbohydrate contents of the diet all influence the activities of these enzymes. The activities of these enzymes are, furthermore, under hormonal control. Little is known regarding the relationship between blood hormone levels, the activities of the gluconeogenic hormones, and the circulating blood glucose level.

CHAPTER III. MATERIALS AND METHODS

A. EXPERIMENT I

Animals

Thirty-six male, CD derived (Sprague-Dawley) rats, weighing 53 to 83 g, were purchased from Charles River Breeding Laboratories, Wilmington, Massachusetts. Upon arrival the rats were caged individually in 7x7x10 inch wire-mesh, stainless steel cages. The animal room in which they were housed was temperature controlled and equipped with an automatic timer set to provide 12 hours of light and 12 hours of darkness. The onset of the light period was 7 A.M.

The rats were offered a commercial laboratory chow (Purina Laboratory Chow) and tap water, ad libitum, for three days. The morning of the fourth day the rats were offered the semi-purified control diet described below. Deionized water was substituted for tap water. The amount of food eaten during the previous twenty-four hours was determined between 10 and 11 A.M. daily. The rats were weighed daily between 9 and 10 A.M.

After four days 24 rats weighing between 113 and 127 g were selected for the study. Excluded from this group were rats with respiratory infections, unexplained growth failure, or weights below 110 g or above 130 g. The weight range of rats used in this study was selected with the expectation that rats of this size would develop deficiency symptoms within five days of the initiation of the study using the experimental diets described below. The 24 rats

were randomly divided into 12 groups of two rats. Each of the four groups of rats with the highest mean weights were randomly assigned to each of four experimental treatments: control-fed (C-F), control-fasted (C-S), magnesium deficient-fed (MD-F), and magnesium deficient-fasted (MD-S). This was considered day 1 of the experiment. On days 2 and 3 the four groups of rats having the intermediate and lowest mean weights (day 1 weights) were similarly randomly assigned to the four treatment groups. This procedure resulted in equalization of the mean starting weights of the four treatment groups.

The rats designated as "control" were fed the control diet described below for 12 full days. Similarly the rats designated as "magnesium deficient" were fed the magnesium deficient diet described below for 12 full days. Rats designated as "fasted" were then denied access to their food beginning at 9 A.M. Rats designated as "fed" were allowed access to their food for an additional 24 hours.

Throughout this period the weight of the food eaten was determined daily as described above. Food spilled was weighed and thus accounted for. Deionized water was given ad libitum. The rats were weighed daily as described above. The amount of food eaten after 12 full days was designated the "total food intake." The weight of each rat after 12 full days was designated the "final weight." The weight of each rat at 8 A.M. on the day of sacrifice was designated the "sacrifice weight."

At the end of the experiment the total food intake of each rat was divided by 12 to give the average daily food intake (ADF). The

difference between the initial and final weight of each rat was divided by 12 to give the average daily gain (ADG).

Diets

The composition of the basal diet is given in Appendix A. This is a modification of that recommended by the American Institute of Nutrition (139). The composition of the mineral mix is given in Appendix B. It, too, is based on that recommended by the American Institute of Nutrition (139) except that MgO was omitted. The mineral mix used in Experiment I was purchased.

For this experiment 10 kg of the basal diet was mixed in 2 kg batches, in a Hobart Kitchen Aid Mixer (Hobart Corp., Troy, Ohio) equipped with a stainless steel bowl and a plastic coated blade. The mean level of magnesium, obtained by analysis, in the five batches of diet was 79.3 $\pm$ 5.3 ppm. Four kilograms of diet were designated as the "magnesium deficient diet." The mean level of magnesium in these two batches of diet was 78.3 $\pm$ 4.7 ppm. To the remaining diet was added an amount of MgO to give approximately 800 ppm. By analysis the mean level of magnesium was 778 $\pm$ 18.2 ppm.

Sacrifice

The groups of rats started on days 1, 2, and 3 were sacrificed beginning at 9 A.M. on days 14, 15, and 16. The sacrifice procedure took approximately two hours. The rats were anesthetized with CO_2, and then blood was obtained by heart puncture using a 10 ml syringe dusted with about 20 mg heparin. Two 0.25 ml aliquots of blood were

transferred to small sampling cups containing 10 to 20 mg, each, heparin and NaF. The samples were mixed then placed on ice. These samples were used for the determination of blood glucose. The remaining blood was placed in a plastic tube containing about 20 mg heparin. The blood was mixed and allowed to stand at room temperature. These samples were used for the determination of plasma magnesium.

After the blood was processed the liver was removed, blotted, weighed, and placed on ice. The samples were covered with cheese-cloth dampened with 0.9 percent saline. One to two minutes elapsed from the time the blood was drawn until the time the samples were placed on ice.

In carrying out the sacrifice one rat from each group was sacrificed first, then the remaining rats. No two rats from the same group were sacrificed successively. This procedure was carried out to minimize the effects of circadian rhythms on the parameters measured.

Tissue Preparation

Immediately after sacrifice the blood was centrifuged at 3500 RPM in an IEC HN-II countertop centrifuge (International Equipment Co., Needham, Mass.) for 30 minutes. The plasma was drawn off and immediately diluted with 0.1 percent La_2O_3 in 0.5 percent HCl. A 1:51 dilution was made of the control plasma and a 1:31 dilution was made of plasma obtained from the deficient animals. Analysis was carried out two days after sacrifice.

While the blood was being processed, about two grams were removed from each liver and weighed. Each liver sample was homogenized in 4 volumes of ice-cold, 0.25 M sucrose. Homogenization was carried out in a Potter-Elvehjem homogenizer equipped with a motor driven teflon pestle. Four milliliters of homogenate were strained through two pieces of cheesecloth into a plastic test tube. Four 0.5 ml aliquots of this strained homogenate were placed in additional tubes. The tubes were capped and frozen at -20 C for assay of G6Pase. The remaining homogenate was centrifuged at 2 C for 60 minutes at 16,000xg in a Sorvall RC-2 centrifuge (DuPont Co., Newtown, Conn.). The supernatant was drawn off and 1 ml aliquots were frozen at -20 C for the assay of FDPase and PEPCK. The total time required for the preparation of the liver samples was about three hours.

The remaining liver was wrapped first in plastic wrap then in aluminum foil. The samples were stored at -20 C in an air tight container. Two weeks later, two 1 g samples of each frozen liver were homogenized in 4 volumes of deionized water. These samples were used immediately for the determination of magnesium and protein.

B. EXPERIMENT II

Animals

Sixty male, weanling, CD derived (Sprague-Dawley) rats were obtained as described above. The initial handling of the rats was the same as in Experiment I. On the fourth day after their arrival the rats were offered the control diet in four, one hour feedings,

at 6 A.M., 12 noon, 6 P.M. and 12 midnight.[1] Deionized water was offered ad libitum. This procedure was carried out for seven days. After this time, designated the adaptation period, 30 rats free of respiratory illness, which exhibited an average daily gain greater than 5.5 g, and which were within the weight range of 108 to 131 g were chosen for study. The choice of this weight range was made on the same basis as that for Experiment I. The 30 rats were randomly assigned to six groups. The three groups having the highest average weights were started on the experiment on day 1; the remaining three groups were started on day 2. On each of the two days each group of rats was assigned randomly to one of three treatments: control (C), pair-fed control (PF) and magnesium deficient (MD). The rats were offered their respective diets beginning 12 noon of days 1 and 2. The meal-feeding schedule followed prior to the experiment was continued.

On days 1 and 2 the pair-fed groups started on these days were not restricted in their food intake. The following day, and throughout the experiment, they were given the average amount of food eaten during the previous 24 hours by the magnesium deficient rats. It was noted that neither the control nor the magnesium deficient rats consumed more than 2 g of food at the 6 A.M. or noon feedings. Therefore, the pair-fed rats were never offered more than this amount of food at these times. Thus the pattern of food intake was similar

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[1]This feeding schedule was based on the recommendation of G. Leveille (140) and a preliminary feeding trial in which rats on a control diet were meal-fed three equally spaced meals per day. This meal feeding schedule resulted in an average daily gain of 5.8 $\pm$ 0.8 g.

among the three treatment groups.

The amount of food eaten by each rat was determined after every meal. The rats were weighed between 10 and 11 A.M. On day 17 the rats were fasted beginning at 1 P.M. Weights obtained that day were considered to be final weights. Weights obtained the following day at 8 A.M. were considered to be sacrifice weights. The amount of food eaten up to and including the meal at 6 A.M. on day 17 (day 18 for rats started on day 2) was considered to be the total food intake. The ADG and ADF were calculated as described for Experiment I using 16 days in the denominator.

Diets

The diets were the same as those used in Experiment I with two exceptions. First, the mineral mix was prepared by the investigator from separately purchased chemicals. Second, the magnesium level of the basal diet was 19.2 $\pm$ 1.01 ppm. After supplementation the magnesium level of the magnesium deficient diet was 76.2 $\pm$ 3.4 ppm and that of the control diet was 777 $\pm$ 23.2 ppm.

Sacrifice

The sacrifice of animals was carried out on days 18 and 19 after the rats had been fasted for 20 hours. The procedures were identical to those used in Experiment I.

Tissue Preparation

The tissue preparation was as described for Experiment I.

C. EXPERIMENT III

Animals

Sixty male, weanling, CD derived (Sprague-Dawley), rats were purchased as described for Experiment I. The initial handling of the rats and the adaptation to meal-feeding were the same as in Experiment II. The experiment was carried out in the same manner as Experiment II except the initial weight range of the rats was 100 to 125 g.

On day 7 of the experiment six healthy rats, not used in the three experimental treatments (but out of the original group of rats purchased) were offered the control diet under the same feeding schedule as that used for the rats in the three original treatment groups. These rats were fed the control diet until day 17 of the experiment. On the morning of that day the rats were sacrificed in the fed state. The livers of these rats were used for the determination of a control value for glycogen in the fed rat.

Diets

The diets were identical to those used in Experiment I with the exception that the mineral mix was prepared from purchased chemicals. The mean magnesium level of the basal diet was 21.2 $\pm$ 0.08 ppm. The magnesium level of the control diet was 768 $\pm$ 19.6 ppm while that of the magnesium deficient diet was 74.3 $\pm$ 5.7 ppm.

Sacrifice

The sacrifice procedure was similar to that used in Experiments I and II with some differences in the handling of the blood and liver.

The syringes used to draw the blood contained sufficient Na_2EDTA to give a final concentration of 2 mg/ml. Heparin was not used as an anticoagulant to avoid interference with the glucagon assay. After taking an aliquot of blood for glucose analysis the remaining blood was transferred to a tube containing sufficient benzamidine to give a concentration of 0.03 M. This blood was placed immediately on ice and used for the determination of glucagon and insulin[2].

After the blood was drawn about a one gram portion of liver was removed prior to the removal of the entire liver. This sample was wrapped in aluminum foil and placed on dry ice for later glycogen assay. The pancreas[3] was next removed and finally the remaining liver was removed. Both tissues were blotted and weighed, then covered with cheesecloth dampened with 0.9 percent saline and placed on ice.

Tissue Preparation

The blood was handled as described previously except it was kept on ice and centrifuged at 2 C. Plasma was frozen immediately after aliquots for magnesium analysis were removed. The frozen liver samples reserved for glycogen assay were quickly weighed and stored at -20 C. Liver preparation for PEPCK assay was as described above.

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[2]The assays for insulin and glucagon were not completed by the time of this writing.

[3]Pancreases were obtained with the intention of measuring pancreatic glucagon. This assay was not completed at the time of this writing.

D. TISSUE ANALYSES

Enzymes

Glucose-6-phosphatase: Glucose-6-phosphatase activity was measured according to the method of Fitch et al. (141) with one modification. Preliminary work indicated that as glucose-6-phosphate (G6P) concentration was increased from 0.01 to 0.08 M there was a linear increase in enzyme activity up to 0.05 M G6P with a given homogenate concentration. This occurred when 0.1 to 0.3 ml of a 1:10 dilution of liver homogenate was used. Therefore a G6P concentration of 0.06 M was used rather than 0.04 M as recommended by Fitch et al. (141).

In final form, the assay was carried out as follows: The liver homogenates were thawed on ice and then diluted 1:10 with 0.25 M sucrose. To duplicate test tubes were added 0.5 ml maleate buffer, and 0.2 ml diluted homogenate. The tubes were placed in a water bath at 37 C for two minutes. One-half milliliter of 0.06 M G6P was added rapidly to each tube and each sample was shaken for two to three seconds then returned to the water bath. After 15 minutes 1 ml of 10 percent trichloroacetic acid was added to each sample; the samples were shaken vigorously then placed on ice. One enzyme blank containing 0.2 ml liver homogenate but no substrate was carried through the entire procedure for each sample. Reagent blanks containing no enzyme were carried through the entire procedure also. Initially a reagent blank was assayed for each sample. However, since assay of successive blanks yielded identical results only one reagent

blank per six samples was run throughout Experiments I and II.

