THE EFFECTS OF MAGNESIUM DEFICIENCY IN THE RAT

A Thesis

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by

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Magnesium deficiency has been found to be more prevalent in man than previously believed, and may, in fact, occur commonly (77,91). Recent research relating to the role of magnesium in metabolism leads Duncan to remark, in regard to the long-term results of magnesium deficiency in metabolism, "that the important part played by this ion in intracellular energy interchange suggests that further research will reveal magnesium depletion to be implicated in certain degenerative conditions" (19). More specifically, Seelig suggests that chronic low-level deficiency states may be an important factor in the pathogenesis of chronic diseases of the cardiovascular, renal, and neuromuscular systems (77).

Magnesium in Metabolism

Magnesium makes up 2.1 percent of the earth's crust, mostly as the insoluble $MgCO_3$ (magnesite) and $MgCO_3$ - $CaCO_3$ (Dolomite). Magnesium is an essential element in both plants and animals, and, according to McIntyre, probably was incorporated into protoplasm when one-celled organisms first evolved in the pre-Cambrian period (54). The cellular concentrations of magnesium and potassium are similar in all animals (about 20 meq/L and 100 meq/L, respectively) and these concentrations are believed to be unchanged from that in the

pre-Cambrian sea. After the time that cellular life evolved, the ionic content of sea water gradually changed, according to McIntyre, and cellular organisms developed an active potassium uptake and sodium extrusion and perhaps an active magnesium transport as well, in order to maintain homeostasis.

The central role of magnesium in metabolism was noted in 1891 by Loew, who reported its importance in phosphate metabolism in plants (51). Later, it was found that magnesium, with phosphate, is concentrated in the seeds of plants and its presence was associated with the synthesis of nucleo-proteins (24). In 1927, Erdtmann reported that mammalian kidney phosphatase was activated by magnesium ion, and other phosphatases were subsequently found to be activated by magnesium also (20). Later, magnesium was discovered to be necessary for the activation of all enzymes transferring phosphate from adenosine triphosphate (ATP), or to adenosine diphosphate (ADP), and is necessary for the oxidative phosphorylation of mitochondria (16). Since ATP supplies the energy for contraction of muscle, synthesis of protein, fat, nucleic acids, and co-enzymes, metabolism of glucose, storage of catecholamines, activation of acetate, sulfate, and other essential functions, this effect on the ATP metabolism is an especially vital role for magnesium.

According to some authorities, almost all cells have the ability of glycolysis because of their origin at a time when the earth's atmosphere contained little oxygen, and anaerobic energy-yielding reactions were essential to life (54). Many of these reactions are

activated by magnesium, - such as those requiring enolase, hexokinase, phosphoglucomutase, 6-phospho-fructose kinase, phosphoglycerate kinase, and pyruvate phosphokinase. Enolase requires magnesium in fairly high concentration, and occurs naturally as a magnesium complex.

Some other enzymes activated by magnesium are the pyrophosphatases, some peptidases, yeast carboxylase, decarboxylases and enzymes involving diphosphothiamine and the pyruvic acid oxidase system of the brain. As Harrison remarks, "Magnesium is implicated in a larger and more diverse number of biochemical reactions than almost any other metal. Despite the importance of magnesium, no more is known about it today than is known about the trace elements" (32).

The body contains about 2000 meq of magnesium (about 24 grams) of which 1 percent (20 meq) is in the extracellular fluid, about 50 percent is held in bone, and the remainder is intracellular, concentrated in the mitochondria and the nucleus where it is likely to be the most essential element in enzyme activity (54,1,16). Magnesium (and strontium and beryllium as well) can replace calcium in the crystal lattice of bone, and may be available for homeostasis in the face of magnesium deficiency in young animals.

Magnesium does not appear to cross the cell membrane freely, since there is a considerable concentration gradient between the cell and the extracellular fluid. The concentration varies from 6 meq/L in red blood cells and 13 meq/L in kidney to 20 meq/L in striated muscle and liver, as compared to 1.5 to 2.0 meq/L for serum. Brain contains

17 meq/L as compared to 2.4 to 3.0 meq/L for cerebrospinal fluid. There are also concentration gradients for magnesium between the mitochondria and nucleus, and the cytoplasm, as has been shown by Mg^{28} turnover studies (18,71,81). As a consequence, cell concentrations change slowly despite fluctuating levels of serum magnesium. A normal serum magnesium concentration may be found with an erythrocytic level 40 to 50 percent below normal (79). A study with Mg^{28} indicates that magnesium uptake is still not complete 60 hours after intravenous administration (81,59). Magnesium turnover is fast in cardiac muscle, kidney, and liver, slow in skeletal muscle (unless exercised) and even slower in other tissues (71). In acute deficiency states, symptoms may not be relieved for 3 to 6 hours after administration of magnesium, and several days may be required for complete remission of symptoms (21,67,79,91).

Like calcium, a portion of magnesium is protein-bound in the plasma. The degree of binding is dependent on thyroxine -- 45 percent normally, but this may be as little as 0 percent in myxedema, or greater than 45 percent in hyperthryoidism (93). The remainder of the plasma magnesium is present as the ion or as a complex (as a hydrate, or absorbed on the surface of colloidal fats) (65). Aikawa has noted the unique complexing ability of magnesium, and suggests that this property which has made the Grignard reagent so useful in organic chemistry syntheses may also be responsible for the integral role of magnesium in plant and animal metabolism (1).

The regulation of plasma levels is not well understood. The average daily intake of magnesium varies from 300 mg (adult human) to 500 mg, according to dietary habits. Magnesium is absorbed in the proximal part of the small intestine, perhaps by the same transport mechanism as for calcium (54). This fact may be responsible for the decreased absorption of magnesium in diets containing ample amounts of calcium (54). A similar common transport mechanism has been postulated for the tubular reabsorption of calcium and magnesium in the kidney (54).

In connection with intestinal absorption, Tidball has found that while either magnesium or calcium are essential for normal intestinal selective permeability, magnesium is the more important of the two (89). When both ions are removed from the lumen of the gut, the mucosa becomes about ten times more permeable than normal, and normal selectivity is best restored by a solution of magnesium.

Intestinal absorption and tubular reabsorption of magnesium are increased in magnesium depletion states (16,17). Urinary excretion in man may drop from 5 to 15 meq/day to as little as 1 to 3 meq (54). On the other hand, the urinary clearance of magnesium approaches the rate for inulin when serum levels are elevated. Parathormone is known experimentally to cause increased urinary excretion of magnesium before affecting plasma calcium levels, but only for periods less than 3 days (1). In hyper-parathyrodism, however, magnesium is retained, as it is also in Addison's disease (95). In primary aldosteronism there is hypomagnesemia, due to increased

urinary loss, and hypomagnesemic convulsions may be produced by injections of aldosterone (75). Growth hormone also causes increased urinary excretion, but it also increases intestinal absorption.

Intestinal absorption is decreased by excessive amounts of dietary phosphate, phytate, calcium, and fat (77). Care and Ross have shown that the high protein content in the diet will also inhibit magnesium absorption (12). This was demonstrated by measuring the quantity of Mg^{28} absorbed from the gut in sheep receiving an adequate amount of dietary magnesium but on a "lush", high-protein pasture. These sheep were compared with a group fed on hay. Vitamin D is thought to increase the urinary excretion of magnesium through its effect on citrate metabolism. Citrate combines with magnesium and is excreted as a "citrate-chelate" (54). However, Richardson and Wells who have produced hypomagnesemia in the rat with injections of Vitamin D, report that Vitamin D causes magnesium to be deposited in the bone, reducing the serum level of magnesium (68). Neal and Neal have suggested an explanation for the apparent increase in magnesium requirements when dietary fat is increased. Magnesium ions in the plasma may be absorbed to the surface of colloidal fats and thus serve to keep those particles in a stable suspension. The fat in the sera of rabbits on an atherogenic diet has been reported to be turbid and to separate on standing, while the sera of comparable rabbits receiving ample dietary magnesium will remain clear and in a stable suspension despite comparable amounts of lipid in both (65).

Magnesium levels are of special interest in connection with smooth muscle. Increased magnesium levels result in relaxation and a decrease in responsiveness of vascular smooth muscle, and decreased levels of magnesium result in an enhanced response (30,31). This fact, coupled with the finding of decreased serum magnesium concentrations in some hypertensive patients, has led to the speculation that hypomagnesemia may play a role in the increased peripheral resistance in hypertensives (2,18,78). The administration of magnesium does, in fact, cause the blood pressure to fall, and magnesium ion has been shown to decrease the coronary vascular resistance (calcium ion, on the other hand, causes an increase in resistance) (76). In uterine smooth muscle, magnesium depressed spontaneous activity and the effect of histamine, but potentiated the effect of posterior pituitary hormones (22). In this connection, it has been noted that estrogen treatment increases the magnesium content of the uterus. These relationships may aid in the explanation of the value of magnesium salts in eclampsia.

Cardiac muscle, which has a rapid turnover of magnesium, also exhibits decreased responsiveness in the presence of increased plasma levels of magnesium. Ventricular arrhythmias, due to digitalis intoxication, can be reversed by the administration of magnesium salts (84). Presumably, decreased plasma levels of magnesium may increase cardiac irritability along with the direct effect on myocardial mitochondria (28).

The neuromuscular action of magnesium given intravenously was first studied by Jolyet and Calhoun in 1869, who reported a curare-like peripheral muscular paralysis in frogs and in dogs (16). In 1906 Meltzer and Aver discovered the depressant effect of magnesium on the central nervous system, and this was later used for local and general anesthesia. Magnesium apparently has two sites of action in the central nervous system, - a peripheral effect, which may be abolished by calcium or by cholinesterase inhibitors (neostigmine) and a more central effect which may be counteracted by analeptics (16). An increase in the concentration of the magnesium ion results in decreased release of acetylcholine at the neuromuscular junction (95).

Increased levels of magnesium are similar clinically to hyperkalemia, although the plasma levels must rise to ten times higher than normal (27-44 meq/L) to produce toxic effects. The heart slows, the blood pressure falls, the P-R interval increases and the T-waves becomes higher, the QRS complex lengthens, the deep tendon reflexes disappear, respiration is depressed and finally, the heart stops in diastole (54).

Hypomagnesemia, on the other hand, is in some respects similar to hypocalcemia. Tetany and convulsions occur in either case. However, tubocurarine will inhibit these effects of hypocalcemia, while increasing the effects of hypomagnesemia (54,28). The actions of the two ions as described by Wacker and Vallee (95) may be summarized as follows.

Effect of Hypocalcemia and Hypomagnesemia on the Neuromuscular System

	Decreased Mg^{++}	Decreased Ca^{++}
Sympathetic Ganglia: Acetylcholine release	Increased	
Nerve Threshold	Decreased	Decreased
Nerve Impulse Transmission	Increased	Increased
Myoneural Junction:		
Amount acetylcholine released	Increased	Decreased
Threhold of muscle membrane (or excitation-contraction coupling)	Decreased	Decreased
Muscle Contraction	Enhanced	Depressed

The tetany of hypomagnesemia seems to differ from that of hypocalcemia in that there is an increase in the amount of acetylcholine released at the myoneural junction and enhanced muscular response to stimuli. Greenberg and Tufts, however, offered evidence that the "tetanic convulsions" were due to effects on the midbrain or pons (28).

It may be seen from this brief review that magnesium is vitally concerned with all phases of cellular metabolism, and that almost all energy exchange in the cell is magnesium-dependent. Presumably, as magnesium concentrations fall in deficiency states, there is a decline in the rate of reaction of the multitude of vital processes essential for continued cellular activity and growth. Perhaps, at the cellular level, the magnesium dependent reactions are retarded in a manner described by the Michaelis-Menten equation (as expressed in terms of concentration of substrate or co-factor). Certainly there is a generalized deleterious effect. The specific effects of magnesium

deficiency in man have aroused little interest until the last ten to fifteen years, and the effects in experimental animals have not yet been fully investigated.

Magnesium Deficiency States

Magnesium deficiency states are much more easily recognized in plants, and were first described by Garner et al. in 1923 (23). Plants growing in light soils, acid soils, or sandy soils are more likely to be magnesium deficient, resulting in stunted growth and "chlorosis". Magnesium is withdrawn from older parts of the plant to supply the growing parts, causing a loss of chlorophyll and a fading of color proceeding from the lower leaves towards the top of the plant (24). Plants may have as little as one-half the normal concentration of magnesium. Deficiency may also occur in soils containing adequate amounts of magnesium if the pH is too low, since roots are unable to absorb magnesium below a pH of 5.4. Furthermore, magnesium in the soil will not be adequately absorbed if the soil potassium-magnesium ratio is more than 4:1, or if the soil is fertilized with inorganic nitrogen, since this results in an elevated soil ammonium level which antagonizes magnesium absorption (41,87). Magnesium-poor soils have been found along the east coast from Maine to South Carolina, and also in Illinois and parts of Ohio, and have caused crop failures and diminished yields (24,41). Acid soils are widely distributed, and, of course, inorganic nitrogen fertilizers are used commonly.

In 1926, Leroy first proved magnesium to be essential in mammalian nutrition by producing an arrest of growth and death in young mice with a low magnesium diet (49). Although McCandlish in 1923 reported growth failure, skin lesions, and neuromuscular irritability leading to convulsions and death in calves on a diet of whole milk, this was not recognized as acute magnesium deficiency until 1935 (53). As many as 5 percent of calves develop this deficiency on farms where calves are fed solely on milk. This type of hypomagnesemia has been shown to occur earlier and to be more severe if calcium supplements are given (54).

A second type of magnesium deficiency was recognized in 1929 by Sjollema and Seekles (82). Cattle turned out on fresh pasture in the spring may become irritable, confused, tremorous, and die in convulsions in a short period of time. The high protein content (30 percent) of fresh pasture apparently induced a state of acute magnesium deficiency in these animals, despite the fact that the forage contained normal amounts of magnesium (16). The high protein content of the "lush" forage seems to increase the magnesium requirement. The bone magnesium content of the cattle affected was normal (0.6 - 0.7%), demonstrating that this store of magnesium was not readily available to mature animals in the presence of acute magnesium depletion. This has been shown recently to occur in sheep also (12).

As early as 1932 Hirschfelder showed that patients with chronic nephritis who had symptoms of twitching and convulsions had a low serum magnesium. In 1944 a child with tetany due to acute magnesium

deficiency was successfully treated with $MgSO_4$ by Miller (38,62). In this interesting case, the child was born with "tetany of the newborn" which did not respond to administration of calcium. Attacks of tremors, muscle cramps, dizziness, and tonic-clonic convulsions occurred at intervals until the patient was seven. Although the serum magnesium was 1.7 mg percent, administration of $MgSO_4$ (0.3 grams) three times daily gave "striking results". When administration was stopped, the tremor and cramps recurred and the serum magnesium fell to 0.6 mg percent. Oral magnesium sulfate (0.3 grams t.i.d.) again gave complete relief, bringing the serum magnesium to 2.6 mg percent. This patient also had an enlarged thymus. More recently, hypomagnesemia in man has been associated with acute pancreatitis, diabetic acidosis and insulin therapy, alcoholism, cirrhosis of the liver, prolonged intravenous therapy and naso-gastric suction, hyperaldosteronism, persistent vomiting or diarrhea, intestinal malabsorption and with other conditions as well (67,91,95).

In 1960, Vallee described magnesium deficiency tetany, a syndrome almost exactly like that in animals, as a specific clinical entity in man (91). The five patients studied had both a dietary inadequacy and a clinical condition resulting in loss of body fluid or in impaired gastro-intestinal absorption. No one has, as yet, given conclusive evidence of acute magnesium deficiency in an otherwise healthy individual, although Seelig and others present evidence, on the basis of a number of magnesium balance studies, that chronic deficiency is rather common (77).

Randall et al. described typical effects on behavior and the peripheral nervous system in acute magnesium deficiency in man (67). The affected patients became "anxious, apprehensive, and confused, and may have had hallucinations." Delirium, delusions and wild combative behavior were reported in some patients. The mental changes preceeded the neuromuscular changes by four to six days in eight out of twelve patients studied by Randall. The neuromuscular changes consisted of tremors, athetoid movements, and convulsions. Four of the twelve patients in this study showed only mild confusion and disorientation in addition to the neuromuscular changes. The signs disappeared in all cases within 12 hours after administration of magnesium salts. In two recent studies, experimentally induced magnesium deficiency in man resulted in the usual tremors, fasciculations, and other neuromuscular changes (79). One patient also developed a persistent adynamic ileus; the other patient became confused, vomited and developed urinary incontinence. The latter had the usual Trousseau and Chvostek signs. An electromyogram showed numerous myopathic-like potentials, intermittent tremor at rest and with contraction, a nd polyphasic potentials at rest. Interestingly, all changes were relieved by $MgSO_4$, except for the myopathic potentials in the proximal muscles. One patient who exhibited a pre-tibial and pedal edema with a transient recurrent purpura showed fragmentation of collagen fibers and acute perivasculitis on biopsy. In another study by Barnes, a patient with the usual clinical signs (including

recurrent tetany, hyper-reflexia, cramps, tingling sensations, and muscular incoordination) had a serum magnesium as low as 0.4 meq/L. Studies like these implicate acute magnesium deficiency states with a number of clinical disorders.

In a prolonged study by Shils, the erythrocytic magnesium level dropped to 3.0 meq/L in the second month, and finally to 2.76 meq/L. After magnesium was added to the diet, three weeks were required for the intracellular content of magnesium to return to normal. The cerebrospinal fluid (normally 2.4 to 3.6 meq/L) was found to be 1.22 meq/L in one patient who had become confused and vomited (79).

Serum calcium levels in this study fell in the second month, despite adequate levels in the diet. Serum potassium gradually fell to 2.3 meq/L, despite an adequate intake. The same phenomenon has been noted for intracellular levels of potassium and calcium.

Magnesium deficiency has been studied in greater detail in the albino rat. The signs and lesion found in this animal are comparable to those found in guinea pigs, rabbits, dogs, cattle and man. In 1932, Kruse et al. induced magnesium deficiency in rats with a diet containing 1 ppm of magnesium (vs. 280 ppm in a commercial rat diet) and noted a peripheral vasodilatation and erythema appearing at 3 to 5 days, followed by increasing neuromuscular irritability, trophic skin lesions, clonicotonic convulsions and death (47). Greenberg et al. later made a more detailed study (27). A diet containing 4 ppm magnesium produced the vascular signs in 7 to 9 days, and death in

21 to 30 days. A diet with 40 ppm resulted in diarrhea and melena in 10 days, followed by hyperemia, hair loss, hyperpnea, and "nervousness". Serum magnesium levels fell to one-half normal in 14 days.

At necropsy, areas of calcification were found in the kidney. The bones were brittle with a decrease in magnesium content of up to two-thirds. The alveolar bone had atrophied and the teeth were white or transparent with a fall in its magnesium content to one-half (22). Ko et al. and others have described the changes in the teeth more specifically (45). Greenberg et al. also found that a slight increase in calcium (from 0.87 to 1.16%) in the diet produced more severe skin changes, and a higher percentage of rats had convulsions. A diet with 130 ppm magnesium did not sustain pregnant rats (27). The litters were either resorbed in utero or eaten after birth. In chronic deficiency, rats developed alopecia, skin lesions, hematomas of the ears and swollen, hyperemic gums.

Vitale et al. demonstrated that 240 ppm magnesium in the diet is inadequate if the diet contains added cholesterol (93). Serum magnesium fell to low levels and a decreased efficiency of oxidative phosphorylation of the mitochondria occurred as early as four days (94). The symptoms of magnesium deficiency which appeared were relieved by increasing dietary magnesium to 1000 to 2000 ppm (about 50 to 100 mg/kg body weight). These higher levels not only prevented the symptoms of magnesium deficiency, but also prevented the development of lesions by the atherogenic diet. Lipid deposits were found in the inner half of the media of the aorta and in the heart valves

in rats receiving "normal" levels of dietary magnesium, and typical lesions and calcium deposits were found in the zona intermedia of the kidney. Similar findings have been shown in rabbits (65). According to Vitale, hypercholesterolemia increased markedly the requirements for magnesium. Vitale and Hellerstein et al. also demonstrated that thyroxine increased the magnesium requirement, while counteracting some of the effects of the magnesium deficiency state that it produced. Increased protein levels had a sparing effect on the renal lesions of magnesium deficiency in this study, although Comar has noted that a high protein intake increased the magnesium requirements (16).

The biochemical and histologic changes of magnesium deficiency are now being studied more extensively. In a study by Ko et al., while serum magnesium fell to a low value (perhaps one-third normal) after one week, it did not continue to fall. Serum phosphorus and protein levels dropped after the second week, but then stabilized. There was a gradual rise in non-protein nitrogen (45). This is due in part to the kidney lesions and perhaps also to less effective glutamine synthesis, since this synthesis requires magnesium. Bois has reported a sharp rise in serum and urinary histamine levels on the fourth day, with a peak at 10 days at which time the blood levels were four times as high as in the control group and urine levels ten times as high (10).

Skin changes consisted of edema, capillary dilatation, endothelial swelling and proliferation, an increase in histiocytes and

eosinophils, and an irregular distribution of areas of hyperkeratosis and mild acanthosis. Ko et al. reported that there was an involvement of connective tissue in magnesium deficiency (45). Others reported a definite arteritis or perivasculitis (27,96).

Diarrhea and melena (occurring in the second week of a low magnesium diet) were found to be associated with lesions in the large intestine, and to a lesser extent the small intestine (34). There was an increase in the number of cells with crowding in the basal areas of the mucosa with enlarged and more vesicular nuclei, increased numbers of mitotic figures, decreased mucus content of mucous cells (other than goblet cells) and a more stringy and uneven staining mucus in the goblet cells.

Mitochondrial swelling occurred in the kidney tubules after one week on a low magnesium diet, apparently followed by mitochondrial distortion and loss, and progressing to nephrocalcinosis (45). In this study by Ko et al., calcium phosphate casts were found at the corticomedullary junction after two weeks. These investigators suggested that since low concentrations of magnesium stabilize calcium phosphate solutions and prevent precipitation, this calcification may be related directly to magnesium deficiency. The calcium content of the kidney may rise to as high as fifteen times the normal level. These casts were in the terminal part of the descending loop of Henle. A decrease of enzymatic activity of the alkaline phosphatase, acid phosphatase and succinic dehydrogenase systems was noted in the involved tubules. A decrease in activity of the diphosphopyridine-

nucleotide (DPN), diaphorase, and succinic dehydrogenase systems in focal lengths of the first part of the proximal tubule in the subcapsular region was noted. Rounded droplets were found in areas where the oxidative enzymes were less active.

After a third week, these changes were more severe, with a larger decrease in enzyme activity and occasional foreign body giant cells around some of the casts. In the involved subcapsular areas, the proximal convoluted tubules had a flattened, basophilic epithelium, with loss of much of the mitochondria and enzyme activity, especially the alkaline phosphatase and the non-specific esterase systems (45). Glomerular degeneration and periglomerular and peritubular fibrosis have also been reported (16).

The liver changes which have been observed consist of prominent lobulation, periportal necrosis with round cell infiltrations, and a homogenous, hyaline-like degeneration of liver cells followed by fibrosis (16). Hypomagnesemia is associated with some cases of cirrhosis of the liver in man, but there is as yet no evidence to show which condition preceeded the other (91).

Although the muscular changes taking place in magnesium deficiency are well described, lesions in the central nervous system have not been well characterized. Clough reports extensive lesions in the cerebellum of the rat with degeneration of the Purkenje cells, - chromatolysis, vacuolations, swelling and a decrease in Nissl staining (15). This change is reported to be the most important lesion of magnesium deficiency in the chick (16). In connection with these

findings, Greenberg and Tufts have produced evidence that the convulsions of hypomagnesemia are due to the effect on the midbrain or pons. No lesions of peripheral nerves have been described (28).

Skeletal muscle changes have been studied by several groups of workers. The magnesium content of muscle declined at a rate of about 2 percent per week on a low magnesium diet accompanied by a loss of potassium (despite adequate serum potassium levels) (54). A progressive muscle weakness in rats, dogs and in human subjects has been reported (77,96). As early as seven days after rats were placed on a low magnesium diet, Heggveit observed swelling of scattered muscle fibers with hyaline degeneration, loss of striations, and vacuolization (35). These lesions then progressed to granular and floccular degeneration and were most severe at 21 days. Nuclear changes occurred after extensive injury to the sarcoplasm. The degenerating myofibers were surrounded by neutrophils, macrophages and some eosinophils. There was little sign of fibrosis or healing. A second type of lesion, apparently due to a progressive deposition of calcium in the muscle fiber was often observed. With the calcification was seen a swelling of the nuclei of the sarcolemma, with an apparent increase in number. These changes are thought to be related to an uncoupling of oxidative phosphorylation by magnesium-dependent enzyme systems, as well as to a concurrent defect in potassium and calcium metabolism (35).

Although one investigator has been unable to demonstrate vascular lesions in magnesium deficiency and has reported only minor

myocardial changes (45), others have reported marked changes in the cardiovascular system (17,16,1). The vascular lesions in rats consisted of perivascular, necrotic foci after one to two weeks, widely distributed throughout the body (16,27). In dogs, the degenerative vascular changes in the small arteries and arterioles were characterized by pyknosis or loss of nuclei in the intimal cells, deposition of a pink-staining, homogenous material in the media with degeneration of smooth muscle cells, complete necrosis of parts of the vessel wall, and "disintegration" of some arteries with extravasation (96). Less severe lesions were seen in the medium-sized arteries. Calcification occurred in the elastica and media of the aorta, coronary and peripheral arteries, and in the endocardium. The myocardium showed areas of necrosis with a patchy distribution of older fibrous lesions. Similar lesions occurred in the myocardium of the rat, with round cell infiltrations and often with calcifications (34). Cold stress increased the severity of the lesions in deficient rats, and the areas of necrosis even extended through the wall of the ventricle (34).

Heggtviet has shown that the earliest lesion was the swelling and distortion of the internal fine structure of the sarcosomes with vacuoles distorting the cristae (35). The swollen and vacuolated sarcosomes accumulated in the cytoplasm of the muscle cells in areas where myofibrils had been lost, and also in a paranuclear position. Clumping of the cristae occurred, with deposition of a particulate dense material (calcium) which eventually filled some sarcosomes.

The calcium content of the heart rose to as much as 60 percent above normal in magnesium deficiency. The fragmentation and loss of myofibrils allowed the sarcoplasmic reticulum and glycogen particles to appear more prominent. Macrophages lined the sarcolemma of degenerating muscle cells. The chromatin of the nuclei at this stage was coarsely clumped, nucleoli disappeared, vesiculation occurred, and rupture of the nuclear membrane followed. Increased numbers of mitotic figures and swelling of the endothelial cells were also observed (35).

No changes of magnesium deficiency per se have been reported for the adrenal and other endocrine glands, for salivary glands, pancreas, spleen, bone-marrow or lungs. It is possible that these organs may be affected if a generalized involvement of the small arteries occurs. Further study of these organs may disclose specific lesions in deficiency states.

The lesions of low grade magnesium deficiency over long periods of time have not been studied in detail in experimental animals until recently (10). While skin lesions of severe magnesium deficiency in man have been studied by biopsy (79), there has not yet been a thorough study of the lesions produced by either acute or chronic magnesium deficiency in man. Seelig and others believe that severe chronic degenerative diseases of the cardiovascular system, the kidneys, and liver may in fact represent lesions of long-term, long-grade magnesium deficiency. Epidemiological studies, studies with experimental animals and medical case reports all suggest that magnesium deficiency

may be a factor in some cases of essential hypertension, chronic renal disease, and arteriosclerosis (including coronary heart disease) (77). More extensive studies of chronic magnesium deficiency in animals would be very rewarding.