Samples and blanks were kept on ice 15 minutes then filtered through a piece of Whatman No. 1 filter paper into a clean test tube. Two-tenths of one milliliter of the clear filtrate were transferred to a test tube. The sample was diluted to 2 ml with deionized water. The method of Chen (142) was used to measure the amounts of phosphorus present. In following this latter method standards ranging from .05 to 5 µg P/ml were used and the absorbance was read at 624 nm.

Protein was determined on 50 µl aliquots of diluted liver homogenate by the method of Lowry et al. (143).

Units of enzyme activity were calculated as described in Appendix E. A "unit" is defined as the amount of enzyme which will release 1 µm phosphorus/minute under the conditions of the assay.

Fructose 1,6-bisphosphatase: The assay used for the determination of the activity of FDPase was based on that of Taketa and Pogell (92). Into duplicate test tubes were pipetted 0.5 ml, 0.1 M cysteine; 0.5 ml, 0.25 M Tris buffer, pH 7.5; and 0.5 ml 0.05 mM $MgSO_4$. Each liver homogenate was diluted 1:5 with 0.25 M sucrose and 0.2 ml was added to the above buffered solution. The volume was adjusted to 2 ml by the addition of deionized water. The tubes were shaken and placed in a water bath maintained at 37 C for two minutes. The reaction was initiated by adding 0.5 ml of 0.5 M fructose diphosphate (FDP) and shaking two to three seconds. The samples were returned to the water bath for 15 minutes. The reaction was terminated by the addition of 0.5 ml 30 percent trichloroacetic acid. The samples were shaken

vigorously and placed on ice. The remaining steps, i.e., filtration and assay for phosphorus and protein were carried out as described for the assay of G6Pase. Enzyme blanks and reagent blanks were carried through the assay for each sample. Units of enzyme activity were calculated as described in Appendix F. A unit is defined as the amount of enzyme which will release 1 μm phosphorus/minute under the conditions of the assay.

Phosphoenolpyruvate carboxykinase: The assay used was that recommended by Wimhurst and Manchester (102). The mixing of the reagents was found to be critical to the success of the assay; therefore, the procedures used will be described.

Reaction mixture A consisted of 0.2 mM glutathione, 6 mM inosine triphosphate, and 9 mM oxaloacetate in 124 mM Tris buffer, pH 8.0. This mixture was prepared fresh daily and refrigerated or kept on ice at all times. Only inosine triphosphate purchased within one month of the assay and shipped in dry ice was used.

Reaction mixture B consisted of 24 mM $MgSO_4$ and 0.4 mM $MnCl_2$ in deionized water. Reaction mixture C consisted of 52 mM NaF in deionized water. Immediately before the assay 10 ml of mixture B was mixed with 10 ml of mixture C. The resulting solution, designated reaction mixture D, was discarded if cloudiness developed.

For the assay 0.4 ml each of reaction mixtures A and D were pipetted into duplicate test tubes. Two tenths of one milliliter of a 1:5 dilution of enzyme homogenate was added to each tube. The tubes were incubated in a water bath at 30 C for 10 minutes. The

reaction was terminated by the addition of 3 ml of 10 percent trichloroacetic acid. The tubes were shaken and placed on ice for 15 minutes. After filtration, as described above, an 0.5 ml aliquot of filtrate was added to 1 ml of 1 percent $HgCl_2$. The tubes were mixed and allowed to stand 15 minutes. This step was carried out to cleave the labile phosphate group from phosphoenolpyruvate as described by Lohmann and Meyerhof (144). Phosphorus was measured by the methof of Chen (142) using 0.5 ml aliquots of the samples treated with mercury and 0.2 ml of those not so treated. Enzyme and reagent blanks were run with each assay. The calculations carried out are shown in Appendix G. A unit is defined as the amount of enzyme which will form 1 μ PEP/min under the conditions of the assay.

Liver and Blood Composition

Magnesium: The procedure used for the analysis of magnesium was one developed and used routinely in this laboratory. One-half milliliter of liver homogenate was pipetted into a 150 ml beaker. To each sample was added 5 ml concentrated HNO_3. The samples were mixed, covered with a watch glass and allowed to stand for 12 to 16 hours. The samples were then placed on a hot plate and heated gently two hours or until foaming had ceased. The temperature was increased to just below the boiling point and the samples were allowed to digest for six hours or until the solution was light amber in color. At this point, 1 ml concentrated H_2SO_4 was added to each sample. The samples were heated for an additional two hours. The watch glass was then

elevated using glass supports, and the nitric acid was allowed to evaporate. Just as the resulting solution began to darken the beakers were removed from the heat and allowed to cool. One-half milliliter of 30 percent H_2O_2 was then added. The samples were returned to the hot plate and allowed to react. The water was evaporated and if the solution darkened again, the peroxidation was repeated until a clear solution was obtained. The samples were cooled, transferred to a 10 ml volumetric flask and made to volume.

For analysis the samples were diluted 1:5 with 0.1 percent La_2O_3 in 0.5 percent HCl. Blank samples containing no homogenate were carried through the entire procedure. The samples were read on a Perkin Elmer model 503 atomic absorption spectrophotometer (Perkin Elmer, Norwall, Conn.) equipped with a Fisher series 5000 Recordall recorder (Fisher Scientific Co., Silver Spring, MD). Magnesium standards ranging in concentration from 0.05 to 1.0 ppm, were read along with the samples.

Diet samples were ashed in a similar fashion except 0.75 to 1.0 g of diet was ashed and 10 ml HNO_3 was used initially. The final dilution ranged from 20 to 1000 fold depending on the sample. Plasma samples prepared as described above were read directly.

The calculations carried out were as follows: The regression of peak height in millimeters on concentration was calculated for the magnesium standards. From the slope of this line the concentration of magnesium in each sample was determined. The concentration of magnesium in the blanks was similarly determined. The value for magnesium in the

blank was subtracted from that for each sample. This value was multiplied by the dilution factor to give the total level of magnesium in the sample.

Protein: Since the total amount of liver available for protein determination was small and since a complete micro-kjeldahl unit was not available, the Nesslers procedure was carried out. The procedure used was based on that described by Oser (145).

One half milliliter aliquots of liver homogenates were pipetted into duplicate thick walled ignition tubes. To each tube was added 2 ml concentrated H_2SO_4. The tubes were placed in a heating block and heated to 180 C. The samples were digested until no frothing or clumped organic material were visible. The samples were cooled and 10 drops of 30 percent H_2O_2 were added. After gentle mixing the samples were heated for 10 minutes at 130 C or until the reaction was complete. The samples were then heated at 180 C for one-half hour. If the samples darkened the peroxidation was repeated until the solution remained clear. Reagent blanks were carried through the procedure.

For Nesslerization the samples were diluted to 100 ml with deionized water. One milliliter of each diluted sample was transferred to a test tube. After the addition of 1 ml water, 5 ml of a 1:1 dilution of Nesslers reagent with deionized water was added to each tube. The samples were shaken and allowed to stand for 10 minutes. The absorbance of each sample was read at 480 mm. Standards containing from 0 to 50 µg nitrogen were Nesslerized and read as above.

A standard curve was constructed from the absorbance readings obtained from the standards. From the slope of this line the concentration of nitrogen was determined in the samples.

Glycogen: The procedure used was that described by Kemp et al. (146). Between 50 and 75 mg of frozen liver was used for the assay of tissues obtained from fed controls and about 250 mg for the assay of tissues obtained from the three experimental treatment groups used in Experiment III.

Blood glucose: The concentration of glucose in the whole blood was determined using a Technicon Auto II Autoanalyzer (Technicon Instruments Corporation, Terrytown, N.Y.). The colorimetric procedure used was based on the method of Brown (147). Glucose standards ranging in concentration from 25 to 150 mg/dl were assayed along with each set of samples. A control serum sample was likewise run with each set of samples. The regression of peak height (mm) on glucose concentration was calculated. The concentration of glucose in each sample was calculated from the slope of the regression line.

E. STATISTICAL ANALYSIS

All values were expressed as mean $\pm$ SEM. The analysis of variance technique was used to test for significant differences between diet, metabolic state, and sacrifice day in Experiment I: and diet and sacrifice day in Experiments II and III. As no significant differences due to sacrifice day were found the treatment

means were averaged and the analysis of variance was used to test for differences between diet and metabolic state in Experiment I and diets in Experiments II and III.

Duncans new multiple range test was used to test for differences between means. Differences between any two groups were considered significant at $P < 0.05$.

CHAPTER IV. RESULTS

The results of each experiment are presented separately. The analysis of variance for each experiment revealed that there was no day to day variation in the results for any parameter measured. Therefore, the results for each sacrifice day were pooled for analysis. For purposes of the following discussion the three starting days of Experiment I and the two starting days of Experiment II and III will be considered day 1 for each respective experiment. The three sacrifice days of Experiment I will be considered day 14 and those of Experiments II and III, day 18. The abbreviations used in the following discussion will be: control, C; magnesium deficient, MD; pair-fed, PF; fed, F; and fasted, S.

A. EXPERIMENT I

External Symptoms

Rats maintained on the magnesium deficient diet developed hyperemia beginning day 3. The hyperemia began at the base of the ear and by days 6 to 8 involved the entire ear. By days 6 to 8 hyperemia was present on all exposed skin areas. The hyperemia persisted throughout the entire experiment. Skin lesions developed beginning day 5. The lesions were scaly, desquamated areas encrusted with dried blood. The lesions were most severe on the head and jaws. It was noted that the rats frequently scratched areas where lesions were present.

The rats remained alert and lively for the first 8 days of the experiment. After this time about 60 percent of the rats appeared to develop an apathy regarding their food and surroundings. They remained gentle and no hyperirratibility or convulsions were noted at any time. These results were noted in both the MD-S and MD-F groups.

No abnormal symptoms such as those appearing in magnesium deficient rats appeared in groups C-F or C-S. These rats were alert and lively with sleek healthy looking coats. No abnormal coloration of exposed skin areas was noted.

Growth and Food Intake

Growth and food intake data are shown in Table 1. Initial weights of the four groups of rats were similar. Final weights of both control groups were similar; there was also no significant difference in final weights of the two magnesium deficient groups. However, final weights of groups MD-F and MD-S were both significantly less than those of groups C-F and C-S. These trends were reflected in the average daily gains of the four groups of rats.

Figure 1 shows in detail the growth of the magnesium deficient and control rats. The growth curves of both control groups and both deficient groups have been combined. It can be seen that the growth of the magnesium deficient rats was less than that of the controls by day 5. Differences in mean weights of the treatment groups became greater as the experiment progressed.

On the day of sacrifice (day 14) group C-S weighed an average of 15 g less than group C-F, but the difference was not statistically

TABLE 1

Experiment I: Effect of magnesium deficiency on growth and food intake

Measurement	Control		Mg Deficient	
	fed	fasted	fed	fasted
Initial weight, g[1]	128 ± 5.3[a]	126 ± 1.8[a]	126 ± 2.1[a]	128 ± 2.1[a]
Final weight, g	215 ± 9.7[a]	217 ± 7.2[a]	187 ± 5.6[b]	181 ± 3.0[b]
ADG, g	7.2 ± 0.71[a]	7.6 ± 0.6[a]	5.0 ± 0.3[b]	5.3 ± 0.2[b]
Sacrifice weight, g	222 ± 11.7[a]	207 ± 7.0[a]	190 ± 6.0[b]	178 ± 8.0[b]
Total food intake, g	250 ± 12.4[a]	230 ± 13.9[ab]	202 ± 7.4[b]	204 ± 6.9[b]
ADF, g	20.8 ± 1.03[a]	19.2 ± 1.16[ab]	16.9 ± 0.62[b]	17.0 ± 0.63[b]

[1]Data are given as means ± SEM. Values in the same row not followed by the same superscript number differ significantly, $P < 0.05$.

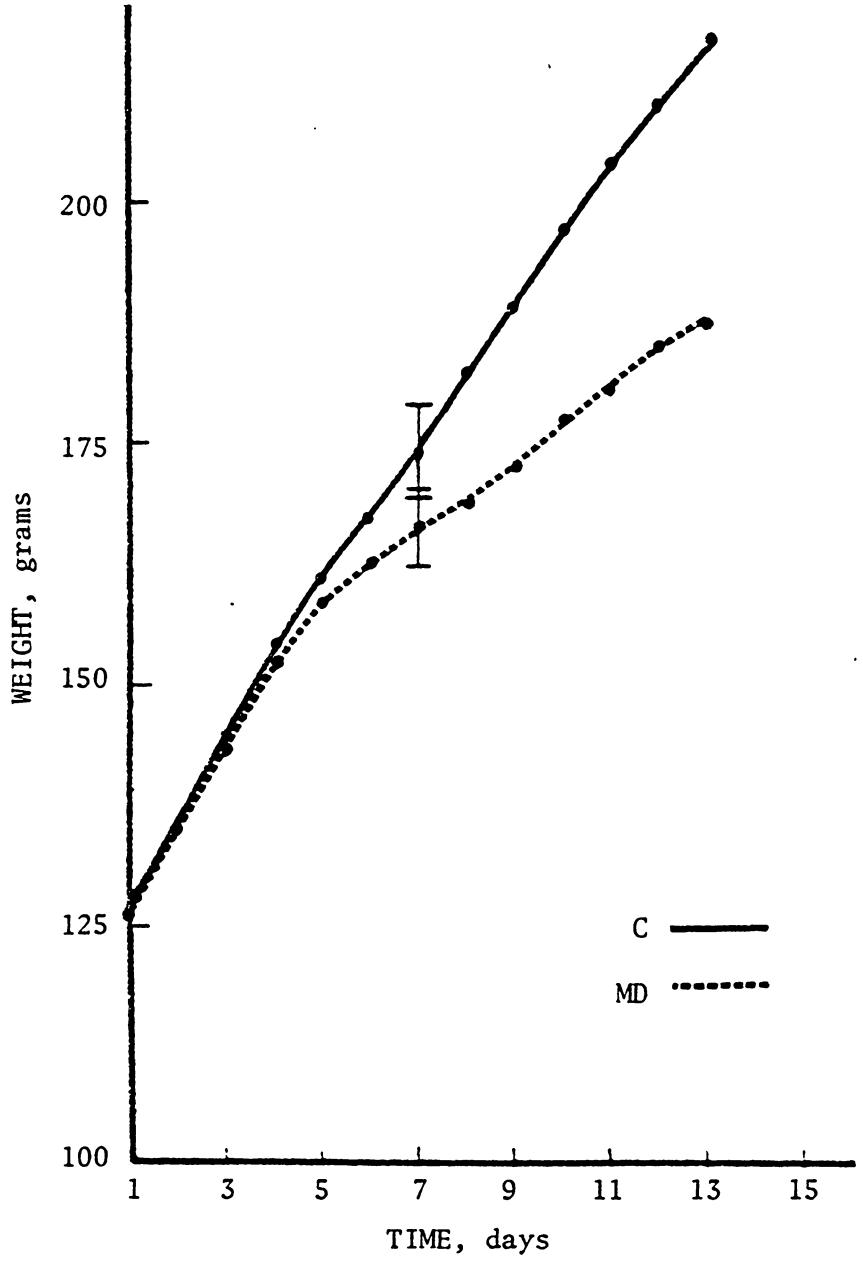

Figure 1. Effect of magnesium deficiency on growth of rats utilized in Experiment I. Each point represents the mean of 10 values. Vertical bars given the mean ± SEM at the midpoint of the experiment. Data for both control groups and both deficient groups have been combined.

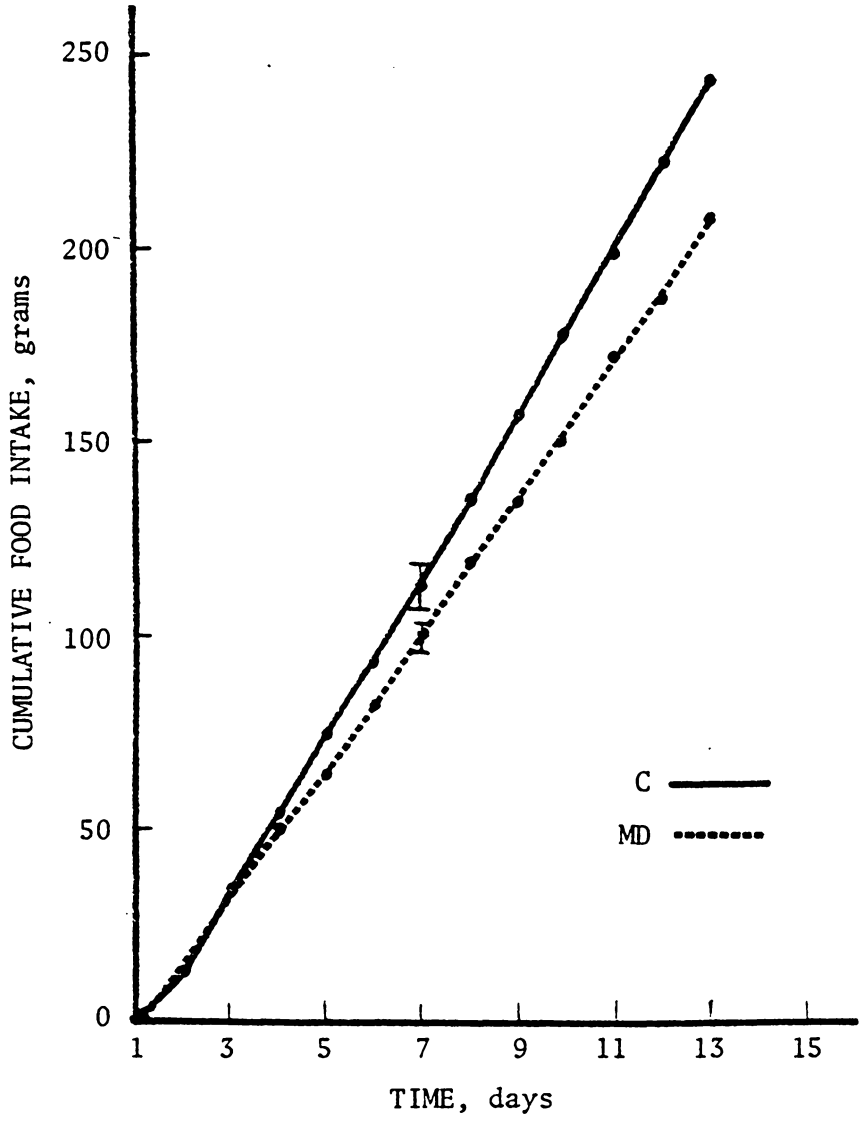

Figure 2. Effect of magnesium deficiency on cumulative food intake of rats utilized in Experiment I. Each point represents the mean of 10 values. Vertical bars give the mean ± SEM for the mid-point of the experiment. Data for both control groups and both deficient groups have been combined.

significant. Group MD-S weighed an average of 12 g less than group MD-F at sacrifice, and, again, the difference was not statistically significant. Sacrifice weights of both control groups were significantly greater than those of both magnesium deficient groups.

Total food intakes of both control groups and both deficient groups were similar. Group C-F exhibited a significantly greater food intake than either group MD-F or MD-S. The food intake of group C-S was greater than that of either group MD-F or MD-S but the differences were not significant. These trends were reflected in the average daily food intake of the rats. In Figure 2 are shown the cumulative food intakes of the animals over the course of the experiment. It can be seen that the total food intake of the deficient rats began to decrease after day 3.

Tissue Analyses

Liver composition: Liver composition of the four treatment groups is given in Table 2. Liver weights of groups C-F and MD-F did not differ significantly. Similarly there were no significant differences in liver weight between groups C-S and MD-S. Liver weights of both fed groups were significantly greater than those of both fasted groups. When liver weight was expressed on a body weight basis, the value obtained for group C-F was similar to those of groups MD-F and MD-S. However, the value of group C-S was significantly less than values obtained for the other three groups.

TABLE 2

Experiment I: Effect of magnesium deficiency on liver composition

Measurement	Control		Mg Deficient	
	fed	fasted	fed	fasted
Total liver weight, g[1]	10.00 ± 0.992[a]	7.46 ± 0.482[b]	9.28 ± 0.284[a]	7.31 ± 0.270[b]
g liver/100 g body weight	4.48 ± 0.25[ab]	3.59 ± 0.14[c]	4.89 ± 0.174[a]	4.10 ± 0.123[b]
mg protein/g liver	202 ± 4.8[a]	210 ± 2.1[a]	206 ± 4.6[a]	204 ± 3.72[a]
Total liver protein, g	2.01 ± 0.178[a]	1.56 ± 0.112[b]	1.92 ± 0.086[a]	1.49 ± 0.060[b]
µg magnesium/g liver	219 ± 6.3[a]	218 ± 3.73[a]	220 ± 3.70[a]	222 ± 4.83[a]
Total magnesium/liver, mg	2.18 ± 0.170[a]	1.62 ± 0.094[b]	2.05 ± 0.091[a]	1.61 ± 0.035[b]

[1]Data are given as means ± SEM. Values in the same row not followed by the same superscript letter differ significantly, $P < 0.05$.

No significant differences were found in the concentrations of protein or magnesium among the four treatment groups. Total liver magnesium and protein values were similar between groups C-F and MD-F and likewise between groups C-S and MD-S. However, both total protein and total magnesium values of groups C-F and MD-F were significantly greater than those of groups C-S and MD-S.

Blood glucose and plasma magnesium: Values obtained for blood glucose and plasma magnesium concentrations are shown in Table 3. In Appendix H are shown individual values for blood glucose concentration.

Plasma magnesium concentrations were similar in groups C-F and C-S and in groups MD-F and MD-S. However, plasma magnesium concentrations of groups MD-F and MD-S were both significantly less than those of groups C-F and C-S. Blood glucose values for groups C-F and MD-F did not differ significantly, not did the values for groups C-S and MD-S. Values obtained for groups C-S and MD-S were significantly less than those obtained for groups C-F and MD-F. Individual values for blood glucose concentration differed widely.

Enzyme activities: Hepatic G6Pase, FDPase, and PEPCK activities of magnesium deficient and control rats are shown in Table 4. Glucose-6-phosphatase activities, when expressed on a gram liver basis, were similar in groups C-S and MD-S. Hepatic G6Pase activities of groups C-S and MD-S were both significantly greater than those of groups C-F and MD-F. The results were similar when enzyme

TABLE 3

Experiment I: Effect of magnesium deficiency on whole blood glucose and plasma magnesium concentrations

Measurement	Control		Mg Deficient	
	fed	fasted	fed	fasted
Blood glucose, mg/dl[1]	164.7 ± 11.57[a]	62.0 ± 5.57[b]	137.6 ± 13.8[a]	76.3 ± 4.95[b]
Plasma magnesium, mg/dl	2.15 ± 0.114[a]	2.05 ± 0.093[a]	1.05 ± 0.078[b]	1.24 ± 0.071[b]

[1]Data are given as means ± SEM. Values within a row not followed by the same superscript letter differ significantly, $P < 0.05$.

TABLE 4

Experiment I: Effect of magnesium deficiency on the activities of liver glucose-6-phosphatase, fructose bisphosphatase and phosphoenolpyruvate carboxykinase

Measurement	Control		Mg Deficient	
	fed	fasted	fed	fasted
Glucose-6-phosphatase[1]				
units/g liver[2]	8.97 ± 0.43^{a}	13.0 ± 0.66^{b}	8.06 ± 0.51^{a}	12.5 ± 0.73^{b}
units/g protein	47.8 ± 2.55^{a}	68.0 ± 3.84^{b}	43.2 ± 2.62^{a}	73.7 ± 3.35^{b}
units/liver	88.6 ± 6.52^{a}	95.9 ± 3.83^{a}	74.2 ± 2.86^{b}	91.2 ± 5.68^{a}
units/100 g body weight	39.8 ± 1.79^{a}	46.4 ± 1.09^{b}	39.1 ± 1.43^{a}	51.1 ± 2.43^{b}
Fructose bisphosphatase				
units/g liver[2]	6.35 ± 0.26^{ab}	5.71 ± 0.315^{b}	6.75 ± 0.27^{a}	6.75 ± 0.21^{ab}
units/g protein	75.6 ± 2.75^{a}	67.1 ± 2.58^{b}	77.8 ± 2.15^{a}	71.8 ± 2.57^{ab}
units/liver	63.7 ± 6.97^{a}	42.2 ± 2.31^{b}	62.5 ± 2.15^{a}	45.0 ± 2.30^{b}
units/100 g body weight	28.4 ± 1.92^{a}	20.4 ± 0.83^{b}	33.8 ± 1.42^{c}	25.2 ± 1.17^{a}
Phosphoenolpyruvate carboxykinase				
units/g liver[3]	7.0 ± 0.53^{a}	19.3 ± 0.27^{b}	10.0 ± 0.26^{c}	22.5 ± 0.43^{d}
units/g protein	92 ± 7.2^{a}	230 ± 7.6^{b}	104 ± 3.4^{a}	263 ± 7.40^{c}
units/liver	75 ± 3.8^{a}	145 ± 9.4^{b}	84 ± 3.2^{a}	164 ± 5.0^{c}
units/100 g body weight	34.0 ± 1.72^{a}	70.1 ± 3.26^{b}	44.3 ± 2.05^{c}	92.3 ± 2.94^{d}

[1]Data are given as means ± SEM. Values in the same row not followed by the same superscript letter differ significantly, $P < 0.05$.