Recently, Bois reported two cases of tumors of the thymus in rats that had been maintained in a state of chronic magnesium deficiency (10). In one case, the normal structure of the gland was replaced by sheets of large lymphoid cells; in another, thymic tissue extended in all directions, infiltrating the base of the heart and wall of the esophagus. The second tumor was composed of large lymphocytes or lymphoblasts with numerous mitotic figures, and the liver, kidney, and adrenal cortex were infiltrated with similar cells. Part of this tumor was transplanted subcutaneously into Sprague-Dawley rats, and killed the new hosts in 20 to 25 days. This tumor resembled lymphosarcoma of the thymus in man (4,70,97). Tumors of the thymus are quite rare in the rat, and apparently occur only in chronic magnesium deficiency, or in rats that have been inoculated with mouse leukemia virus immediately after birth (29). The type occurring in magnesium deficiency is quite similar to that induced by mouse leukemia virus in the new-born rat.

In addition to the tumors of the thymus as described by Bois, Jasmin describes a case of "lymphoblastic adenoma" of the thymus in a small number of chronically magnesium-deficient rats. Jasmin and Bois suggest that in the face of prolonged magnesium deficiency, magnesium may be mobilized from the nucleus, resulting in chromosomal

aberrations and cell mutation (10). Why the thumus should be the site of neoplastic degeneration is a subject for speculation. As early as 1934, Mendel commented on a possible association between magnesium deficiency and thymic tumors (58). At that time he also advanced the idea that the thymus had something to do with magnesium metabolism. There have been various other reports cited by Pleshchitser of an association between magnesium deficiency and an increase in incidence of neoplasms, and some investigators have noted that neoplastic growths have a lower magnesium concentration than normal tissue (66).

Thymoma in humans is associated with symptoms of myasthenia gravis in 75 percent of all cases. This is a syndrome consisting of symptoms of muscular weakness and fatiguability, with "lymphorrhages" or perivascular infiltrations in skeletal muscle. There apparently is an interference with the transmission of impulses across the myoneural junction (25). More recently, Zacks suggests that some factor interferes with the synthesis or the release of acetylcholine by the pre-synaptic nerve terminal, leading eventually to a type of disuse atrophy of the motor end-plate (98). The weakness is temporarily relieved by prostigmine and may be simulated by administration of curare (25). Animals with chronic magnesium deficiency (and thymoma) have similar muscular weakness and myopathy. One may speculate that either a defect in magnesium metabolism or low-grade magnesium deficiency may cause both conditions as Mendel suggested in 1934 (58).

As illustrated by the preceeding account of the widespread lesions and changes produced by magnesium deficiency in the rat and other species, almost all metabolic processes must be impaired in one way or another. This impairment probably reflects a decrease in the rate of reaction of enzymes dependent on magnesium, such as the ATP:Mg complex. Since magnesium is required specifically in all reactions transferring phosphate to ADP or from ATP, it seems reasonable to suspect that a decrease in intracellular magnesium in deficiency states would be accompanied by a rapid decrease in the concentrations of ATP and consequently by decreased activity of all reactions requiring ATP as a source of energy. ATP is, of course, produced primarily, if not entirely, in the mitochondria. The resulting depletion of ATP and pyrophosphate reserves may be the most important basic defect in magnesium deficiency.

In addition to the depletion of ATP or decreased ability to use pyrophosphate stores, the ionic changes may be a factor in pathogenesis of cardiovascular disease. An elevated calcium:magnesium ratio results in heightened irritability or responsiveness of vascular smooth muscle and cardiac muscle (skeletal muscle as well). Vascular resistance increases as the magnesium level falls (76). Calcium levels, which usually rise as a result of a fall in serum magnesium, are in part responsible for this increase in resistance, since the effect of the two ions on vascular smooth muscle normally balance each other.

In addition to the fall in ATP concentration and an imbalance of Ca:Mg effects on vascular smooth muscle and other tissues, a third metabolic disorder may occur in magnesium deficiency states. The ATP:Mg complex may be intimately associated with the synthesis, storage, or binding of catecholamines in the nerve endings or in amine granules in the adrenal medulla. As Hillarp has demonstrated, catecholamines are found in the adrenal medullary amine granules in a molar ratio with ATP:Mg of about 4:1 (presumably four molecules of catecholamine cations bound to one molecule of ATP having four negative charges) (37,57,86). Perhaps, as ATP levels drop in magnesium deficiency, there is a decrease in the storage capacity for catecholamines in the adrenal medulla, in the nerve endings in the heart, and elsewhere in the cardiovascular system. In addition, magnesium-dependent enzymes such as catechol-o-methyl transferase may be less effective in the inactivation of catecholamines. As a result, blood levels of catecholamines may remain at higher than normal levels for prolonged periods of time, perhaps contributing to the degenerative changes that are found in the cardiovascular and renal systems of animals in magnesium deficiency states.

Statement of Problem

Because of the integral role of magnesium in all processes, the concentration of magnesium is a useful parameter against which to study metabolism. If, in addition, it is true that magnesium deficiency states (subclinical or severe) are of common occurrence, then the role of magnesium in disease and in health deserves even greater interest.

Since there is a paucity of information relating to the clinical and pathological changes of magnesium depletion with specific biochemical changes, it was decided to study the relationship of magnesium deficiency to the levels of ATP and subsequently the content of the catecholamines. Because of the high levels of the catecholamines and the constancy of the molar ratio of ATP/catecholamine in the adrenal medulla as worked out by several previous workers (11,13, 84), it was decided to study the changes in the magnesium, ATP, and catecholamine concentration of the adrenal in rats maintained on a low magnesium diet and distilled water.

Changes in the concentration of magnesium in the serum and in the adrenal, heart, kidney, skeletal muscle, brain, nerves, and thymus were noted, and an attempt made to study the urinary excretion of magnesium in the deficient rats. Levels of ATP and catecholamines in the heart were investigated, since the turnover of magnesium is fairly high in this organ and catecholamines are integrally associated with its function. Since a fall in ATP levels in magnesium deficiency states is the principal hypothesis advanced in the plan for this study, the kidneys, skeletal muscle, and brain was assayed for ATP, in addition to the heart and adrenal.

Throughout this study the clinical signs and changes in growth rate were noted. Necropsies were performed on all animals, and tissues placed in formalin for subsequent histologic preparation and study. Where possible, tissues were prepared for electron microscopy.

In this manner, an attempt was made to correlate the specific biochemical changes with the course of events taking place in the development of the magnesium deficient state, and this hopefully will lead to better insight into the role of magnesium in health and disease.

CHAPTER II

MATERIALS AND METHODS

Experimental Animals

Most of the experiments were carried out with five week old female and male albino Wistar rats (from Harlan Industries, Inc., Cumberland, Indiana) and all weighed between 90 and 110 grams at the start of the experiment, unless otherwise indicated. The rats were held for a minimum period of one week after shipment before being placed on the test diet. All were kept in a constant environment room[1] at a temperature of 24.5° C. and humidity of 40 percent. Unless otherwise indicated, the rats were housed four to each cage, and conditions were made as identical as possible for test and control groups.

Ten rats weighing 220 to 240 grams (ten weeks of age) were utilized in the earlier stages of this study. These rats were housed in the regular animal quarters. Two served as controls, two were sacrificed after two weeks, two after four weeks, and four were used to study chronic effects of magnesium deficiency. For the rest of the investigation, the rats were arranged in experimental groups of two rats each. For part of the work, comparable stock rats were also studied in groups of two.

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[1] Lab-Line-C.S.&E.

The test and control groups received the identical diet in all cases, making the conditions as uniform as possible. A commercial diet for rats low in magnesium but otherwise nutritionally adequate was obtained from General Biochemicals, Chagrin Falls, Ohio, and contained the following ingredients:

Casein	25.00%	Biotin	0.140 g/100 lb
Dextrose	58.00%	B_{12} with Mannitol	2.00 "
Gelatine	5.00%	Vitamin A concentrate (200,000 u/g)	9.08 "
Corn Oil	5.00%		
DL methionine	136.20 g/100 lb	Vitamin D concentrate (400,000 u/g)	0.92 "
Choline Cl.	113.52 "		
Inositol	45.40 "	Alpha Tocopheral	0.92 "
Thiamine HCl	0.272 "	Calcium Carbonate	905.84 "
Riboflavin	0.272 "	Monobasic Potassium Phosphate	981.169 "
Calcium Pantothinate	1.140 "	Monobasic Calcium Phosphate	226.44 "
Nicotinic acid	3.175 "	Sodium Chloride	507.20 "
Pyridixine HCl	3.175 "	Iron Citrate	84.52 "
Folic acid	0.113 "	Potassium Iodide	2.40 "
Zinc Carbonate	0.76 "	Manganous Sulfate	13.08 "
Copper Sulfate	0.92 "		
Menadione	0.227 "		

This diet contained about 4 ppm magnesium. It was well accepted, and was fed ad. libitum.

Both test and control rats were given demineralized double-distilled water to drink. However, 15 grams of $MgCl_2 \cdot 6H_2O$ were added

per liter of water for the control rats. This supplied about 1770 mg of magnesium per liter, or about 20 mg of magnesium per rat per day (or roughly 200 mg of Mg/kg body weight per day). With the drinking water as the source of most of their dietary magnesium, the control rats gained weight normally and were maintained in apparent good health. Body weight and clinical signs were noted at intervals.

The stock rats were given the Purina Laboratory chow diet, which contains 2300 ppm magnesium (roughtly equivalent to 15 to 20 mg of magnesium per rat per day, or about 200 mg/kg of body weight). The stock rats received tap water to drink; this water contained about 0.4 mg percent Mg, or 4 ppm.

Procedures

Collection of specimens

1. Blood was collected in centrifuge tubes after decapitation. The clot was allowed to retract before centrifuging. Specimens were refrigerated prior to assay.

2. Twenty-four hour urines were obtained from some of the rats by means of stainless steel metabolism cages. The rats were housed singly in these cages, and food and water consumption was also observed.

3. Adrenals were taken immediately after collection of blood, weighed, and then one adrenal was placed immediately in a glass-stoppered test tube containing boiling glass-distilled water for

subsequent assay of ATP. The other adrenal was immediately homogenized in cold 0.01 N HCl for catecholamine and magnesium determinations. In some groups, the adrenals were heated in dilute acetic acid to extract the magnesium according to the method of Steinbach (72).

4. Heart, kidney, and skeletal muscle were taken in several groups for ATP and magnesium determinations. A portion weighing between 500 to 1000 mg was weighed and extracted in boiling water as with the adrenals (86,87).

5. Brain, heart, kidney, thymus, nerve, and testes in several groups were weighed and frozen for subsequent magnesium determinations.

Necropsy procedure

The rats were killed by decapitation. After collection of blood and tissue for assay, a necropsy was performed. For selected groups organ weights were determined and sections of all organs were placed in formalin solution for histological preparation and study. Microphotographs were taken of some tissue sections with a Zeiss photomicroscope.

One test rat and one control rat was selected for an electron microscopic study of the adrenal medulla. These animals were killed quickly and the adrenals removed at once and fixed in cold buffered osmium tetroxide containing sucrose.

Magnesium determinations

Magnesium determinations were made with Schacter's method, which is based on the pH-dependent fluorescence of ethanolic solutions of

magnesium 8-hydroxyquinolinate (6,72). Measurements of fluorescence were made with the Turner Fluorometer.[2]

Determination of Magnesium Concentration in Serum and Urine

Demineralized double-distilled water was used throughout. Reagents were analytical reagent grade unless specified. Five grams of 8-hydroxyquinoline (free base) were dissolved in 100 ml of absolute ethanol. This solution was kept in a brown bottle in the refrigerator and was replaced when it became discolored. Two molar acetate buffer was prepared by adding 11.5 ml of gracial acetic acid to 70 ml of water, and the pH adjusted to 3.5±0.2 with about 10 ml of 2 N NaOH. This solution was then diluted to 100 ml with water. The pH 6.5 acetate buffer (also 2 N) was prepared by dissolving 27.2 g of reagent grade sodium acetate ($NaOOC\ CH_3 \cdot 3H_2O$) in 70 ml of water. The pH was adjusted to pH 6.5±0.1 with less than 1.0 ml of glacial acetic acid (72).

"Serum reagents" were prepared fresh daily by mixing two volumes of the appropriate buffer with two volumes of the oxime (8-hydroxyquinoline) solution, thirty volumes of absolute ethanol, and five volumes of water. A 0.1 ml aliquot of the serum was added to 3.9 ml of each reagent (pH 3.5 and pH 6.5). After shaking for two minutes and centrifuging for twenty minutes, the solutions were poured into

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[2]Model 111, G.K. Turner Associates, 2524 Pulgas Avenue, Palo Alto, California.

12 X 75 mm cuvettes. The pH 3.5 solution served as the blank since magnesium hydroxyquinolinate has minimal fluorescence below pH 5.0.

Fluorescence is high at pH 6.5 which is critical in this analysis. A 405 mu primary filter and a 520 mu secondary filter were used.[3] After reading the amount of fluoresence in each cuvette, the samples were compared to standards and the magnesium content of the samples were found by graphical methods. Recoveries and internal standards were run periodically. The internal standards were especially useful in those determinations where quenching of fluorescence occurred. Recoveries were comparable to those reported by Batsakis et al. (6) using this method.

"Urine reagents" were also prepared fresh daily with the same buffers. These reagents differ from the serum reagents only in the propertions used. Two volumes of oxime solution, five volumes of buffer (at pH 3.5 and 6.5, respectively) and 31 volumes of absolute ethanol are used. Schacter describes a modification of this method to correct for non-specific fluorescence, by preparing a duplicate set of blank and sample diluted 1:5 with 0.01 M sodium versenate. This modification did not give good results, and so the original method was used. In these urine determinations, the urine was diluted 1:5 and 0.2 ml aliquots were taken. The magnesium concentration was determined by comparing the dial readings with those of magnesium standards, by means of a group. Here again, internal standards and

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[3] Turner Filters #110-813 (47B) and #110-818 (2A-12).

recoveries were used, with results comparable to those obtained by Batsakis et al. (6).