[2]A unit is defined as the amount of enzyme which will release 1 μmole phosphate/minute under the conditions of the assay.

[3]A unit is defined as the amount of enzyme which will form 1 μm PEP/minute under the conditions of the assay.

activity was expressed on a unit protein or unit body weight basis. Total hepatic G6Pase activities did not differ significantly among groups C-F, C-S, and MD-S. Values obtained for all three groups were significantly greater than that obtained for group MD-F.

Fructose bisphosphatase activities, expressed on a gram liver basis, were similar in groups C-F and C-S, and in groups MD-F and MD-S. There were no significant differences between fed control and magnesium deficient groups or between fasted control and deficient groups. When enzyme activity was expressed on a unit protein basis values obtained for groups C-F and M-F were similar. Enzyme activities were significantly greater in groups C-F and MD-F than in group C-S. Enzyme activities were similar in groups MD-F and MD-S. Fructose bisphosphatase activities, when expressed on a total liver basis, were similar in groups C-S and MD-S and in groups C-F and MD-F. Enzyme activities were significantly greater in both fed groups than in both fasted groups. Enzyme activities, when expressed on a unit body weight basis, were significantly greater in both fed groups than in both fasted groups and in both magnesium deficient groups relative to both control groups.

Phosphoenolpyruvate carboxykinase activities, expressed on a unit liver basis, were significantly greater in both magnesium deficient groups than in the respective control groups and greater in both fasted groups than in their fed counterparts. Trends were similar when activities were expressed on protein and body weight bases except, when activities were expressed in the former manner, values obtained

for groups MD-F and C-F were similar. When expressed on a total liver basis, PEPCK values were similar in both fed groups. Enzyme activity per liver was significantly greater in group MD-S than in group C-S.

B. EXPERIMENT II

External Symptoms

The symptoms observed in magnesium deficient rats were similar to those observed in Experiment I. Hyperemia first appeared on day 3 and increased in intensity to days 10 to 12. The redness of the exposed skin areas then slowly faded over the duration of the experiment. Skin lesions, identical to those observed in Experiment I were first noted on day 5. As the experiment progressed lesions appeared on all parts of the body. No healing was observed. Hyperirritability was noted in one animal beginning day 16. Rats in groups C and PF remained healthy throughout the experiment.

Growth and Food Intake

Weight gain and food intake data are shown in Table 5 and Figures 3 and 4. Initial weights were similar among the three treatment groups. Final weights, average daily gains, and sacrifice weights of group C were significantly greater than those of group PF. Values obtained for the latter group were all significantly greater than those obtained for group MD. Differences in weight gains of the rats were apparent by day 5.

TABLE 5

Experiment II: Effect of magnesium deficiency and food restriction on growth and food intake

Measurement	Control	Mg deficient	Pair-fed
Initial weight, g[1]	121 ± 1.4[a]	121 ± 1.4[a]	121 ± 1.4[a]
Final weight, g	236 ± 4.4[a]	180 ± 3.1[b]	196 ± 3.2[c]
ADG, g	7.2 ± 0.25[a]	3.6 ± 0.23[b]	4.7 ± 0.17[c]
Sacrifice weight, g	223 ± 4.5[a]	169 ± 2.9[b]	187 ± 3.6[c]
Total food intake, g	297 ± 5.9[a]	234 ± 5.8[b]	233 ± 3.3[b]
ADF	18.6 ± 0.36[a]	14.6 ± 0.36[b]	14.6 ± 0.20[b]

[1]Data are given as means ± SEM. Values in the same row not followed by the same superscript letter differ significantly, $P < 0.05$.

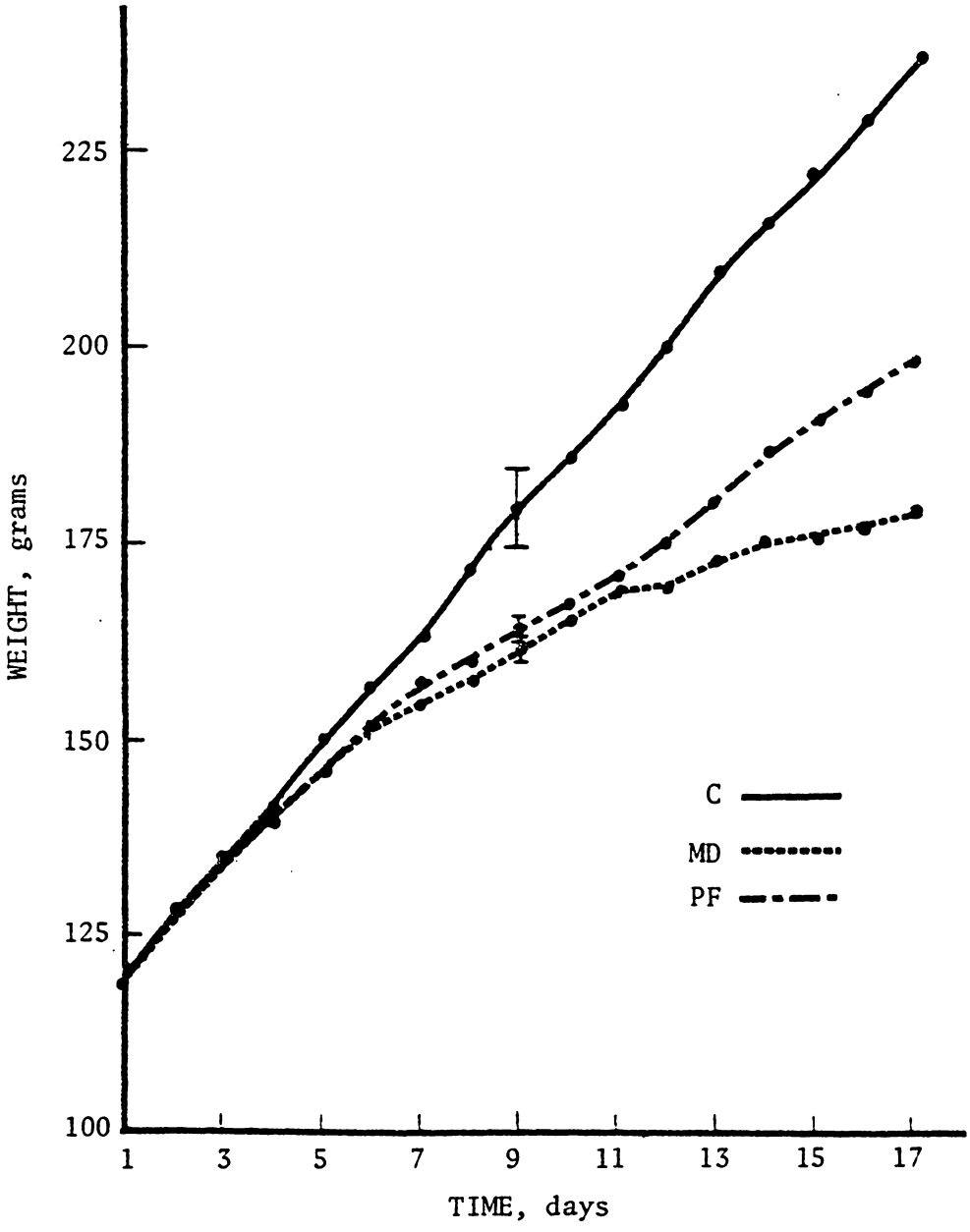

Figure 3. Effect of magnesium deficiency and food restriction on growth of rats utilized in Experiment II. Each point represents the mean of 10 values. Vertical bars give the mean ± SEM at the midpoint of the experiment.

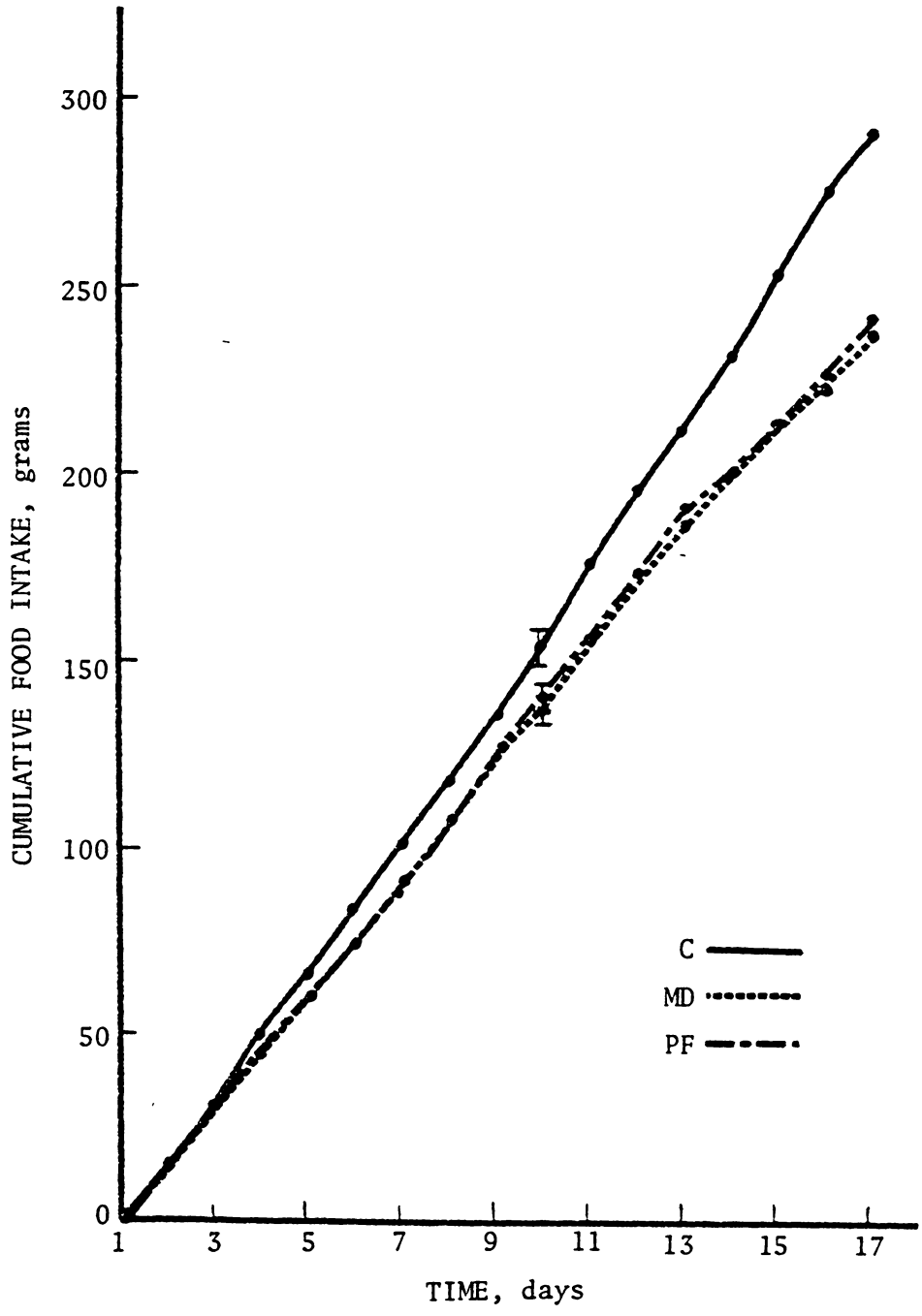

Figure 4. Effect of magnesium deficiency and food restriction on cumulative food intake of rats utilized in Experiment II. Each point represents the mean of 10 values. Vertical bars give the mean ± SEM at the midpoint of the experiment.

Total and average daily food intakes of groups PF and MD were almost identical. Values obtained for group C were significantly greater than those obtained for groups MD or PF. Differences in food intake were apparent by day 5.

Tissue Analyses

Liver composition: Liver composition is shown in Table 6. Total liver weight of group C was significantly greater than that of either group PF or MD. Liver weights of the two latter groups did not differ significantly. Liver weight, expressed on a body weight basis, was significantly greater in group MD than in groups C or PF. Values obtained for the latter two groups did not differ significantly.

Protein concentration did not differ significantly among the three treatment groups. Comparison of groups C and MD revealed no significant differences in total protein or magnesium values. Likewise, there were no significant differences between values obtained for groups MD and PF. Total liver protein and magnesium values were significantly lower in group PF relative to group C.

Blood glucose and plasma magnesium: Blood glucose and plasma magnesium values are shown in Table 7. Individual values for blood glucose concentration are shown in Appendix I. Plasma magnesium concentration was significantly decreased in group MD relative to values obtained for groups PF and C. Values obtained for groups C and PF did not differ significantly.