Standard solutions of magnesium were prepared by drying MgO at 110° C. for 24 hours, dissolving this in the minimum amount of concentrated HCl, followed by appropriate dilutions with water.

Determination of Tissue Magnesium

Acetic acid extracts were used in most cases, using a slight modification of the method used by Steinbach and later by Sparrow and Johnstone (83,85). One ml of glacial acetic acid was added to 200 ml of water to make the stock solution. The adrenals were weighed and placed in 2.0 ml of the dilute acid in glass-stoppered centrifuge tubes. The tubes were brought to a boil in a water bath and then allowed to stand overnight to complete the extraction. Aliquots of 0.1 ml acetic acid extracts and the urine reagents (because of the larger amount of buffer) were used in the determination of magnesium. Otherwise, the method of Schacter was followed, as described for urine.

Portions of heart, brain, and kidney were homogenized in dilute acetic acid and brought to a total volume of 10 ml, and the magnesium was then determined in the same manner. In some cases where these tissues were homogenized in glass-distilled water for ATP, the homogenates were acidified with glacial acetic acid for the magnesium determination. Magnesium standards were prepared in the same concentration of dilute acetic acid, as used for the tissue extracts.

In some groups, the adrenals were homogenized in 0.01 N HCl for determination of catecholamines. Aliquots of these homogenates were taken for the adrenal magnesium determinations, and these were compared to magnesium standards make-up in 0.01 N HCl. In general, the results agreed well with those obtained with acetic acid extraction.

Standards

Dilutions of the magnesium standards usually plotted out as a straight line on a group, sometimes with a slight diminution in fluorescence at the higher concentrations. Internal standards and duplicate readings usually fell within the plus or minus 12 percent range described by Batsakis et al. (6). Recoveries were usually within ± 20 percent, with occasional determinations exceeding this range when the procedures were first performed.

Adenosine triphosphate determinations

Immediately after sacrifice one adrenal was removed, quickly trimmed of fat, weighed, and dropped into 2.0 ml of boiling water in a glass-stoppered centrifuge tube in a water bath after the method of Strehler and McElroy (87). After boiling for ten minutes, the tube was cooled in cold water for 15 seconds and then placed immediately in dry ice. After freezing in dry ice the adrenals and other tissues could be stored in a deep freeze. Occasional tubes cracked when frozen. In these cases the ice plug was removed and placed in another tube. Breakage may be avoidable by pouring the boiling extract slowly

into another tube pre-cooled in dry ice. Heart, skeletal muscle, brain, and kidney were also extracted in a similar manner. Five to eight hundred mg of tissue were weighed, sliced 2 to 3 mm thick, and placed in 2.0 ml of boiling water. The muscle was taken from the quadriceps femoris muscle group.

ATP levels were determined by a modification of the method of Strehler and McElroy (87). Frozen, desiccated firefly tails[4] were ground in a chilled mortar with ice-cold 0.1 M sodium arsenate buffer, and poured into 50 ml centrifuge tubes standing in ice. Two mg of tail per ml of solution were required to obtain good readings. After grinding, the tubes were shaken for 30 seconds, and extraction was allowed to continue (with the tubes packed in ice) for an hour or more, until needed (86). Immediately before use, one tube was centrifuged for 10 minutes at low speed, and then placed in ice throughout the determination.

At the time of the determination, several samples were thawed at a time and homogenized with a Ten Broek homogenizer. The heart, skeletal muscle, brain, and kidneys were brought to a total volume of 10 ml with glass-distilled water, shaken well, centrifuged for 10 minutes, and packed in ice. Adrenal extracts were not diluted beyond 2.0 ml.

ATP standards were prepared just before use by adding 5 mg of ATP to 50 ml of glass-distilled water to make a concentration of 100 ug per ml. This solution was kept in a 50 ml tube packed in ice.

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[4]Sigma Chemical Co.

The Turner Model 111 Fluorometer with the high sensitivity attachment was adapted for use as a photometer by masking the ultraviolet light source. The UV light source was turned on, in order to permit the inner light path to calibrate the instrument. A Sola voltage regulating transformer was used to avoid error in readings resulting from fluctuations in line voltage. A stock solution for the luciferin-luciferase-ATP reactions was made by adding 10 ml of 0.1 M $MgSO_4$ to 150 ml of 0.05 M glycine. Three ml of this solution were placed in the 12 X 75 mm cuvettes with a burette.

0.2 ml aliquots of the tissue extract were added to 3.0 ml of the $MgSO_4$-glycine mixture in a cuvette. 1.0 ml of firefly extract was then drawn into a 1.0 ml syringe through a piece of polyethylene tubing, and ejected quickly into the sample. The cuvette was then stoppered with a piece of parafilm, inverted five times, and then placed quickly into the cuvette holder of the fluorometer. The fluorometer was attached to a recording milliammeter.[5] A peak was reached in about ten seconds. It was found convenient to take the deflection recorded one minute after the peak deflection as a measure of the amount of ATP present in the sample. The sample readings were compared with ATP standards by a method suggested by Moos (63). Six or more aliquots in duplicates of a sample were read, - one alone, one with one ug of ATP standard, one with two ug, one with three, another aliquot of the sample alone again. Readings as a rule were quite

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[5]Texas Instruments, Inc., Rectilinear Recording Milliammeter, Houston, Texas.

reproduceable. Average readings were graphed, and the line drawn through these determinations was carried beyond the ordinate to the abscissa, extended to the left of the ordinate. The average amount of ATP present in the six aliquots was then read off the abscissa.

A recovery carried through the boiling procedure gave a 95 percent yield. In the beginning, several trials were made with adrenal homogenates in 0.5 N perchloric acid, brought to neutrality with 2 N $KHCO_3$. Other trials were made with homogenates in 0.01 N HCl, brought to neutrality with Na OH. The boiling procedure was found to be as efficient in extracting ATP, and seemed to be a simpler procedure. Boiled extracts also appeared to be much more stable after thawing than the perchloric acid extracts, and last only 10 to 30 percent of the ATP present after standing at room temperature for five hours.

Catecholamine determinations

A slight modification of the method of Shore and Olin was used to determine the adrenal concentration of epinephrine and norepinephrine (80). Samples were read in a Model 111 Turner Fluorometer.

Reagents

All reagents were analyzed reagent grade. 0.1 N iodine was prepared by dissolving 1.27 grams of iodine in 100 ml of absolute ethanol. A 0.05 N sodium thiosulfate solution was made up by dissolving 1.24 grams of reagent grade $Na_2 S_2O_3 \cdot 5H_2O$ in 100 ml of water.

A 2 M pH 3.0 acetate buffer was prepared by adding 1 volume of 2 M sodium acetate to 50 volumes of 2 M acetic acid. A 2 M pH 5.0 buffer was prepared by adding 1 volume of 2 M acetic acid to 2 volumes of 2 M sodium acetate. Alkaline ascorbate solution was made fresh as needed by adding 1 volume of aqueous solution of ascorbic acid[6] to 2 volumes of 5 N NaOH. Spectro-analyzed n-butanol and n-heptane were used in the initial extraction, and no further purification seemed necessary. Five mg of norepinephrine and 5 mg of epinephrine were each dissolved in 100 ml of 0.01 N HCl and stored in the refrigerator for use as standards.

Procedure

The adrenal or other tissue was taken immediately from the animal after death, weighed, homogenized in 0.01 N HCl with a Ten Broek homogenizer, and made up to volume. 2.0 ml of the homogenate was then added to a 125 ml glass-stoppered Pyrex bottle containing 5 to 6 grams solid NaCl and 25 ml of n-butanol. These bottles were shaken for 20 minutes on a shaking apparatus, centrifuged for 15 minutes, and then a 5 ml aliquot was transferred to a 50 ml polyethylene-capped tube containing 6 ml of 0.01 N HCl and 27 ml of heptane. These tubes were shaken by hand for three minutes by the clock, and then centrifuged for 15 minutes. Following centrifuging, 2.0 ml aliquots of the aqueous part were pipetted into cuvettes.

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[6] Ascorbic acid, Merck, U.S.P., in 10 mg/ml solution.

One recovery was attempted for each group of test animals, and one for the controls. Only 1.0 ml of a sample was pipetted into a duplicate sample bottle of butanol and salt, and to this was added 1.0 ug of epinephrine and 1.0 ug of norepinephrine for recovery. The determinations for this sample were then compared with the duplicate sample. Recoveries in general were near the 65 percent figure obtained by Shore and Olin (80).

Internal standards were prepared by pipetting a duplicate set of cuvettes with a sample, with only 1.0 ml aliquots in place of two, and then placing 0.5 ml of each standard (i.e. 1/2 ug each of epinephrine and norepinephrine) into each cuvette.

After extraction into butanol and separation by heptane into 0.01 N HCl, the catecholamines were oxidized to trihydroxyindoles (highly fluorescent) by a method modified after Lund, as used by Shore and Olin (68). One ml of the pH 3.0 buffer is added to a 2 ml sample in a cuvette, and one ml of pH 5.0 buffer is added to a duplicate sample in a second cuvette. After mixing, 0.1 ml of iodine reagent is added to each cuvette, which is mixed again. After about 6 minutes (by the clock) 0.3 ml of thiosulfate solution is added to reduce the excess iodine. After mixing again, the alkaline ascorbate solution is prepared and 1 ml is added to each cuvette. After a final mixing, the cuvettes are read 45 minutes later. The amount of epinephrine is calculated from the reading for the aliquot oxidized at pH 3.0 (norepinephrine shows negligible fluorescence when oxidized

at pH 3.0) by comparison with a standard curve. Both catecholamines fluoresce at pH 5.0 and so the total catecholamines are determined by comparison with a standard curve of the catecholamines together oxidized at pH 5.0. The amount of epinephrine determined to be present in the first cuvette is subtracted from the total amount of catecholamines present in the second aliquot. Specimens were read in a Turner fluorometer, with an activating wavelength of 520 mu, and the fluoroscence of the sample was read at 400 mu. Recoveries and internal standards were usually comparable with that obtained by Shore and Olin.

CHAPTER III

RESULTS

Morbidity and Mortality

Acute effects of magnesium deficiency were best observed in the five-week old rats which became hyper-reactive about four days after starting on a low magnesium diet. Moderate hyperemia of the ears appeared in most cases at about the sixth day, and at eight days, slight edema of the ears was apparent, with crusty excoriations in some cases. The hyperemia appeared to involve the skin over all the body, though to a lesser extent than the ears. A crusty dermatitis of feet and tail gradually appeared, and in many rats the skin of the tail developed scabby ulcers. These lesions occurred in some rats at eight days, and the majority of the rats had marked lesions at 12 days (Figure 1). Piloerection was noted as early as five to six days, becoming more prominent later, and some of the rats developed areas of relative alopecia. A red crusty discharge was observed around the eyes of some of the rats as early as 10 days. These general effects can be seen in Figure 1.

The rats in general were not active unless disturbed, and then they were hyper-reactive. Generalized convulsions were observed as early as 10 days. These were preceeded by piloerection, exophthalmus, an aimless, agitated wandering about the cage, and then a collapse

Figure 1. Clinical Signs of Acute Magnesium Deficiency

Lower Left: Typical apprehensive stance, with pilo-erection.

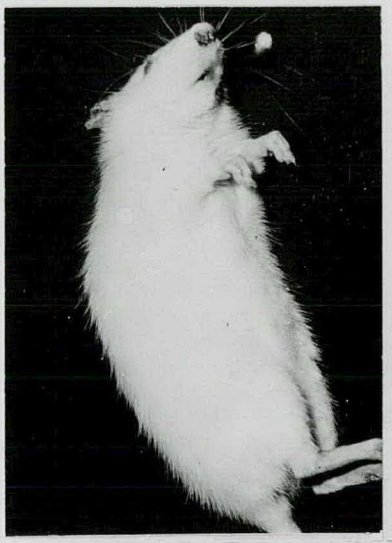
Center Left: Pilo-erection, with mild tonic-clonic convulsions involving fore-legs, head and neck.

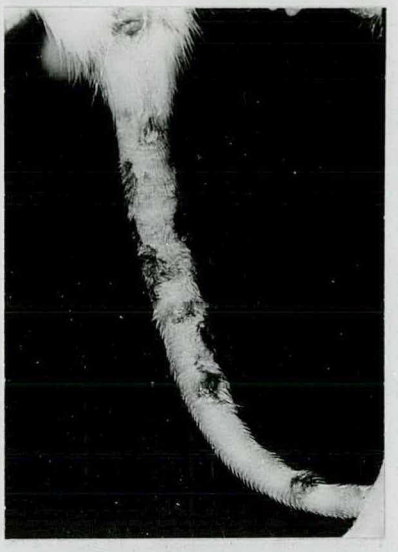
Upper Left: Generalized tonic-clonic convulsions, with pink frothy exudate at nostrils. The cartilage at the tip of the ear of this rat has been partially destroyed, in addition to the skin lesions.

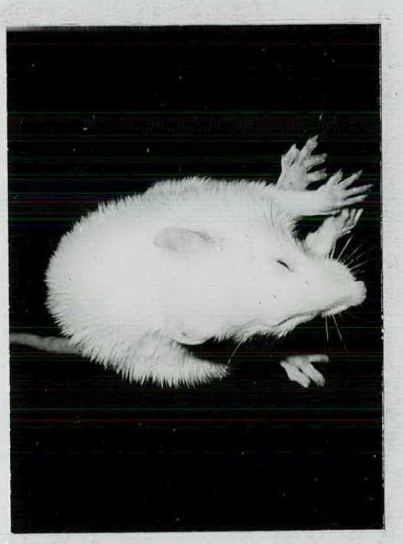
Lower Right: Rat standing up with mild tonic-clonic convulsions of the fore-limbs, head and neck. Ears show the typical lesions of magnesium deficiency, - dry, crusty sores, edema, hyperemia.

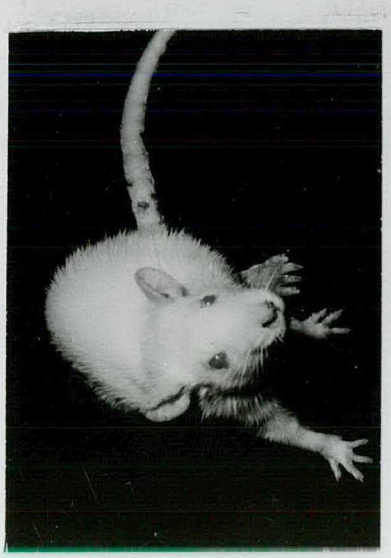
Middle Right: Early signs of magnesium deficiency, - ears hyperemic and edematous, with similar but less pronounced changes in the feet. Pilo-erection is prominent.

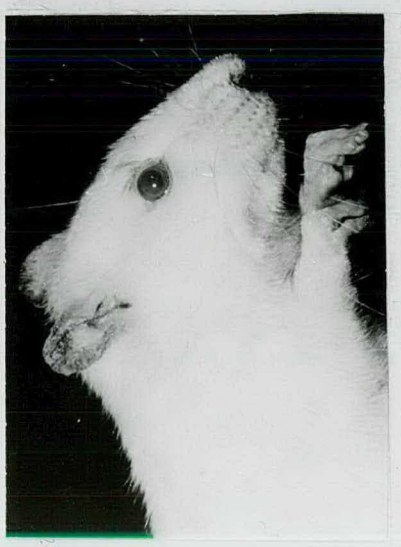
Upper Right: Typical areas of alopecia and ulceration occurring in rats on a low magnesium diet.











into tonic-clonic convulsions, which were observed to last 20 to 40 minutes, sometimes ending in death (Figure 1). One rat in an open box suddenly jumped about 18 inches into the air, and fell in a convulsive seizure. During the convulsions, a pink froth would usually appear at the nostrils, suggesting pulmonary edema. Defecation and urination did not occur, and the rats appeared to remain conscious. The convulsing rats were more likely to recover if placed alone in a dark, quiet place. Handling the rat during weighing did not cause an attack immediately, but those rats that seemed most apprehensive during weighing would often have convulsions about 5 to 15 minutes later.

Some rats experienced mild convulsions limited to one fore-leg or the neck and jaws. The head would be drawn back with a biting motion of the jaws. Some of these rats would then rise on their hind legs during one of these attacks and fall over backward. These rats would then get up and repeat this procedure several times.

In general, all deficient rats seemed to become progressively weaker, and many developed tremors. More severely depleted rats become obviously incoordinated, some to a degree which made walking difficult.

Diarrhea and melena were observed in some rats, but after a longer time on the low magnesium diet than previously reported (45). This did not usually appear until the third or fourth week. The test rats seemed to eat and drink about as much as the control rats, until they became severely affected, but even then continued eating the test diet until death.

In contrast to the control rats which approximately doubled their body weight over a 30 day test period, the test rats gained slowly until the 12th day and then consistently lost weight (Table I, Figure 2). At the end of the 30 day test period, two of the rats were given magnesium in their drinking water. Clinical signs faded within three days, and the growth rate accelerated so that the body weight became comparable with controls in about ten days (Table II, Figure 3).

In one study of 18 test rats, two died at 15 days, one at 16 days. Three of the survivors selected at random were kept on the diet with a recovery group and a control group, but all had died by the 40th day. In another grouping of 12, two died at 11 days, two at 14 days, and two at 15 days.

Ten-week old rats were found to be slightly more resistant to magnesium deficiency than five-week old rats, and the typical clinical signs did not become severe until later. The earliest signs of hyper-reactivity, piloerection, and erythema began to appear at seven days. The erythema became marked in several older rats at eight days, but in general, the clinical signs did not become severe until 14 days had passed, and about half of the rats did not show severe changes at all. Confulsions were noted in one rat which died after 25 days. No other rat in a group of eight had convulsions.

The four rats (ten-weeks old) in the chronic deficiency group gained weight slowly on the low magnesium diet until the 11th day,

TABLE I

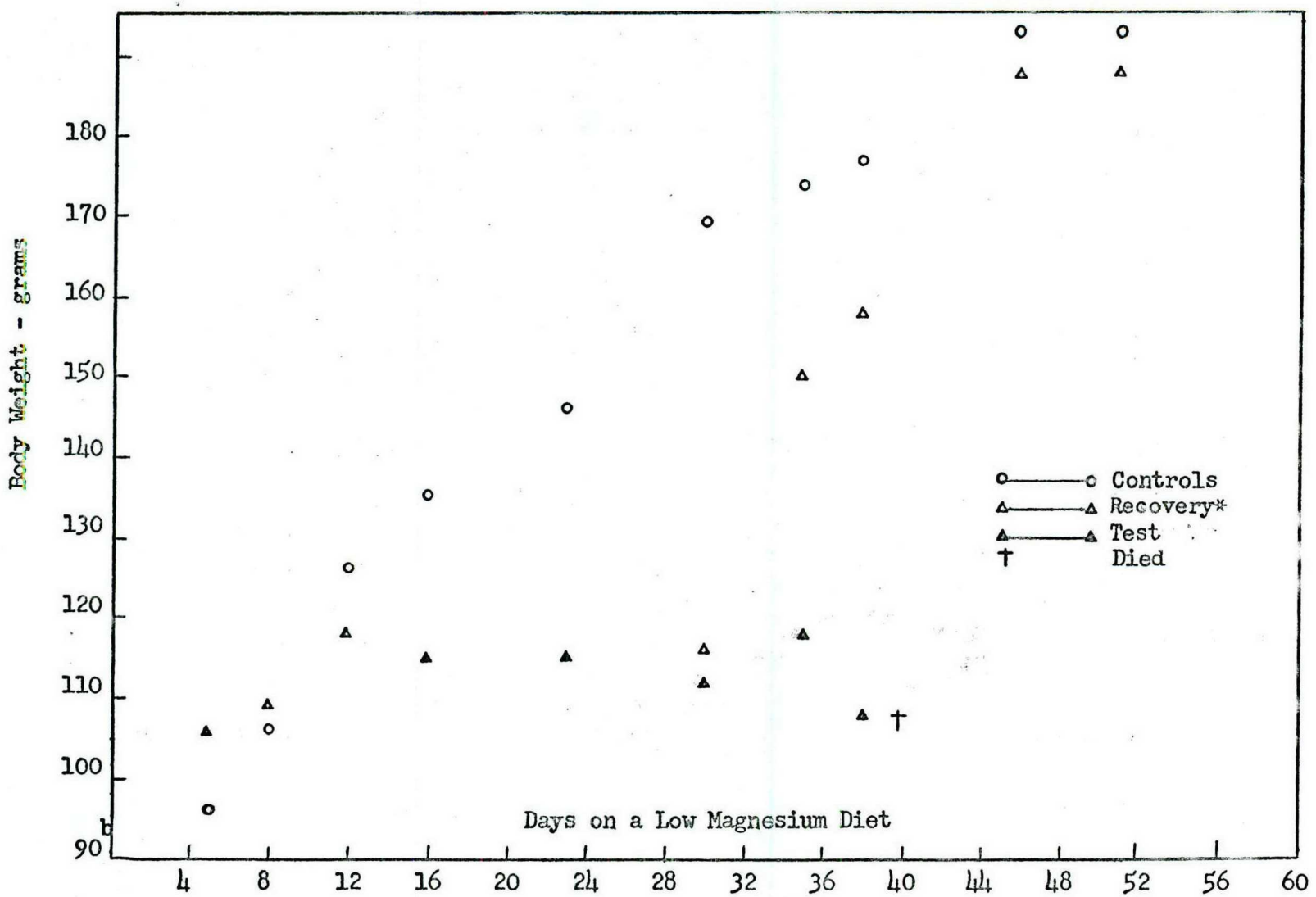
Effect Of Magnesium Deficiency On Growth
Of Five Week Old Female Rats

Rat No.	Days on Low Magnesium Diet: 0	3	5	8	12	16	23	30	35	38
	control									
19	98	102	122	127	158	164	175	199	212	(217)
21	97	97	113	120	133	144	155	172	155	157
20	102	105	124	132	162	167	176	205	210*	
22	116	114	138	140	167	170	182	206	215*	
32	71	68	68	84	113	117	130	160*		
34	94	91	94	106	113	135	142	193*		
31	92	65	86	99	102	113	122*			
33	96	100	103	120	136	147	164*			
	test									
38	86	84	88	100	110	108	113	117	113	107†
37	99	97	102	110	118	120	117	133	127	128†
35	102	98	108	126	140	139	145	150	143†	
11	87	87	102	104	106	98	99	88*		
12	102	103	105	110	107	102	113	102*		
13	109	114	125	126	131	126	134	129*		
14	108	104	113	124	133	128	137	147*		
24	108	120	128	134	132	127	120	113*		
23	102	104	117	122	127	115	113*			
25	105	110	125	130	134	135	141*			
27	108	115	127	130	145	139	147*			
30	99	98	108	104	122	120	126*			
15	112	117	130	140	156	155*				
16	104	109	118	132	144	140*				
17	95	100	112	116	121	121†				
18	89	101	114	120	125	128*				
29	100	107	116	114	132	132†				
36	95	95	100	104	117	117†				

* Sacrificed

† Died or Moribund

Figure 2
Effect of a Low Magnesium Diet on Growth of Five Week Old Female Rats, and Recovery



* Test animals given 15 g $MgCl_2$/liter drinking water after 30 days (Average of two animals)

TABLE II

Recovery From Magnesium Deficiency in Five Week Old Female Rats

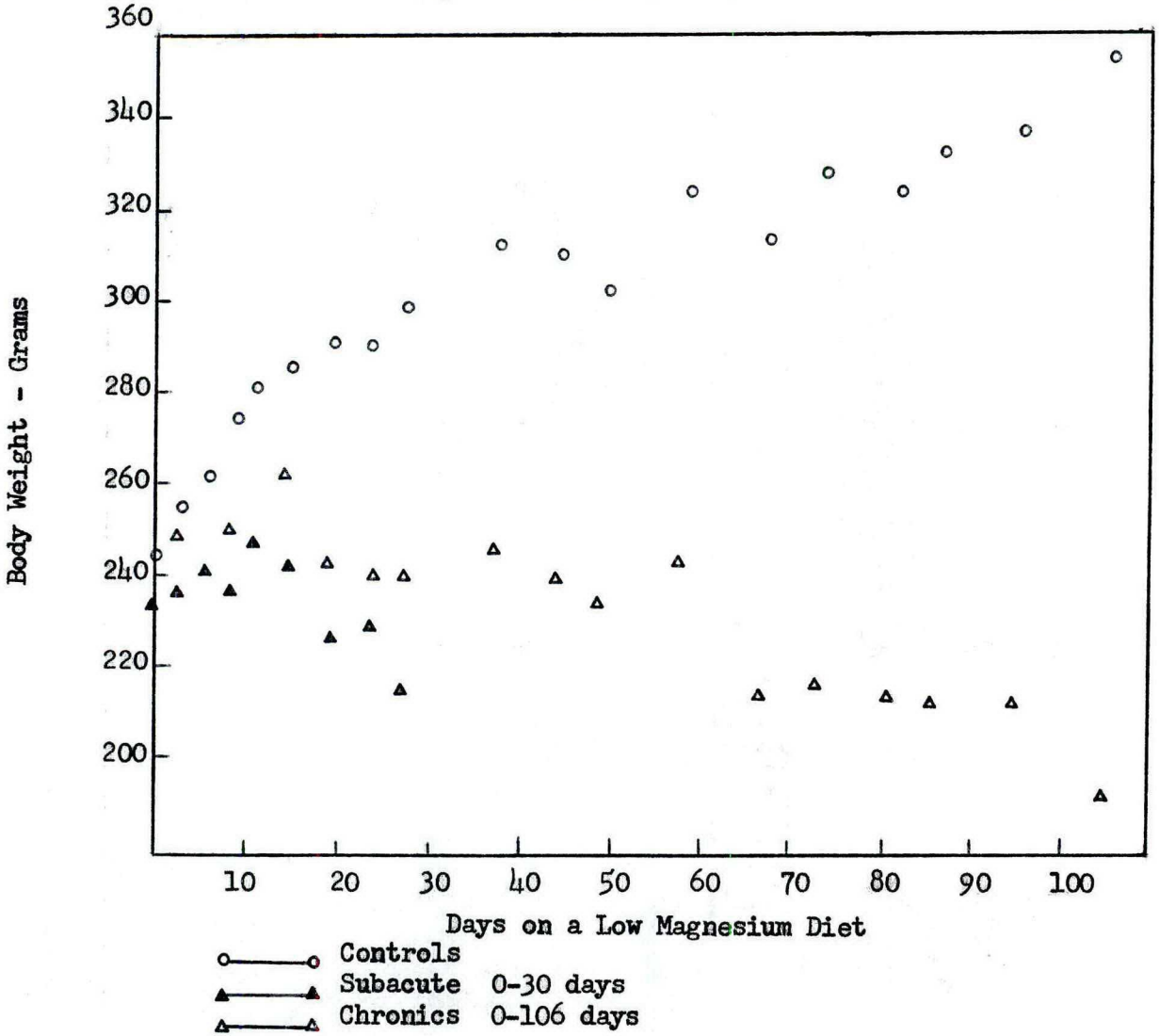
Rat No.	Group	Days on Low Magnesium Diet: 0	3	5	8	12	16	23	30	35	38	46	51
		weight in grams											
19	Control	98	102	122	127	158	164	175	199	212	(216)	231	232
21	Control	97	97	113	120	133	144	155	172	155	157	175	174
26	Test and Recovery	100	103	116	122	128	125	(118)	108	153*	157	186	186
28	Test and Recovery	101	112	119	120	134	135	151	145	168*	178	211	210
35	Test	102	98	108	126	140	139	145	150	143†			
37	Test	99	97	102	110	118	120	117	133	127	128†		
38	Test	86	84	88	100	110	108	113	117	113	107†		

* 15 grams of $MgCl_2 \cdot 6H_2O$ was added per liter to the drinking water of test rats #26 and #28 after 30 days on a low magnesium diet. The control rats received the identical diet, but received the same concentration of $MgCl_2 \cdot 6H_2O$ in their drinking water throughout the experiment.