TABLE 6

Experiment II: Effect of magnesium deficiency and food restriction on liver composition

Measurement	Control	Mg deficient	Pair-fed
Total liver weight, g[1]	7.50 ± 0.319[a]	6.34 ± 0.312[b]	5.89 ± 0.148[b]
g liver/100 g body weight	3.35 ± 0.087[a]	3.72 ± 0.146[b]	3.16 ± 0.092[a]
mg protein/g liver	188 ± 2.0[a]	201 ± 2.36[a]	203 ± 1.94[a]
Total liver protein, g	1.46 ± 0.092[a]	1.27 ± 0.059[ab]	1.19 ± 0.034[b]
µg magnesium/g liver	210 ± 21.6[a]	233 ± 3.71[a]	225 ± 4.28[a]
Total magnesium/liver, mg	1.62 ± 0.098[a]	1.47 ± 0.057[ab]	1.32 ± 0.033[b]

[1]Data are given as mean ± SEM. Values in the same row not followed by the same superscript letter differ significantly, $P < 0.05$.

TABLE 7

Experiment II: Effect of magnesium deficiency and food restriction on whole blood glucose and plasma magnesium concentrations

Measurement	Control	Mg deficient	Pair-fed
Whole blood glucose, mg/dl[1]	81.2 ± 4.56[a]	113.5 ± 10.22[b]	82.9 ± 5.27[a]
Plasma magnesium, mg/dl	2.00 ± 0.196[a]	1.07 ± 0.031[b]	2.06 ± 0.043[a]

[1]Data are given as means ± SEM. Values in the same row not followed by the same superscript letter differ significantly, $P < 0.05$.

Whole blood glucose concentration was significantly increased in group MD relative to the values obtained for groups PF and C. Values obtained for groups PF and C did not differ significantly. Individual blood glucose values varied widely.

Enzyme activities: Hepatic G6Pase, FDPase, and PEPCK activities are shown in Table 8. Activities of G6Pase expressed on a unit liver or protein basis were similar in groups C and PF. Enzyme activity was significantly decreased in group MD relative to the value obtained for either group C or PF. When expressed on a total liver basis enzyme activity was significantly greater in group C than in either group MD or PF. Enzyme values did not differ significantly between the two latter groups. There were no significant differences among the treatment groups when enzyme activity was expressed on a body weight basis.

Fructose bisphosphatase activities, expressed on a unit liver or unit protein basis, were significantly greater in groups PF and MD relative to values obtained for group C. Values obtained for groups PF and MD did not differ significantly. Enzyme activities, expressed on a total liver basis, were similar among the three treatment groups. Expressed on a body weight basis, the activities of FDPase were similar in groups C and PF. Fructose bisphosphatase activity was significantly increased in group MD relative to the value obtained for group PF.

Phosphoenolpyruvate carboxykinase activities, expressed on a unit liver, protein, or body weight basis, were significantly greater in group MD relative to values obtained for groups C or PF. Enzyme

TABLE 8

Experiment II: Effect of magnesium deficiency and food restriction on the activities of liver glucose-6-phosphatase, fructose bisphosphatase, and phosphoenolpyruvate carboxykinase

Enzyme	Control	Mg Deficient	Pair-fed
Glucose-6-phosphatase			
units/g liver[1,2]	14.0 ± 0.33[a]	12.1 ± 0.32[b]	14.0 ± 0.41[a]
units/g protein	55.0 ± 1.16[a]	48.1 ± 1.46[b]	54.8 ± 1.42[a]
units/liver	105 ± 4.25[a]	77.2 ± 5.44[b]	82.1 ± 0.75[b]
units/100 g body weight	46.8 ± 1.18[a]	45.2 ± 2.61[a]	43.9 ± 1.01[a]
Fructose bisphosphatase			
units/g liver[2]	5.45 ± 0.231[a]	6.34 ± 0.083[b]	6.19 ± 0.121[b]
units/g protein	78.0 ± 2.66[a]	90.4 ± 1.37[b]	87.2 ± 1.85[b]
units/liver	47.0 ± 2.74[a]	40.0 ± 1.61[a]	36.4 ± 0.94[a]
units/100 g body weight	18.3 ± 4.56[a]	23.5 ± 0.77[a]	19.5 ± 0.44[b]
Phosphoenolpyruvate carboxykinase			
units/g liver[2]	14.4 ± 0.84[a]	19.8 ± 0.51[b]	15.2 ± 0.48[a]
units/g protein	207 ± 11.6[a]	283 ± 9.7[b]	214 ± 8.1[a]
units/liver	110 ± 10.2[a]	125 ± 5.4[a]	89.5 ± 2.21[b]
units/100 g body weight	50.8 ± 3.76[a]	73.8 ± 3.07[b]	48.3 ± 1.54[a]

[1]Data are given as means ± SEM. Values in the same row not followed by the same superscript letter differ significantly, $p < 0.05$.

[2]"Unit" is defined as in Table 4.

values were similar in groups C and PF. When enzyme activity was expressed on a total liver basis there was no significant difference between the values obtained for groups C and MD. Enzyme activity was significantly decreased in group PF relative to values obtained for groups C and MD.

C. EXPERIMENT III

External Symptoms

The onset of magnesium deficiency in group MD, the symptoms, and the course of the deficiency were identical to those observed in Experiment II except no hyperirritability was observed.

Growth and Food Intake

Initial weights of the rats were similar. Final weights, average daily gains and sacrifice weights for the three treatment groups all differed significantly. These data are shown in Table 9. Highest values for all parameters were observed in group C followed by groups PF and MD in that order. There were no significant differences in total food intake or ADF between groups MD and PF. Values obtained for both latter groups were significantly less than those obtained for group C. Additional information is given in Figures 5 and 6.

TABLE 9

Experiment III: Effect of magnesium deficiency and food restriction on growth and food intake

Measurement	Control	Mg Deficient	Pair-fed
Initial weight, g[1]	111 ± 1.5[a]	111 ± 1.71[a]	112 ± 1.7[a]
Final weight, g	212 ± 6.1[a]	171 ± 5.11[b]	192 ± 5.1[c]
ADG, g	6.3 ± 0.36[a]	3.7 ± 4.9[b]	5.0 ± 0.31[c]
Sacrifice weight, g	202 ± 5.9[a]	159 ± 5.3[b]	185 ± 5.1[c]
Total food intake, g	293 ± 5.8[a]	242 ± 3.8[b]	239 ± 2.0[b]
ADF	18.3 ± 0.36[a]	15.1 ± 0.24[b]	14.9 ± 0.12[b]

[1]Data are given as means ± SEM. Values in the same row not followed by the same superscript letter differ significantly, $p < 0.05$.

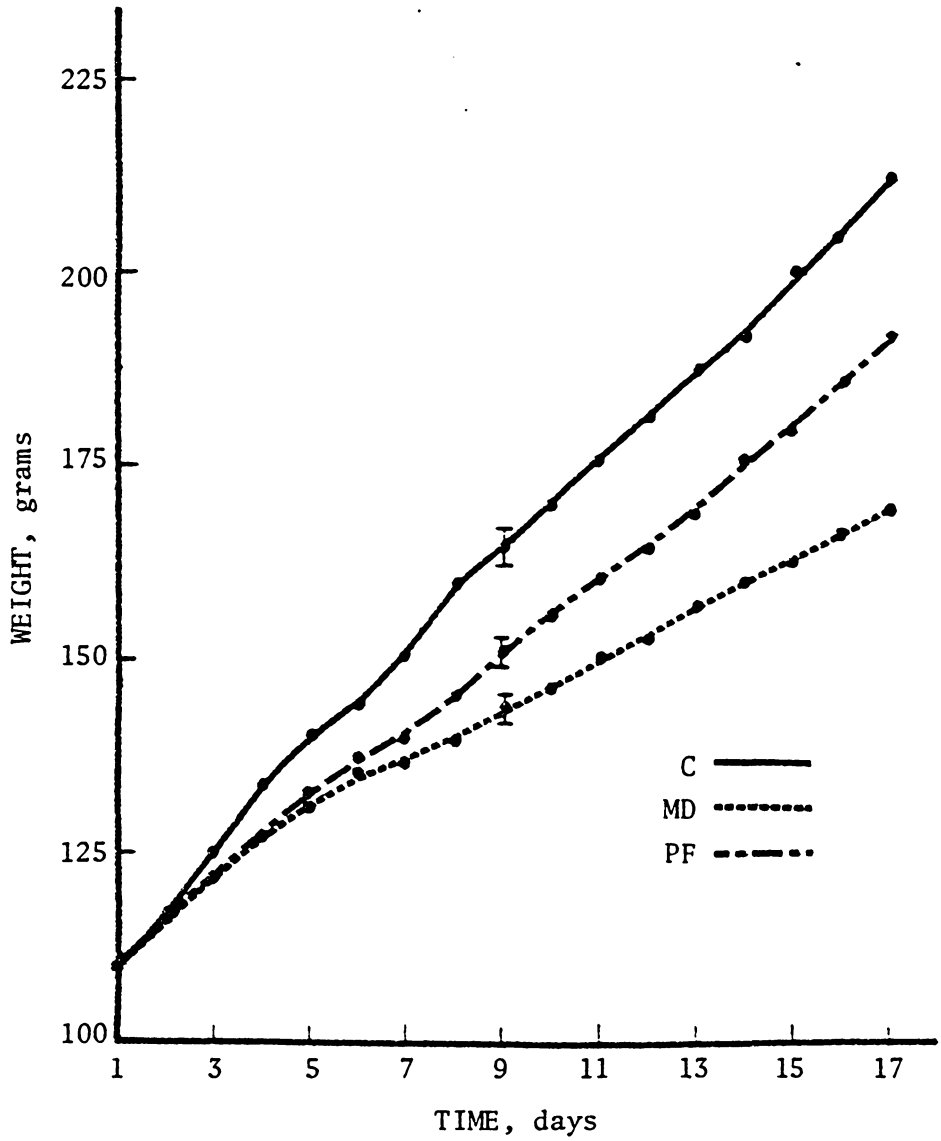

Figure 5. Effect of magnesium deficiency and food restriction on growth of rats utilized in Experiment III. Each point represents the mean of 10 values. Vertical bars give the mean ± SEM at the midpoint of the experiment.

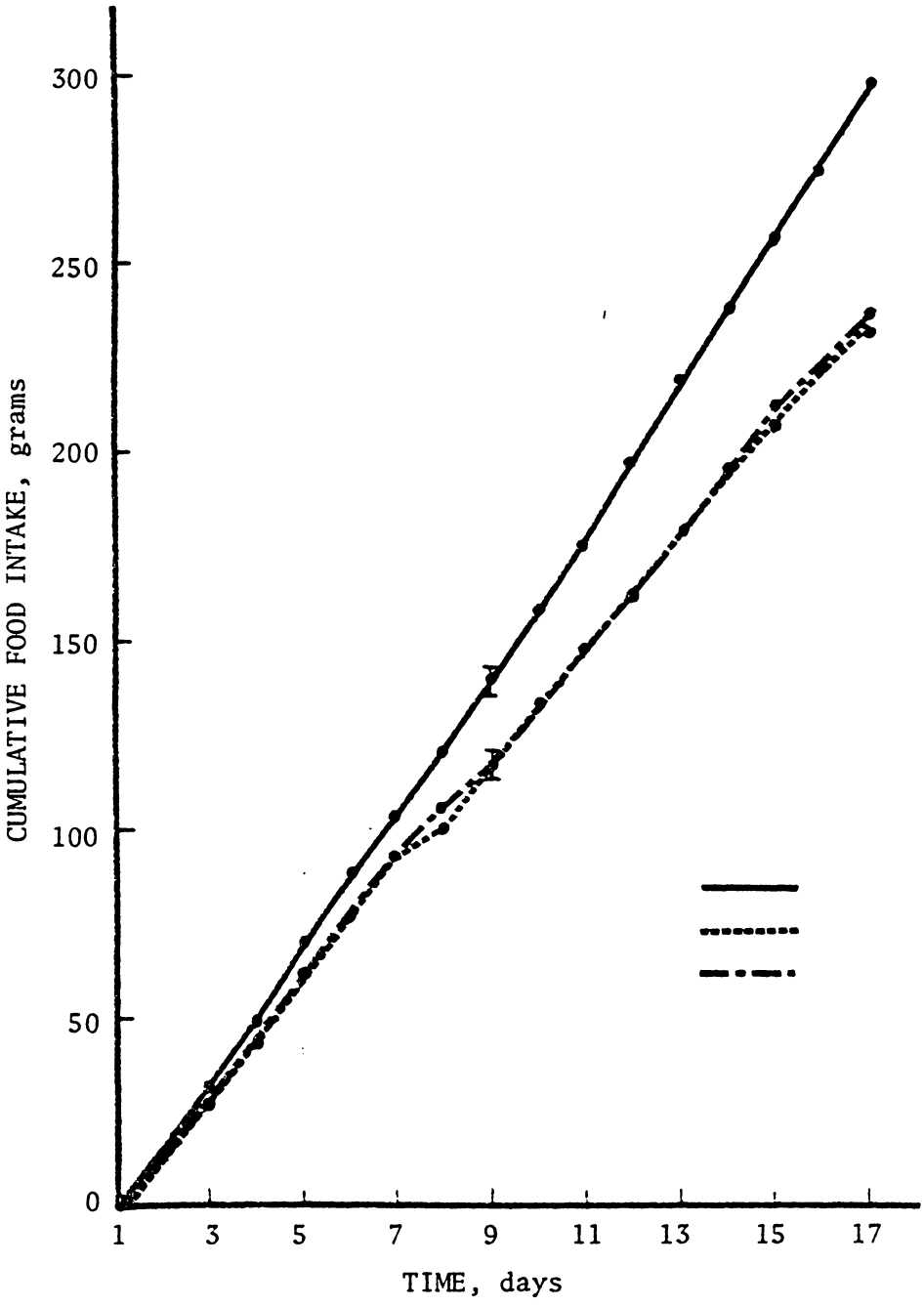

Figure 6. Effect of magnesium deficiency and food restriction on cumulative food intake of rats utilized in Experiment III. Each point represents the mean of 10 values. Vertical bars give the mean ± SEM for the midpoint of the experiment.

Tissue Analyses

Liver weight and glycogen: Liver weight data are shown in Table 10. Liver weights, expressed on a total liver or a body weight basis, all differed significantly among the three treatment groups. That of group C was greater than that of group MD which was, in turn, greater than that of group PF.

Liver glycogen was measured in all animals; however, no glycogen was found. In the six fed control rats a value of 23.8 $\pm$ 5.6 mg glycogen/g liver was found.

Enzyme activity: The results of the enzyme analyses are shown in Table 10. The activity of PEPCK, when expressed on a unit liver, unit protein, or total liver basis was significantly increased in group MD relative to the values obtained for groups PF or C. There were no significant differences in the activities of this enzyme between groups C and PF when the activity was expressed on these bases. The activity of PEPCK expressed on a body weight basis was significantly increased in group MD relative to values obtained for groups C or PF. The activity of PEPCK expressed on a body weight basis was significantly decreased in group PF relative to the values obtained in the other two treatment groups.

Blood glucose and plasma magnesium: Plasma magnesium values are shown in Table 11. As in Experiment II plasma magnesium concentrations were similar in groups C and PF. Both latter values were significantly greater than that obtained in the magnesium deficient group.

TABLE 10

Experiment III: Effect of magnesium deficiency and food restriction on liver composition and the activity of phosphoenolpyruvate carboxykinase

Measurement	Control	Mg Deficient	Pair-fed
Total liver weight, g[1]	6.94 ± 0.205[a]	6.27 ± 0.176[b]	5.60 ± 0.19[c]
g liver/100 g body weight	3.44 ± 0.051[a]	3.94 ± 0.108[b]	3.04 ± 0.108[c]
Phosphoenolpyruvate carboxykinase			
units/g liver[2]	13.9 ± 0.98[a]	20.0 ± 0.55[b]	14.9 ± 0.34[a]
units/g liver protein	180 ± 8.0[a]	265 ± 6.20[b]	185 ± 4.5[a]
units/liver	95.6 ± 7.37[a]	125.3 ± 4.59[b]	83.5 ± 3.34[a]
units/100 g body weight	47.2 ± 3.14[a]	78.6 ± 2.35[b]	45.3 ± 1.64[a]

[1]Data are given as means ± SEM. Values in the same row not followed by the same superscript letter differ significantly, $p < 0.05$.

[2]"Unit" is defined as in Table 4.

TABLE 11

Experiment III: Effect of magnesium deficiency and food restriction on whole blood glucose and plasma magnesium concentrations

Measurement	Control	Mg Deficient	Pair-fed
Whole blood glucose, mg/dl[1]	86.2 ± 8.31[a]	83.2 ± 5.42[a]	76.5 ± 7.85[a]
Plasma magnesium, mg/dl	2.59 ± 0.052[a]	0.74 ± 0.019[b]	2.39 ± 0.046[a]

[a]Data are given as means ± SEM. Values in a row not followed by the same superscript letter differ significantly, $p < 0.05$.

Mean whole blood glucose values are shown in Table 11, and individual values are shown in Appendix J. There were no significant differences in the whole blood glucose means for the three treatment groups. As in Experiments I and II wide variation in individual values was observed.

CHAPTER V. DISCUSSION

A. GROWTH, FOOD INTAKE AND LIVER SIZE AND PROTEIN

Growth, as indicated by weight gain, is a commonly used indicator of the nutritional status and general health of experimental animals. Control rats studied in Experiments I and II gained over 7 grams per day. Control rats used in Experiment III gained 6.3 grams per day. These values are comparable to those reported in the literature and listed in handbooks (31,32,148) for growing rats on an adequate diet.

Average daily food intake of control rats used in Experiment I was comparable to literature values for control rats eating a semi-purified diet (31,32); it was greater than values obtained for control rats studied in Experiments II and III. Rats used in these latter two studies were meal-fed; those in the former study were fed ad libitum. Leveille and coworkers (149) have shown that meal-fed animals gain weight at an equal or greater rate than ad libitum fed controls, but consume less food. This is accompanied by changes in fat and carbohydrate metabolism leading to increased lipogenesis (150). Differences in food intakes between rats used in Experiment I and those used in Experiments II and III were similar to differences reported by Leveille et al. (149) for ad libitum versus meal-fed rats.

Comparison of growth rates of magnesium deficient rats used in Experiment I with those of the deficient rats used in Experiments II and III indicated that growth rate of rats used in the former

experiment was greater than that obtained in the latter two experiments. This was apparently not related to the greater length of Experiments II and III: Analysis of data presented in Table 1 and Figures 3 and 5 indicated that deficient rats studied in Experiment II gained 41 grams, and those used in Experiment III gained 46 grams over the first 12 days of the studies. Magnesium deficient rats studied in Experiment I gained 62 grams over the corresponding time period. These results may indicate that rats used in the latter two studies were more deficient in magnesium than those used in the first study. On the other hand, the metabolic mechanism responsible for the greater feed efficiency in control meal-fed rats may not have been operative in magnesium deficient meal-fed rats.

Comparison of growth rates of experimental rats observed in all three experiments with those of their respective controls indicated that feeding a magnesium deficient diet was accompanied by a significant reduction in growth rate and final body weight. Comparison of food intakes of magnesium deficient rats with those of their respective controls indicated that anorexia was present in the former groups. In Experiments II and III pair-fed controls were used to assess effects of anorexia. Growth in pair-fed rats was significantly reduced relative to values obtained in control rats but significantly greater than those obtained in magnesium deficient rats. These data suggest that anorexia contributed to decreased growth in magnesium deficiency. The greater decrease in growth observed in magnesium deficient rats relative to values observed in pair-fed controls suggests the presence

of an effect on growth specifically due to magnesium deficiency.

It is tempting to assign a specific percentage of the decreased growth rate observed in magnesium deficiency to anorexia and the remainder to unavailability of magnesium for anabolic processes. This assumes that food restriction in a control rat and anorexia in a deficient rat produce decreases in weight gain through the same mechanism. It also suggests that magnesium deficiency per se affects growth through a second mechanism. There are no data in the literature to support these assumptions. Future studies using a combination of pair-feeding and force-feeding techniques may provide insight into this problem.

Changes in body weight reflect changes occurring in organ size and composition. Total liver weight of experimental rats used in Experiment I was similar to that of the controls in both fed and fasted rats. Significant decreases in liver weights of deficient rats relative to control values were observed in Experiments II and III. Differences in response of liver weight to magnesium deficiency between the first and latter two experiments may be related to differences in experimental design.

Liver weight with respect to body weight was significantly increased in all fasted magnesium deficient groups relative to values observed in their respective controls. This was not a consequence of decreased food intake; pair-fed rats had liver to body weight ratios comparable to control values. Protein concentration was unaffected by dietary treatment. A relative increase in tissue

protein was not therefore associated with an increase in liver mass.

The increase in the ratio of liver to body weight observed in magnesium deficiency is striking. The increase was not significant in fed magnesium deficient rats studied in Experiment I. This, together with the results obtained for the fasted rats, indicates that in magnesium deficiency liver growth occurred in proportion to body growth, but in fasting, liver mass was not lost in proportion to body mass. Zieve et al. (33) observed an increase in liver weight of magnesium deficient rats (fed or fasted state unspecified) relative to values observed in pair fed controls. DNA and RNA synthesis, as assessed by isotope techniques, were increased in livers of magnesium deficient rats relative to values observed in pair-fed controls. Protein synthesis was unaffected by dietary treatment. This study suggests a mechanism whereby magnesium deficiency altered the liver to body weight ratio, but it does not explain differences between fed and fasted rats.

Tissues and organs, other than the liver, and their composition with respect to protein, fat, water, and nucleic acids were not assessed in the experiments carried out here. Such measurements would have provided information on the contribution of each component of the body to the growth reduction observed in magnesium deficiency. Such information is presently unavailable in the literature.

B. THE SYMPTOMS OF MAGNESIUM DEFICIENCY

Growth reduction and anorexia alone are not specific indicators of the presence of magnesium deficiency. However, symptoms characteristic of this state were observed in the experimental rats used in these studies. Hyperemia appeared about day 3 and skin lesions were noted two days later. Pruritis was observed and this may have led to the development of skin lesions. Convulsions were not noted. These symptoms and their pattern of development are similar to literature descriptions of the course of magnesium deficiency (54-57).

Plasma magnesium concentration is invariably depressed by magnesium deficiency in the rat. Values for plasma magnesium comparable to values reported in the literature were observed here (15,17,18). Liver magnesium concentration was unchanged by dietary treatment in these experiments. This is consistent with literature reports (20,31).

Whether plasma magnesium levels are indicative of the degree of magnesium deficiency may be questioned. Smith and Nisbet carried out serial determinations of plasma magnesium in mature rats fed a magnesium deficient diet for five weeks (diet level of magnesium not stated). Plasma magnesium concentration declined significantly four days after the start of the experiment. After eight days it reached a level of approximately 0.5 mg/dl. It remained at this level for the remainder of the experiment. Clinical signs of increasing magnesium depletion, e.g., increased susceptibility to convulsions, were noted as the experiment progressed. Thus the plasma magnesium concentration reflected the presence but not the degree of deficiency.

The relationship of body stores of magnesium to plasma magnesium concentration has been explored by Alfrey et al. (151). Muscle, erythrocyte, and bone magnesium were correlated with plasma magnesium in hypo- normo-, and hypermagnesiemic patients. A significant positive correlation was noted between plasma magnesium and bone magnesium concentration. No correlations were noted between erythrocyte or muscle magnesium and plasma magnesium concentrations. This indicated that the level of magnesium in the soft tissues did not accurately reflect the presence of magnesium deficiency while that of the bone did. Studies carried out in the rat support this conclusion (15-20).

In the experiments carried out here clinical symptoms of magnesium deficiency, and plasma and liver magnesium concentrations were used to evaluate the presence of magnesium deficiency. The symptoms and plasma magnesium concentration did indicate that magnesium depletion had occurred. No conclusions regarding the extent of the deficiency can be made. The unresponsiveness of liver magnesium concentration to dietary treatment indicates only that feeding a magnesium deficient diet had an effect on this parameter comparable to literature reports.

C. LIVER ENZYMES AND BLOOD GLUCOSE

Enzyme data obtained here were expressed in four ways. Values were first expressed as units per gram liver. This is essentially a concentration value. It is a measure of the activity of the enzyme in question in a unit of tissue regardless of tissue composition.

Use of these values permits comparisons between animals with different organ sizes. These values do not represent activity per cell as cell size and number may vary with dietary treatment.

Enzyme values were also expressed on a protein basis. This corrects for the amount of protein in the tissue and differences in extract preparation.

Expression of enzyme data on a total liver basis gives an indication of the total enzyme activity available to the organism. When corrected for body weight, the amount of enzyme activity available to each unit of the organism may be assessed. This allows comparison of animals differing in body weight.

The question arises as to which method of expressing enzyme results is "best." This depends on the purpose for which the data are to be used. If results are to be used to assess liver metabolism, then measures based on weight or protein should be used. Expressing data in terms of DNA would also be helpful. If the data are to be related to the metabolism of the animal as a whole, then expressing results on a body weight basis would be most appropriate.

In all three experiments results for a single enzyme differed depending on the manner in which values were expressed. Analysis of results obtained in Experiment I indicates that G6Pase activity was generally increased in fasting in magnesium deficient and control rats. A loss of liver mass as a result of fasting evidently prevented the results from reaching statistical significance when values were expressed on a total liver basis.