† #35 died after 32 days on the low magnesium diet, #37 after 39 days, and #38 after 40 days.

Figure 3

Effect of a Low Magnesium Diet on Growth of Ten Week Old Female Rats



after which they lost weight steadily (Table III, Figure 3). At the time of death, all weighed less than at the start of the test. After about three weeks, the skin lesions began to fade slowly, though piloerection and the hyper-irritability reamined fairly prominent. The rats becamse difficult to handle, and often squealed when picked up for weighing. Weakness, inactivity, and poor coordination became progressively more noticeable, and several of the rats had persistent diarrhea. All exhibited red crusts about the eyes, some degree of exophthalmus, and edema of the head and feet. One rat of the four survived to 106 days, when the test was ended.

Gross Changes and Lesions

There were few obvious lesions found on gross examination of the young female rats, aside from the skin lesions previously described. A few had pale kidneys, with a light grayish-white band at the cortico-medullary junction, without evidence of calcification. Kidney weights were 35 percent heavier than those of the control group at 23 days, on the basis of body-weight. The changes in the kidneys of the male rats were much more prominent than in the females. They were markedly pale, swollen, soft, and had a much more pronounced greyish-white band at the corticomedullary junction. Kidney weights were nearly twice as great as in the controls, both on an absolute and a relative basis. The testes of the test rats were soft as compared to the controls, and in some instances weighed considerably less than in controls.

TABLE III

Effect Of Magnesium Deficiency On Growth Of Ten Week Old Female Rats

Rat No.	Days on low magnesium diet																		
	0	3	6	9	11	15	20	24	28	38	45	50	59	68	74	82	87	96	106
	Weight in grams								CONTROL GROUP										
9	244	260	268	280	282	286	294	292	295*										
10	246	250	256	268	280	284	286	286	292	312	310	302	324	314	328	325	333	337	353*
									TEST GROUP										
1	220	222	222	218	228	222*													
3	244	254	252	244	248	238*													
6	250	258	258	258	264	256	234	240†											
6 2	222	212	234	232	234	230	220	220	216*										
									CHRONIC TEST GROUP										
7	244	254	251	250	258	250	246	246	252	250	256	254	253	210	222	208†			
5	230	239	246	246	258	248	236	234	227	242	234	210	228	220	220	214	212†		
8	252	267	270	278	282	280	270	258	260	262	272	272	276	220	222	230	211	200	179†
4	228	242	243	230	240	234	226	226	224	234	230	206	224	215	212	210	223	230	211*

* Sacrificed

† Died or Moribund. #7 died after 79 days, #5 after 84 days, and #8 after 98 days.

The brains of the deficient rats appeared more hyperemic than those of the controls, and despite the wide discrepancy in body weight (120 grams vs 194 grams in the 30 day group) the average brain weight was about the same. On an organ weight-body weight basis, the brains of the deficient rats were proportionately much heavier. The spleens were also much heavier, - 0.66 grams as compared to 0.45 grams for the much larger control rats. Heart, lung, liver, pancreas, and adrenals showed variable weight changes. The hearts of the test rats appeared somewhat more distended at necropsy, and were more flaccid when examined and sectioned. No grossly visible lesions were found. The liver in some test animals seemed small, pale, and softer, but this finding was variable. The adrenals in some test animals were enlarged, but not in others. Examination of the adrenals after fixation disclosed a reduction in the size of the medulla. While this may represent in part only an apparent reduction due to hypertrophy of the cortex, in many of the test rats the medulla had been reduced to only a narrow band. The thymus was normal in gross appearance, and in general was equal in weight or heavier than that of the controls, especially when compared on the basis of body weight. The entire left lung of a test rat at 30 days was found to be consolidated with dense, light grey tissue, and there were extensive pleuritic adhesions. A nodule of similar tissue was found embedded in the liver. Sections of these organs were placed in formalin for subsequent histologic study.

In general, the viscera of the test rats appeared more congested than those of the controls. The stomach mucosa in some test rats was ulcerated and streaked with blood, and in some cases the intestines were hyperemic and distended with gas. Many had semi-liquid dark feces.

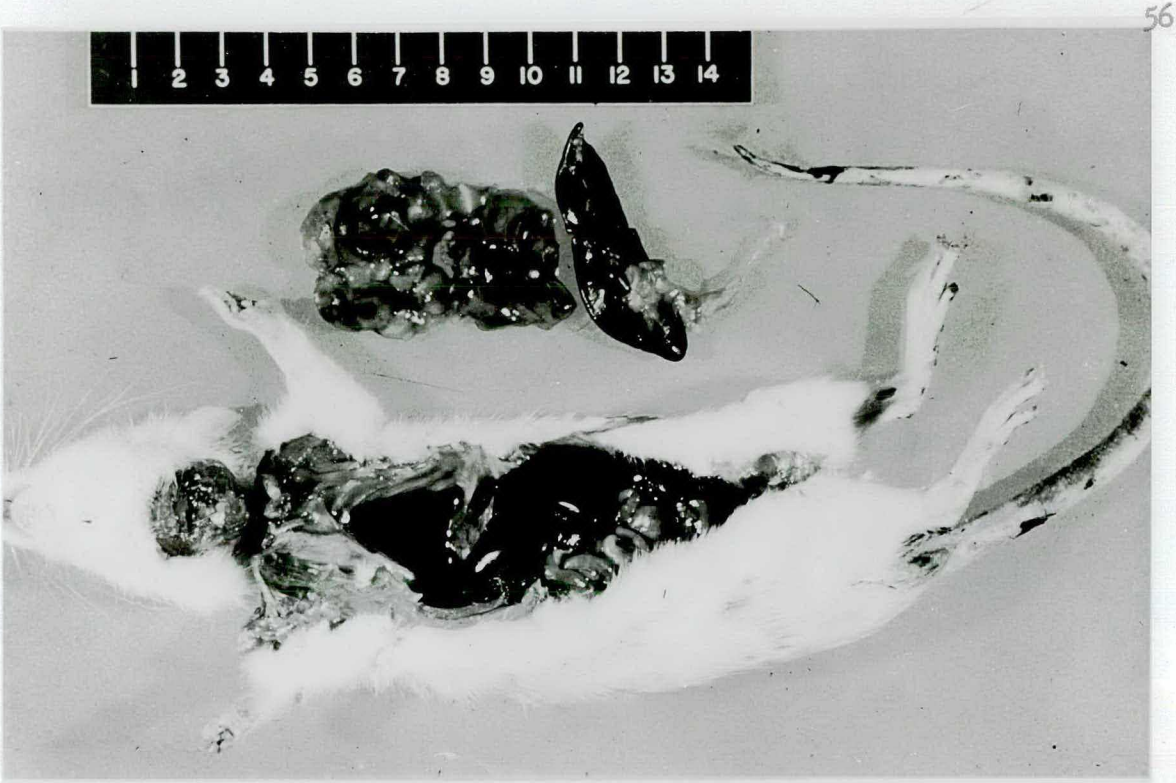
The bone marrow was usually dark and more fluid than that of the controls. The strength of the tibia did not seem to differ (subjectively) from that of the controls. Skeletal muscle appeared slightly pale and atrophic. In some animals at 16 days and in a larger proportion of the animals at 30 days the sciatic, optic, and trigeminal nerves appeared swollen, softer, and dull.

In the ten-week old rats on a low magnesium diet, few changes were seen at 14 and 30 days, except for the skin changes previously described. In the chronically deficient group (four rats) the first death occurred at 79 days. This rat became progressively more weak, emaciated, dyspneic, and uncoordinated, and finally collapsed. The outstanding finding at necropsy of this rat was an 8.5 gram tumor of the thymus (body weight was 208 grams, normal thymic weight is 0.15 to 0.25 grams). Normal thymus tissue was replaced by a large, multilobulated tumor mass firmly adherent to the chest wall and mediastinal structures, including the heart, and extending down to the diaphragm and anteriorly and dorsally to nearly surround the trachea and esophagus (Figure 4). On section, the tumor was composed of a number of firm, somewhat round, greyish-white nodules up to 1 cm in diameter

Figure 4. Thymoma in a Rat With Chronic Magnesium Deficiency

Upper: 150 day-old white female rat, after 79 days on a low magnesium diet. A malignant thymoma was adherent to the chest wall and all mediastinal structures. The liver was congested in appearance. The tail is smeared with dark semi-liquid stool.

Lower: The thymoma is shown attached to part of the chest wall, aorta, heart, and diaphragm. Anterior mediastinal structures were also imbedded in tumor tissue. The reddish-grey color of the heart is due to a thin layer of tumor tissue over the pericardium. Several of the firm, greyish-white nodules of the tumor may be identified in the soft matrix, which appears hemorrhagic in some areas. Note the size of the tumor and the congested spleen in relation to the heart.


1 2 3 4 5 6 7 8 9 10 11 12 13 14


ETRIC 1 2 3 4 5 6 7 8 9

embedded in a softer greyish-red matrix. The pleural cavity contained 3 to 4 ml of sanguinous fluid. The lungs were moderately edematous, congested, and heavy, but otherwise normal in appearance. The heart was congested, but appeared grossly normal.

The periaortic nodes were markedly enlarged and adherent to the adrenals and the abdominal aorta and postcava, and weighed well over one gram. Some of the mediastinal nodes were markedly enlarged as well. The spleen was dark, soft, congested, and enormous in size, weighing 4.57 grams, as contrasted to that of a much larger control rat which weighed 0.57 grams. A cachetic rat might be expected to have a spleen weighing 0.15 to 0.25 grams. The adrenals were moderately enlarged and pale. The kidneys and liver were somewhat congested, but were grossly normal. The brain was large for the size of the rat (2.12 grams with a body weight of 208 grams, as compared to an older rat with a brain weight of 2.00 grams and a body weight of 337 grams). The muscles were soft and pale and the bone marrow was pale, in contrast to the dark bone marrow of other test rats.

Formalin-fixed, hematoxylin and eosin-stained sections of this rat were studied in order to describe the neoplasm and to corroborate the tissue changes previously reported for magnesium deficiency. At low power, the thymus appeared to have been replaced by a follicular lymphoma or lymphosarcoma (Figure 5). In some areas there were many irregularly-shaped, large follicles without areas of necrosis or fibrosis. Many of these had the typical "cracking artifact" of giant

Figure 5. Histopathology of a Rat With Chronic Magnesium Deficiency and a Thymoma

Lower Right: Lymphomatous nodule in the thymus (X20).

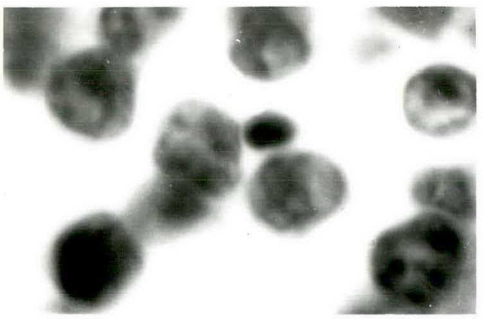
Lower Left: Nodule on the left with incomplete capsule. Parietal pleura on the right (X40).

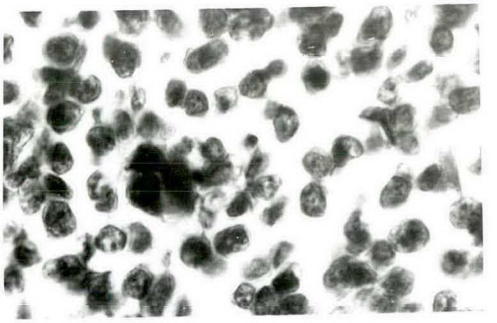
Middle Right: Arteriole in tumor mass, with a thymoma cell (or lymphoma cell) in the media (X160).

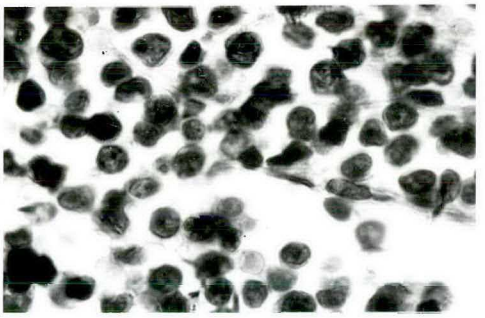
Middle Left: Vein in thymus, containing thymoma cells (X400).

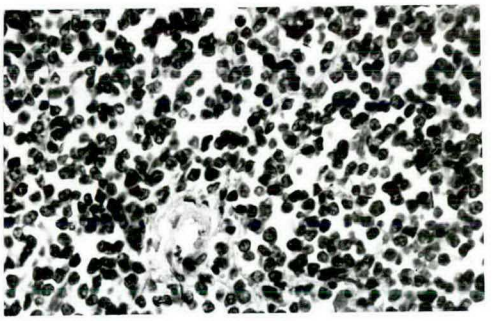
Upper Right: Giant cell in the lymphomatous nodule (X400).

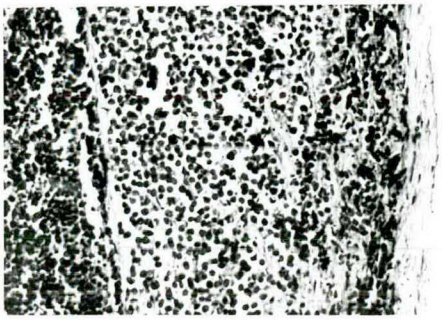
Upper Left: Thymoma cells with lymphocyte. Note large, irregularly-shaped nuclei containing large nucleoli and clumps and strands of chromatin (X1280).