There was a decline in FDPase activity with fasting in control and experimental groups. This decline reached statistical significance consistently only when expressed on total liver and body weight bases. This reflects loss of enzyme activity in each unit of tissue plus additional loss of liver mass.

Phosphoenolpyruvate carboxykinase activity was increased by fasting in control and magnesium deficient rats regardless of how the values were expressed.

Comparisons between fasted deficient and control rats may be made for all three experiments. Glucose-6-phosphatase activity was unaltered by feeding a magnesium deficient diet in Experiment I regardless of how the results were expressed. In Experiment II enzyme activity decreased when values were expressed on all bases except body weight. Decreases in enzyme activity seen in Experiment II relative to those seen in Experiment I may reflect differences in experimental design. The data indicate that relative enzyme activity available to the organism was unchanged by dietary treatment regardless of experimental design. In Experiment II decreases in G6Pase activity in magnesium deficient rats relative to values obtained in pair-fed controls were observed when results were expressed on a concentration or protein basis. On a total liver basis values obtained in magnesium deficient rats were similar to those obtained in pair-fed controls. There were no differences between pair-fed and magnesium deficient groups when values were expressed on a body weight basis. These results indicate that although decreases in

G6Pase activity, unrelated to food intake, did occur in magnesium deficiency, these did not result in a decrease in the level of enzyme activity available to each unit of the organism.

Fructose bisphosphatase activity generally increased in deficient rats relative to control values in Experiment I. The results did not reach statistical significance except when expressed on a body weight basis. The pattern of enzyme changes differed in Experiment II. Here enzyme activity increased in deficient rats relative to control values when expressed on a concentration or protein basis. This was evidently due to anorexia as values for pair-fed rats were similar to those for deficient rats. In spite of these increased values FDPase activities expressed on total liver or body weight bases were similar in the three groups of animals. Thus total and relative FDPase activities were unchanged by magnesium deficiency or food restriction.

Phosphoenolpyruvate carboxykinase activity increased in magnesium deficiency when values were expressed in any manner except on a total liver basis in Experiment II. This was the only enzyme which increased in activity when expressed on a body weight basis. Food restriction did not account for the changes observed.

Several generalizations may be made based on the above analysis. First, while changes in enzyme activity may occur in a unit of tissue they need not be reflected in a change in the relative amount of enzyme available to the organism. In some cases a change in tissue enzyme activity is accentuated by loss of organ or body mass, but in other cases the change in enzyme activity may be offset. A decrease

or increase in enzyme activity may not result in a change in the overall metabolism of the organism. This may represent a homeostatic mechanism. There is, unfortunately, no data in the literature to support this supposition.

The pattern of enzyme changes produced by fasting and food restriction differed from each other and from changes produced by magnesium deficiency. This may indicate that these three dietary variables effect changes in the gluconeogenic enzymes through different mechanisms. The mechanism responsible for changes in enzyme activities effected by fasting may involve changes in the insulin:glucagon ratio (152) as well as alterations in the secretion of the glucocorticoid hormones (153). The mechanism by which food restriction and magnesium deficiency produce changes in the gluconeogenic enzymes have not been investigated here or elsewhere. Regardless of mechanism it appears that decreased food intake does not account for the changes in enzyme activity induced by magnesium deficiency.

A third generalization is that changes observed in activities of the gluconeogenic enzymes in response to fasting in both the control and deficient rats are consistent with literature reports not only with respect to direction of changes but also with respect to differences in results with manner of expression. These literature results have been reviewed in detail in Chapter II and will not be covered again.

Another generalization is that magnesium deficient rats respond to fasting in the same manner as control rats. This may indicate that magnesium is not required for the response to fasting. On the other

hand magnesium may be required for the response to fasting, but since this element is not lost from the liver in magnesium deficiency, the response proceeds normally.

The final generalization is as follows. Under conditions of fasting or magnesium deficiency the gluconeogenic enzymes did not show synchronous changes in their activities when either the liver itself or the liver in relation to the entire organism is considered. The three enzymes studied, and in addition pyruvate carboxylase, are rate limiting for gluconeogenesis. If these enzymes collectively control the flux of precursors through the pathway (given an adequate substrate supply), then it might be expected that changes in their activities would occur simultaneously and in the same direction.

Weber and coworkers (6,13) have proposed that the key gluconeogenic enzymes are synthesized on the same genome unit. Enzyme synthesis is induced by cortisol and suppressed by insulin. This results in a synchronous response of the enzymes to various stresses. Support for this hypothesis has been derived mainly from studies of the diabetic and cortisol or insulin treated rat.

Weber's hypothesis does not appear to be consistent with the results obtained here. Several factors may account for this. First, fasting, and possibly magnesium deficiency, are accompanied by changes in hormone levels other than cortisol and insulin. Glucagon secretion is enhanced as the blood glucose level falls (152). Hypoglycemia, and possibly magnesium depletion, are accompanied by increased secretion of epinephrine (10). Both glucagon and epinephrine increase

gluconeogenesis (125,131). Thus actions of hormones other than insulin are cortisol must be considered.

In the cell synthesis and degradation of enzyme molecules occur simultaneously. A net rise in enzyme activity may represent increased synthesis and/or decreased degradation. In the experiments carried out here increased degradation of FDPase in fasted versus fed rats and of G6Pase in magnesium deficient versus control rats may have been responsible for the results obtained.

Enzyme modulation is an important factor involved in the response of enzymes to experimental treatments. Dietary or hormonal variables may result in activation of a given enzyme. For instance, glucagon activates PEPCK through a cyclic-AMP mediated mechanism (131). Inhibition of enzyme activity may also occur. For instance, FDPase is inhibited by AMP (92). Patterns of enzyme activity seen in experiments such as those carried out here may reflect levels of activators and inhibitors in the enzyme system and not the actual level of enzyme protein. The combination of enzyme synthesis, degradation, and modulation may lead to asynchronous behavior of the gluconeogenic enzymes to dietary stresses.

Weber's hypothesis is a worthy attempt to explain the response of the gluconeogenic enzymes to various stresses and disease states. It is, however, simplistic. There is need for the development of a model for the control of the gluconeogenic enzymes which integrates dietary and hormonal variables with enzyme synthesis, degradation, and modulation occurring simultaneously in the organism.

Regardless of the particular pattern of enzyme changes induced by magnesium deficiency, the question remains as to the mechanism involved. Roobol and Alleyne (38) observed that neither the presence nor absence of magnesium from the incubation medium affected the rate of glucose synthesis from pyruvate by liver slices. In the experiments carried out here, increased PEPCK activity was not accompanied by magnesium loss from the liver. Given these findings, a theory proposing a direct action of magnesium on the activity of hepatic PEPCK appears untenable.

An effect on hormone secretion or circulating hormone levels could be a means by which magnesium deficiency affects the activity of PEPCK. This is supported by studies showing that both glucagon and epinephrine secretion were increased by the absence of magnesium from the medium bathing the pancreas and adrenal medulla, respectively (9,10). Also, Shrago et al. (11) and Wicks et al. (119) observed that injections of either of these hormones increased PEPCK activity.

A direct effect of magnesium on the pancreas or adrenal medulla is just one mechanism by which magnesium deficiency could induce hormone mediated changes in PEPCK activity. Kahil et al. (39) observed increase glucose uptake by the diaphragm in the magnesium deficient rat. In magnesium deficient puppies, changes in the glucose tolerance curve suggested increased peripheral uptake of glucose. These findings suggest that increased gluconeogenesis from such precursors as alanine or pyruvate, may be required for the maintenance

of the blood glucose concentration in magnesium deficiency. This could be mediated by a decreased insulin:glucagon ratio (4) which, in turn, could lead to increased PEPCK activity (11).

Irrespective of the initial stimulus, it is clear that, since magnesium was not lost from the liver of experimental rats in the experiments carried out here, magnesium deficiency induced an increase in the activity of PEPCK by indirect means. A hormone mediated mechanism appears plausible given the role of magnesium in hormone secretion. A hormone mediated mechanism is also suggested by a consideration of changes occurring in glucose utilization.

A final question raised by the results presented here regards the consequences of alterations in enzyme patterns observed in magnesium deficiency for the organism as a whole. An increase in PEPCK activity could lead to an increased flux of metabolites through the gluconeogenic pathway. However, if FDPase and G6Pase were more rate limiting than PEPCK, increased glucose production may not be realized.

As discussed in Chapter II the relationship between the activities of the gluconeogenic enzymes to glucose production is not well defined. The two studies which have been carried out indicate that increases in the activity of PEPCK and possibly FDPase, are accompanied by increased glucose production (12,124). The results of Experiment II support this; in this experiment blood glucose concentration in magnesium deficient rats was elevated along with elevated PEPCK values. The results of the three experiments were not consistent, however:

in Experiments I and III elevations in the activity of PEPCK were not accompanied by elevated blood glucose values.

Individual blood glucose values varied widely in all experiments. All rats were handled similarly with respect to sacrifice procedures but the factor of stress was nevertheless uncontrolled. Stress, leading to increased secretion of epinephrine and corticosterone, could decrease peripheral glucose uptake (153) leading to increased blood glucose levels. Such elevated blood glucose concentrations occurring in a proportion of rats in each treatment group could mask the actual effects of magnesium deficiency. Use of a less stressful method for obtaining blood for glucose analysis may lead to clarification of the results.

Control of the gluconeogenic enzymes is important in the control of glucose production and its circulating level, but other factors are also involved. These include the supply of gluconeogenic precursors to the cell, uptake of precursors by the cell and mitochondria, and the redox state of the cell (4). Any theory accounting for the effects of magnesium deficiency on gluconeogenesis and the blood glucose level must consider the role of these variables in glucose production.

In any consideration of glucose production, glycogenolysis as a factor must be recognized. In Experiment III an attempt was made to explore the relationship between the glycogen content of the liver and the blood glucose concentration. The results indicated that glycogen was absent from the livers of all three treatment groups.

This may have been the result of the method used for sacrifice. Specifically, carbon dioxide was used as an anesthetic; anoxia is known to accelerate the breakdown of glycogen (154). Furthermore, four to five minutes elapsed from the time the anesthetic was administered to the time the liver was frozen. Glycogen levels are low in the fasted animal (155). It is likely that such low levels of glycogen together with the anoxia and time factors were responsible for the results. Due to these circumstances no conclusions can be drawn regarding the role of glycogenolysis in the production of blood glucose in magnesium deficiency.

D. DIRECTIONS FOR FUTURE RESEARCH

The response of the gluconeogenic enzymes to nutritional deficiencies have not, in general, been fully explored. That the enzymes respond to metabolic stresses, e.g., diabetes, in a synchronous fashion has been questioned by Wimhurst and Manchester (102). The results presented here indicate that nutritional stresses do not result in synchronous changes in the activities of the gluconeogenic enzymes. These findings indicate the need for a theory of control which takes into account such factors as enzyme synthesis, degradation, and modulation. In future studies an attempt should be made to identify the role of each of these factors in producing the enzyme changes observed in magnesium deficiency. This would involve the use of tracer techniques and protein isolation methods. Time course studies using inhibitors of protein synthesis would also be

helpful. Measurement of activators and inhibitors of the gluconeogenic enzymes would give insight into the role of enzyme modulation in producing the enzyme results.

Endocrine studies will be essential to an understanding of the mechanism by which magnesium deficiency alters the activities of the gluconeogenic enzymes. It will be necessary to measure insulin, glucagon, epinephrine, and corticosterone in single samples. Chronic treatment of normal rats with these hormones may provide additional information.

It will be necessary to address the question regarding whether glucose production is altered in magnesium deficiency. Three techniques offer the means for understanding this problem. The use of labeled precursors would permit the calculation of fluxes through individual steps of the gluconeogenic pathway. In the whole organism isotope techniques can be used to measure glucose influx, efflux, and turnover. Using the perfused liver preparation basal and maximal rates of glucose production can be measured. Measurements of intermediates in the gluconeogenic pathway can lead to the identification of rate limiting steps.

Studies of gluconeogenesis in the magnesium deficient rat have practical aspects. Uncontrolled diabetes is a condition characterized by increased gluconeogenesis (4) urinary magnesium loss and hypomagnesemia (156). It is possible that a relative magnesium deficiency, through an action on PEPCK, may contribute to the increased glucose production. A study of the gluconeogenic enzymes in the magnesium depleted diabetic rat may provide information on this problem.

CHAPTER IV. SUMMARY AND CONCLUSIONS

Gluconeogenesis refers to the process of glucose synthesis. Through this process glucose is provided to the body during fasting or under conditions where glucose absorption from the alimentary tract is limited. Gluconeogenesis is controlled, in part, by the activities of the four "key" gluconeogenic enzymes: pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase (FDPase), and glucose-6-phosphatase (G6Pase). These enzymes are in turn controlled by insulin, glucagon, corticosterone, and epinephrine. Dietary variables, e.g., fasting and the protein, carbohydrate and fat contents of the diet, influence the activities of the gluconeogenic enzymes.