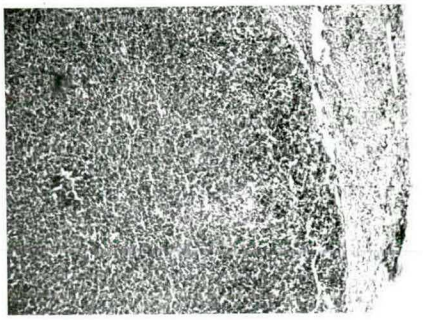












follicle lymphoma as described by Robbins (70). The cells in the follicles were atypical-appearing pleomorphic lymphoblasts and reticulum cells, with large, light, foamy-appearing nuclei and little cytoplasm. Within the nuclei were irregular masses and strands of chromatin material and numerous nucleoli.

The pulp around the nodules in many areas appeared compressed. Mitotic figures were seen frequently, and neoplastic cells were found in large numbers in the veins and could be seen passing through the walls of veins. In some areas a fine stroma was observed. Although a thin capsule was found in some parts of the tumor, in other areas the neoplasm was observed breaking through capsule and infiltrating muscle. The aorta was entirely surrounded by tumor tissue, and the pericardium also was infiltrated. The periaortic nodes were filled with tumor masses, which here also had extended beyond the capsules of the nodes and had surrounded the aorta. Neurons in a ganglion in the tumor mass had very pale, foamy nuclei, - this may have been due primarily to magnesium deficiency, or perhaps to interference with the vascular supply by the neoplasm. An area of the tumor near the abdominal aorta had clearly evident rosette formation, and resembled neuroblastoma tissue (Figure 6). A nodule of tumor tissue was also present in the spleen, which was also diffusely infiltrated. The bone marrow appeared to have been replaced by neoplastic cells (Figure 7). There was marked perivascular and diffuse infiltration of the liver with neoplastic cells, but little or no infiltration of

Figure 6. Histopathology of a Rat With Chronic Magnesium Deficiency: Brain, Lymph Nodes, Blood Vessels

Lower Left: Metastatic lymphoma to the periaortic nodes and surrounding aorta (left). Note area of apparent rosette formation on the left (X10).

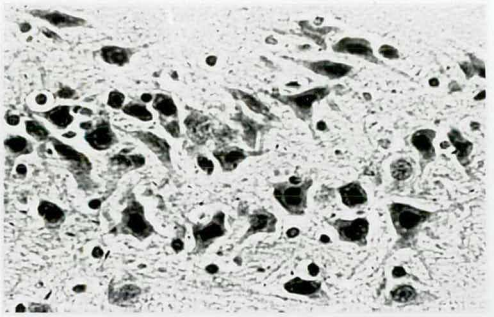
Lower Right: Area of tumor mass resembling neuroblastoma, with rosettes (X10).

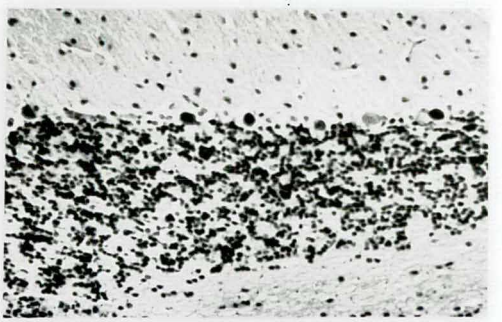
Middle Right: Ganglion in tumor mass near periaortic nodes. Note perineural infiltration and ganglion cells with pale, vacuolated nuclei (X10).

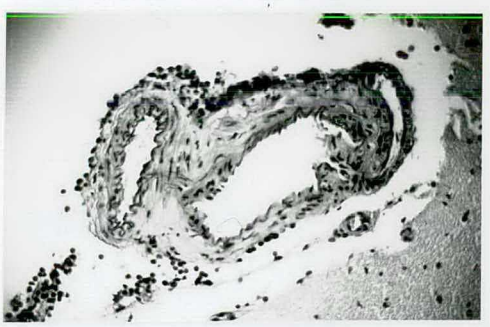
Middle Left: Meningeal artery with perivascular infiltrate of round cells, consisting mostly of lymphoma (thymoma) cells. Some proliferation and piling up of the intima is apparent, with pyknosis of some of the endothelial cells (X64).

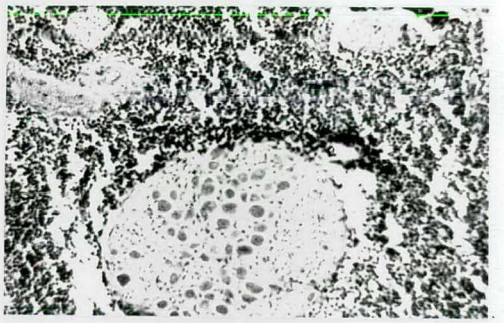
Upper Right: Cerebellum with pyknosis of some Purkinjie cells and chromatolysis and loss of Nissl substance in others (X10).

Upper Left: Hippocampus with pyknosis in many neurons and chromatolysis in others. There is some evidence of satellitosis and neuronophagia (X40).









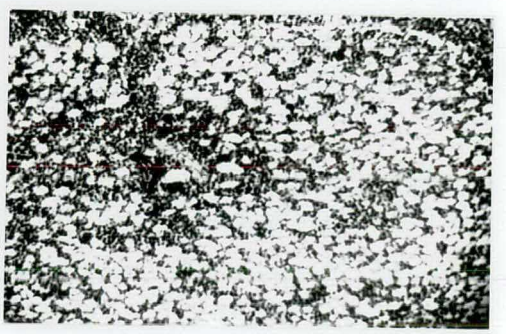


Figure 7.

Lower Left: Bone marrow with lymphoblasts (X160).

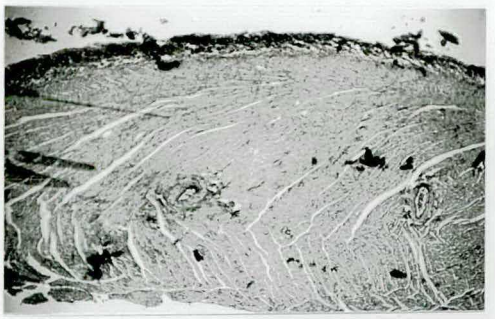
Lower Right: Adrenal sectioned through the cortex. The medulla appears atrophic (X10).

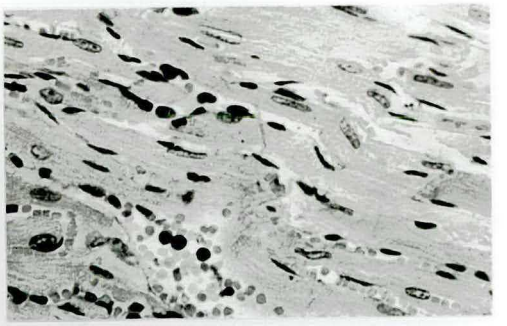
Middle Left: Liver, with marked perivascular and interstitial infiltration with lymphoma cells. Hepatocytes are atrophic, with pale, homogeneous-appearing cytoplasm and nuclei that are pyknotic and vacuolated in varying degrees (X40).

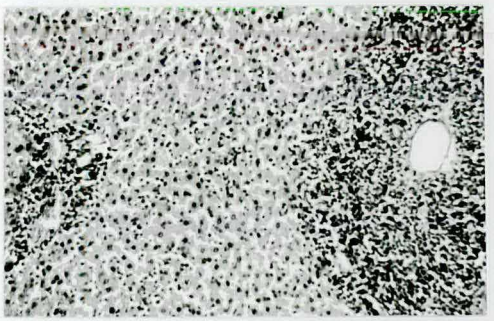
Middle Right: Calcification of renal tubules in the kidney. To the right above the artifact in the film may be seen a sheet of epithelial cells, the apices of which are calcified, the nuclei pyknotic. Different stages of degeneration and some regeneration of tubular epithelial cells are seen elsewhere (X64).

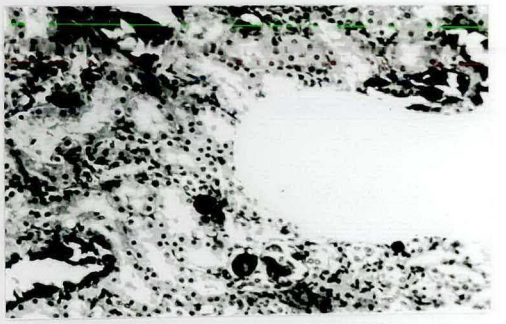
Upper Left: Section of left ventricle. Note layer of lymphoma cells overlying pericardium and infiltrating myocardium. Media of arteries here appear increased in thickness (X10).

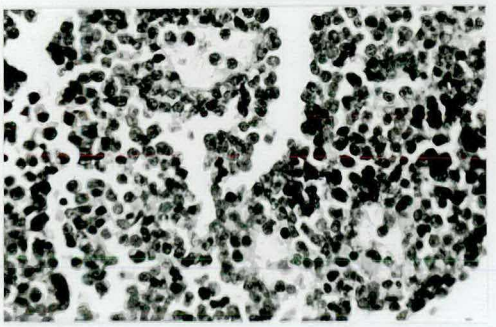
Upper Right: Myocardium. Note lymphoma cells in capillaries and pyknosis of endothelial cells. Muscle fibers are atrophic, cross-striations are not apparent, nuclei show chromatolysis, and the cytoplasm appears floccular and stains poorly (X160).

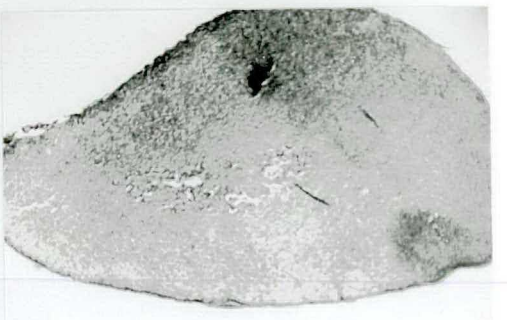












the kidney, brain and adrenal. In addition to the pericardial extension of the tumor, there was a diffuse infiltration of the myocardium.

Other changes previously described for magnesium deficiency were observed. Most striking of these changes was the calcification of the renal tubular epithelium. Areas were observed where the apices of the epithelial cells had calcified in sheets, with the pyknotic nuclei of the cells still visible. In other fields the calcification had progressed to form dense deposits with much destruction of tissue. Arterial and glomerular changes described by others were not clearly demonstrated in this animal.

The adrenal medulla appeared to have shrunken to a small fraction of the size found in normal rats (Figure 7). Skeletal muscle and myocardial changes were seen, - irregular staining, atrophy, focal infiltrations. However, no calcification was observed here, and most of the infiltrating cells could not be distinguished from tumor cells. There was a marked perivascular and peribronchial infiltration in the lungs -- these cells also appeared to be mostly tumor cells. Several sections of the gastrointestinal tract were examined, and did not appear remarkable, except that mitosis in the crypts of Lieberkuhn seemed to be decreased, and the mucosa was slightly atrophic.

Although perivascular infiltration of a meningeal artery was found, no infiltration of the brain itself was observed. As previously described, degenerative changes in the Purkinjie cells were found. The nuclei of some neurones showed marked chromatolysis or

pyknosis. Satellitosis and neuronophagia were observed. Similar changes were found in the hippocampus (Figure 7) but not in the cortical cells.

Vascular changes, aside from perivascular infiltration, were not very striking. Some proliferation and piling up of the intima were found. The hematoxylin and eosin stain did not demonstrate changes in the media.

Two of the three surviving rats died at 80 days and 98 days, respectively. Here again, the brains were heavier than normal, hyperemic, and appeared swollen. The liver of one of these animals was markedly edematous, mottled, pale and firm, suggesting a diffuse cirrhosis. No obvious lesions were found in the hearts, which were heavier than the control rat when compared on the basis of body weight. One of the rats also had a sanguinous pleural effusion similar to the rat with the thymic tumor. Similarly, the bone marrow in this rat was moderately pale and edematous. Aside from congestion, the kidneys showed little change grossly. Adrenals were enlarged. In one rat the gastrointestinal tract was hyperemic and distended with gas, but the rat had had no diarrhea.

One rat of the group of four survived to 106 days. The thymus was slightly enlarged and hyperemic. Again, the heart was congested and relatively heavy. The spleen was moderately congested and markedly enlarged. The liver and kidneys were pale but with no other obvious change. The adrenals were large and pale. As noticed in the other

deficient rats, the brain was swollen, hyperemic, and heavy (2.15 grams as compared to 2.00 grams for the much larger matching control rat). The trigeminal, optic, sciatic and other nerves were dull, swollen, and softer, and the skeletal muscles were pale and atrophic.

Serum and Urine Magnesium Levels

The mean serum magnesium value for control rats in this study was 1.89 mg percent ± .022 (s.e.m.). After 24 hours on a low magnesium diet the mean serum magnesium had dropped 36 percent, to 1.21 mg percent (Table IV). The animals were then able to maintain this level for about six days, until clinical signs of deficiency began to appear. By the eighth day, however, the serum magnesium dropped again, to only 32 percent of normal (0.66 mg percent) and clinical signs became more severe. Although clinical signs became more marked at 12 and 16 days, the serum magnesium level rose to about 1.1 mg percent, followed by a second fall in the serum magnesium. Approximately 50 percent of the rats died during this period. Three of the rats in this age group that were allowed to continue past the termination of the 30 day test period had all died by 40 days.