Magnesium appears to be yet another dietary variable influencing the activities of the gluconeogenic enzymes. In the experiments carried out here the effects of feeding a magnesium deficient diet to the rat on the activities of G6Pase, FDPase, and PEPCK were investigated. The effects of fasting and food restriction were also observed.

Feeding a diet deficient in magnesium to the rat resulted in symptoms characteristic of the deficient state. These symptoms included hyperemia, skin lesions, anorexia, decreased weight gain, and decreased plasma magnesium levels. Anorexia accounted for part, but not all of the reduced weight gain. The concentration of magnesium in the liver of the magnesium deficient rats was unchanged relative to control values.

In the fasted rat relative to the fed rat, the activities of G6Pase and PEPCK were increased; that of FDPase was decreased. The response to fasting was similar in control and magnesium deficient rats. In the magnesium deficient rat, relative to the control rat, the activities of G6Pase and FDPase were unchanged, while that of PEPCK was increased. Anorexia was not responsible for these results.

Since magnesium was not lost from the liver in magnesium deficiency, a direct action of PEPCK appears untenable. Magnesium is involved in the secretion of insulin, glucagon, corticosterone, and epinephrine. A change in the circulating level of one or more of these hormones may be responsible for the effects of magnesium deficiency on the activity of PEPCK.

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APPENDICES

APPENDIX A. COMPOSITION OF BASAL DIET

Ingredient	Percent
Casein	25
Alpha-Cellulose	5
Corn Oil	5
Vitamin Mix	1
Mineral Mix	4
Choline chloride	0.2
Corn Starch	59.8
TOTAL	100.0

APPENDIX B. COMPOSITION OF MINERAL MIX

Ingredient[1]	g/kg
$CaHPO_4$	500
NaCl	74.0
$K_3C_6H_5O_7 \cdot H_2O$	220.0
K_2SO_4	52.0
$MnCO_3$	3.5
$FeC_6H_5O_7$	6.0
ZnO	1.6
$CuCO_3$	0.3
KIO_3	0.01
$Na_2SeO_3 \cdot 5H_2O$	0.01
$CrK(SO_4)_2 \cdot 12H_2O$	0.55
Sucrose	142.0

[1]All chemicals supplied by American Scientific Products, 1430 Waukegan Road, McGaw Park, Illinois 60085.

APPENDIX C. COMPOSITION OF VITAMIN MIX

Ingredient	per kg mixture
Thiamine HCl	600mg
Riboflavin	600mg
Pyridoxine	700mg
Nicotinic acid	3mg
d-Calcium pantothenate	1.6mg
Folic acid	200mg
d-Biotin	20mg
Cyanocobalamin	1mg
Retinyl Palmitate, pre-mix	800mg
dl-α-tocopheryl acetate, pre-mix	20g
Cholecalciferol	2.5mg
Menaquinone	5.0mg
Sucrose	972.9g

[1]Supplied pre-mixed by ICN Nutritional Biochemicals, 26201 Miles Rd., Cleveland, Ohio.

APPENDIX D. CHEMICALS USED IN ASSAYS

Assay	Chemicals	Source
Glucose-6-phosphatase	Sucrose	(1)
	Maleic acid	(2)
	Glucose-6-phosphate	(2)
	Trichloroacetic acid	(1)
Fructose 1,6-biphosphatase	Cysteine	(2)
	Tris	(2)
	$MgSO_4$	(1)
	Sucrose	(1)
	Fructose diphosphate	(2)
	Trichloroacetic acid	(1)
Phosphoenolpyruvate carboxykinase	Glutathione	(2)
	Inosine triphosphate	(2)
	Oxaloacetate	(2)
	Tris	(2)
	Trichloracetic acid	(1)
	$MnSO_4$	(1)
	$MgSO_4$	(1)
	NaF	(1)
Phosphorus	H_2SO_4 (conc.)	(1)
	Ammonium Molybdate	(1)
	Ascorbic acid	(2)
	Na_2HPO_4	(1)
Protein	NaOH	(1)
	$CuSO_4$	(1)
	Potassium sodium tartrate	(3)
	Folin reagent	(2)
Nitrogen	H_2SO_4 (conc.)	(1)
	H_2O_2 (20 percent)	(3)
	NH_4Cl	(3)
	NaOH	(1)
	Nesslers reagent	(3)
Magnesium	HNO_3 (conc.)	(1)
	H_2SO_4 (conc.)	(1)
	H_2O_2 (20 percent)	(3)
	Magnesium standard	(1)

(1) Fisher Scientific Company, 7722, Fenton St., Silver Spring, MD.

(2) Sigma Chemical Company, P.O. Box 14508, St. Louis, MO.

(3) American Scientific Products, 1430 Waukegan Rd., McGaw Park, IL.

APPENDIX E. CALCULATION OF GLUCOSE-6-PHOSPHATASE ACTIVITY

The activity of glucose-6-phosphatase in liver was calculated as follows:

1) $$OD = OD_{\substack{\text{sample} \\ \text{blank}}} - OD_{\substack{\text{reagent} \\ \text{blank}}} - OD_{\substack{\text{enzyme} \\ \text{blank}}}$$

2) $$\frac{\text{Units G6Pase}}{\text{g liver}} = OD \times \frac{\text{ug P Standard}}{\text{OD unit}} \times \frac{1500}{10 \text{ min} \times \frac{30.975 \text{ µg}}{\text{um P}}}$$

3) $$\frac{\text{Units G6Pase}}{\text{g protein}} = \frac{\text{units G6Pase/g liver}}{\text{g protein/g liver}}$$

4) $$\frac{\text{Units G6Pase}}{\text{liver}} = \frac{\text{Units G6Pase}}{\text{g liver}} \times \text{total g liver}$$

5) $$\frac{\text{Units G6Pase}}{\text{100 g body weight}} = \frac{\text{units G6Pase/g liver}}{\text{body weight (g)}} \times 100$$

APPENDIX F. CALCULATION OF FRUCTOSE BISPHOSPHATASE ACTIVITY

The activity of fructose bisphosphatase in liver was calculated as follows:

1) $$OD = OD_{sample} - OD_{reagent\ blank} - OD_{enzyme\ blank}$$

2) $$\frac{\text{Units FDPase}}{\text{g liver}} = OD \times \frac{\frac{\mu g\ P\ Standard}{OD\ unit}}{} \times \frac{1875}{15\ min \times \frac{30.975\ \mu g}{um\ P}}$$

Units/g protein, units/liver and units/100 g body weight were calculated in a manner similar to that for G6Pase.

APPENDIX G. CALCULATION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY

The activity of phosphoenolpyruvate carboxykinase in liver was carried out as follows:

1) $$\frac{\text{Units}}{\text{g } HgCl_2 \text{ treated sample}} =$$

$$OD \times \frac{\mu g\ P \text{ Standard}}{OD \text{ unit}} \times \frac{3000}{10 \text{ min} \times \frac{30.975\ \mu g}{um\ P}}$$

$$(OD = OD_{sample} - OD_{reagent\ blank} - OD_{enzyme\ blank})$$

2) $$\frac{\text{Units}}{\text{untreated sample}} =$$

$$OD \times \frac{\mu g\ P \text{ Standard}}{OD \text{ unit}} \times \frac{2500}{10 \text{ min} \times \frac{30.975\ \mu g}{um\ P}}$$

$$(OD = OD_{sample} - OD_{reagent\ blank} - OD_{enzyme\ blank})$$

3) $$\frac{\text{Units PEPCK}}{\text{g liver}} = (1) - (2)$$

The calculations of units PEPCK/g protein, units/liver, and units/100 g body weight were carried out as for G6Pase.

APPENDIX H. EXPERIMENT I: EFFECT OF MAGNESIUM DEFICIENCY ON INDIVIDUAL BLOOD GLUCOSE VALUES

Rat No.	Day	Diet	Blood glucose mg/dl
14	1	C-F	145.3
18	1	C-F	164.6
40	2	C-F	125.8
11	2	C-F	215.8
32	3	C-F	143.4
25	3	C-F	143.4
39	1	C-S	69.1
30	1	C-S	44.7
21	2	C-S	81.7
44	2	C-S	69.4
17	3	C-S	52.4
27	3	C-S	55.1
23	1	MD-F	116.9
7	1	MD-F	123.0
38	2	MD-F	200.6
22	2	MD-F	143.5
13	3	MD-F	136.9
34	3	MD-F	104.8
5	1	MD-S	70.1
20	1	MD-S	82.3
6	2	MD-S	84.6
45	2	MD-S	67.5
35	3	MD-S	60.7
11	3	MD-S	92.8

APPENDIX I. EXPERIMENT II: EFFECT OF MAGNESIUM DEFICIENCY AND FOOD RESTRICTION ON INDIVIDUAL BLOOD GLUCOSE VALUES

Day 1			Day 2		
Rat No.	Diet	Blood Glucose mg/dl	Rat No.	Diet	Blood Glucose mg/dl
44	C	89.8	17	C	107.6
12	C	65.0	39	C	86.7
34	C	93.5	24	C	72.6
41	C	70.8	30	C	72.6
37	C	107.6	27	C	63.1
47	MD	196.0	19	MD	86.4
11	MD	99.2	8	MD	117.7
6	MD	117.6	36	MD	106.4
5	MD	123.9	38	MD	86.3
60	MD	114.4	40	MD	86.7
42	PF	95.4	13	PF	65.1
46	PF	93.5	28	PF	58.5
1	PF	110.6	18	PF	95.1
4	PF	76.5	26	PF	90.9
29	PF	67.0	31	PF	76.4

APPENDIX J. EXPERIMENT III: EFFECT OF MAGNESIUM DEFICIENCY AND FOOD RESTRICTION ON INDIVIDUAL BLOOD GLUCOSE VALUES

Day 1			Day 2		
Rat No.	Diet	Blood Glucose mg/dl	Rat No.	Diet	Blood Glucose mg/dl
3	C	83.8	49	C	65.5
6	C	125.8	20	C	116.6
17	C	73.4	57	C	51.7
22	C	89.1	34	C	55.2
48	C	83.8	15	C	116.6
43	MD	112.5	52	MD	65.5
24	MD	99.0	1	MD	75.9
51	MD	81.0	13	MD	89.6
58	MD	72.0	39	MD	89.6
25	MD	54.0	21	MD	93.1
36	PF	94.5	16	PF	58.6
4	PF	103.5	28	PF	75.9
30	PF	90.0	2	PF	113.6
29	PF	49.5	42	PF	86.2
47	PF	45.0	31	PF	48.3

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THE EFFECTS OF MAGNESIUM DEFICIENCY ON THE GLUCONEOGENIC ENZYMES WITH EMPHASIS ON THE EFFECTS OF FASTING AND ANOREXIA

by

Deborah Anne McNeill

(ABSTRACT)

Three experiments were carried out to explore the effects of magnesium deficiency on the activities of the hepatic gluconeogenic enzymes. In Experiment I rats were fed, ad libitum, diets adequate (control) or deficient in magnesium for 12 days. One half of the rats from each treatment group were then fasted for 24 hours. The remaining rats were allowed to eat. The rats were subsequently sacrificed and the following parameters were measured: blood glucose and plasma magnesium, liver magnesium and protein, and the activities of liver glucose-6-phosphatase (G6Pase), fructose 1,6-bisphosphatase (FDPase), and phosphoenolpyruvate carboxykinase (PEPCK). In Experiments II and III rats were meal-fed diets adequate or deficient in magnesium; in addition, a group of rats were pair-fed to the magnesium deficient group to test for the effects of anorexia. After 17 days the rats were fasted for 20 hours then sacrificed. The parameters measured in Experiment I were again assessed except liver FDPase and G6Pase were not measured in Experiment III.

Feeding a diet deficient in magnesium to the rat produced symptoms characteristic of the deficient state. These symptoms included hyperemia, skin lesions, anorexia, decreased weight gain, and decreased plasma magnesium levels. Anorexia ccounted for part, but not all of the decreased weight gain. The concentration of magnesium in the liver of the magnesium deficient rat was unchanged relative to control values. In the fasted rat, relative to the fed rat, the activities of liver glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) were increased while that of FDPase was decreased. The response to fasting was similar in magnesium deficient and control rats. In the magnesium deficient rat, relative to the control rat, the activities of G6Pase and FDPase were unchanged, while that of PEPCK was increased. Anorexia was not responsible for the changes in the activity of PEPCK. Since magnesium was not lost from the liver in magnesium deficiency a direct action of this cation on the activity of PEPCK appears untenable. Magnesium is involved in the secretion of insulin, glucagon, epinephrine and corticosterone. These hormones all affect the gluconeogenic enzymes. A change in the circulating level of one or more of these hormones may be responsible for the effects of magnesium depletion on PEPCK.