Mean 24 hour urine magnesium was 0.75 ± .07 mg. Rats on a low magnesium diet excreted only 0.16 mg/24 hours after two days. Urinary magnesium remained at about this figure for about eight days. On the 12th day, the test animals only excreted 0.08 mg/24 hours. At this point urinary excretion of magnesium began to rise, until by 23 days nearly as much was excreted as was excreted by the controls. At 30

TABLE IV

Correlation of Clinical Signs With Serum And Urine Magnesium Levels
Of Five Week Old Female Rats On A Low Magnesium Diet

Group	Urine Mg/24 hrs. In Mg	Serum Mg mg/100 ml	Clinical Signs
Stock	0.75 ± S.E.M. 0.07 (6)	1.89 ± S.E.M. 0.22 (6)	none
2 days on diet	0.16* (2)	1.21* (2)	none
4 days "	0.17* (2)	1.24* (2)	slight
6 days "	0.16* (2)	1.06* (2)	slight to moderate
8 days "	0.16* (2)	0.61* (2)	slight to moderate
10 days "	0.08* (2)	0.82* (2)	marked
12 days "	0.04* (2)	1.08* (2)	marked
16 days "	0.38* (2)	1.05* (2)	marked
23 days "	0.66* (2)	0.92* (2)	marked
30 days "	0.30* (2)	0.82* (2)	marked

* Average value

Numbers in parentheses represent animals in each group.

days, however, the magnesium excretion had again dropped to less than one-half the normal value.

The serum magnesium values for the entire group of five-week old rats used in this study followed a similar course during the first 30 days (Figure 8).

Magnesium, Adenosine Triphosphate, and Catecholamine Concentrations in Tissues

Control rats were matched with test rats in all phases of this study in order to demonstrate that the effect of the low magnesium diet was due to magnesium deficiency alone. In general, the control rats were comparable with stock rats receiving Purina Laboratory Chow.

Data collected from the five-week old control rats was pooled for comparison with test rats, since the control rats showed no ill effects from the diet when a magnesium supplement was given in the drinking water, no matter how long they remained on the diet.

The adrenals of the control rats contained 0.40 ± 0.2 (s.e.m.) mg/g of magnesium. Rats on the test diet for two days had an adrenal magnesium of 0.26 ± 0.1 (s.e.m.) mg/g. At eight days the concentration was still essentially unchanged, 0.29 ± 0.02 (s.e.m.) mg/g (Table V) ($p < 0.001$). At twelve days the concentration of magnesium reached its lowest point, 0.23 ± 0.03 (s.e.m.) mg/g ($p < 0.001$). At 16 days, the magnesium concentration returned to normal levels, 0.37 ± 0.03 mg/g, and the concentration remained high at 23 days. However, at 30 days the magnesium level had fallen again to less than one-half the

Figure 8
Serum Magnesium Levels in Five Week Old Female Rats

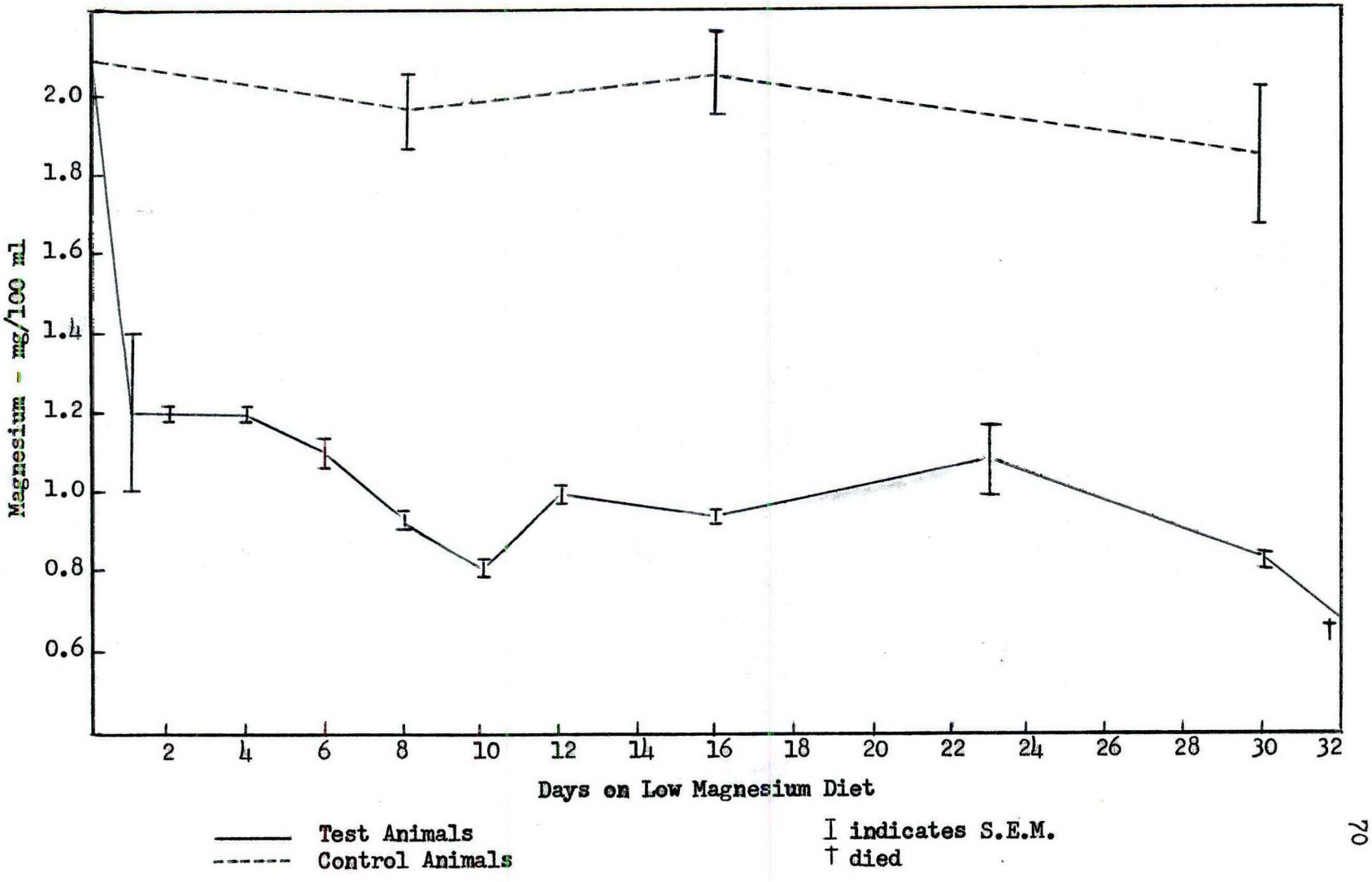


TABLE V

Magnesium Content In Serum and Tissue Magnesium Concentration
In Five And Ten Week Old Rats On A Low Magnesium Diet

Age	Group	Serum Mg mg/100ml	Adrenal Mg mg/g	Adrenal Wt. mg	Heart Mg mg/100g	Muscle Mg mg/100g	Kidney Mg mg/100g	Brain Mg mg/100g
5 wks	Stock	2.0 ± 0.3 (23)	0.39 ± 0.03 (18)	9-30				
	Controls	2.1 ± 0.1 (30)	0.40 ± 0.02 (30)	11-31	22.6 ± 1.4 (14)	24.0 ± 0.2 (4)	32.0 ± 2.2 (4)	10.7 ± 1.3 (6)
	2 days	1.2 ± 0.1 (2)	0.26 ± 0.07 (2)	17-21				
	4 days	1.2 ± 0.03 (2)	0.28 ± 0.04 (2)	16-20				
	6 days	1.1 ± 0.04 (2)	0.25 ± 0.03 (2)	15-17				
	8 days	0.93 ± 0.08*** (14)	0.29 ± 0.02*** (10)	9-16	23.2 ± 1.0 (4)		23.4 ± 1.2* (4)	
	10 days	0.8 ± 0.2** (2)		24-25				
	12 days	1.00 ± 0.06*** (11)	0.23 ± 0.03*** (11)	28-36				
	16 days	0.94 ± 0.05*** (21)	0.37 ± 0.03 (15)	17-41	17.1 ± 0.8** (12)		19.2 ± 0.1** (2)	10.4 ± 1.0 (4)
	23 days	1.08 ± 0.11 (4)	0.36 ± 0.03 (4)	19-31				
	30 days	0.84 ± 0.04	0.18 ± 0.04 (4)	19-36	23.9 ± 1.5 (3)			9.3 ± 0.9 (4)
	33 days	.60 (1)		19				
10 wks								
	79 days	0.32 (1)		34-35				
	106 days	0.86 (1)	0.41 (1)		20.9 (1)	23.0 (1)	24.1 (1)	

Values are the Mean ± S.E.M.

Numbers in parentheses indicate number of animals in each group.

P (Students' t test) **<0.01 ***<0.001
*<0.05

normal value, 0.18 ± .04 (s.e.m.). One of the ten-week old group of rats surviving to 106 days had an adrenal magnesium concentration of 0.41 mg/g, higher than the control figure, and this is a good demonstration of how the adrenal may be maintained while other tissues are depleted (Table V and Table VI).

The magnesium content of the heart was essentially unchanged in all test groups except for the 16 day groups of female rats (Table VI). Here the magnesium concentration in the heart was significantly lower than in the controls ($p < 0.01$). A fall in the magnesium content of the heart was also recorded for the male rats, but the number of rats studied were too small to be significant (Table VI).

It was anticipated that muscle magnesium levels would fall, and a significant drop in the muscle concentration of magnesium was noted in the male rats ($p < 0.01$), but not in the females (Table V and VI).

The kidney showed a remarkable rate of magnesium depletion, dropping from a normal level of 32.0 ± 2.2 (s.e.m.) mg/100 g to 23.4 ± 1.2 (s.e.m.) in the female rats at eight days ($p < 0.05$) and later to 19.2 ± 0.1 (s.e.m.) at 16 days ($p < 0.01$) (Table V). The effect on the kidneys of the male rats was even more pronounced, the magnesium level dropping from 16.7 ± 0.1 (s.e.m.) to 8.1 ± 1.0 (s.e.m.) by 16 days ($p < 0.001$).

The magnesium concentrations in brain showed a slight but not significant drop in the female rats at 16 days (Table VI). However, in the male rats a significant fall was observed at 16 days, from 11.4 ± 0.6 (s.e.m.) to 9.5 ± 0.2 (s.e.m.) ($P < 0.05$).

TABLE VI

Serum And Tissue Magnesium Concentration In Five-Week Old Male And Female Rats On A Low Magnesium Diet For Sixteen Days

Group	Serum Mg mg/100ml	Heart Mg mg/100g	Kidney Mg mg/100g	Brain Mg mg/100g	Nerve Mg mg/100g	Thymus Mg mg/100g	Testes Mg mg/100g
Controls (female)	2.1 ± 0.1 (30)	22.6 ± 1.4 (14)	32.0 ± 2.2 (4)	10.7 ± 1.3 (4)			
Controls (male)	1.4 ± .0 (2)	16.0 ± 1.3 (2)	16.7 ± 0.1 (2)	11.4 ± 0.6 (2)	34.3 ± 0.7 (2)	26.3 ± 0.2 (2)	9.0 ± 0.1 (2)
Test (female)	0.94 ± 0.05*** (21)	17.1 ± 0.8** (12)	19.2 ± 0.1** (2)	10.4 ± 2.0 (4)			
Test (male)	0.86 ± .04***	14.4 ± 1.2 (4)	8.1 ± 1.0*** (4)	9.5 ± 0.2* (4)	37.4 ± 0.7 (4)	24.8 ± 1.5 (4)	11.1 ± 1.3 (4)

Values are the Mean ± S.E.M.

Numbers in parentheses indicate number of animals in each group.

P (Students' t test)
* <0.05
** <0.01
*** <0.001

TABLE VII

Magnesium Concentration Of Skeletal Muscle Of Five Week Old Male And Female Rats On A Low Magnesium Diet For Sixteen Days

Group	mg/100 g Wet Weight	mg/100 g Dry Weight	% Water Content
Control Male	24.0 ± 4.0 (2)	123.5 ± 3.6 (2)	76.6 ± 3.0 (2)
Control Female	24.0 ± 0.2 (4)		
Test Male	17.3 ± 2.4 (4)	70.0 ± 11.6* (4)	75.0 ± 1.3 (4)
Test Female	28.5 ± 2.0 (2)		

Values are the mean ± S.E.M.

Numbers in parentheses indicate number of animals in each group.

P (Students' t test)

* = 0.01

Because of the changes in nerve, thymus, and testes found at necropsy, it was decided to assay these tissues for magnesium (Table V). Nerve and testes showed an apparent slight increase in magnesium concentration (not significant). These animals appeared slightly dehydrated, - perhaps, as was observed with the skeletal muscle, a better picture of the change in magnesium concentration might have been obtained on a dry weight basis (Table VII). The thymus showed a decline in magnesium concentration, from 26.3 ± 0.7 (s.e.m.) mg/g for the control group to 24.8 + 1.5 (s.e.m.) for the male rats on a low magnesium diet (Table VI). With the few numbers of rats tested, these figures were not significantly different.

Thirty of the control rats were compared to 18 stock rats (receiving Purina Lab Chow and tap water to drink, but otherwise treated in the same manner). The serum and adrenal magnesium were each slightly higher in the controls than in the stock rats. Surprisingly, the total catecholamines were 44 percent higher in the control rats than in the stock rats. Norepinephrine constituted 20 percent of the total catecholamines in each case (Table VIII).

While adrenal magnesium levels fell 37 percent in the group on the test diet for eight days, adrenal ATP levels rose to 75 percent above normal. The ratio of magnesium to ATP changed from 10:11 in the controls (on a weight basis) to 10:27 in the eight day test group. During this period total catecholamines dropped about 15 percent, from 0.63 mg/g to 0.54 mg/g, and the molar ratio of catecholamines to ATP

TABLE VIII

Effect Of A Low Magnesium Diet On The Concentration Of ATP And Catecholamines In The Adrenals Of Young Female Rats

Age	Group	Adrenal Mg mg/g	Adrenal ATP mg/g	Total Catechol. amine mg/g	Epin. mg/g	Norepin mg/g	% Norepin	Molar Ratio Cat./ATP
5wks								
	Stock	0.39 ± 0.03 (18)		0.44 ± 0.04 (7)	0.35 ± 0.05* (7)	0.09 ± 0.04 (7)	20.4	
	Control	0.40 ± 0.02 (30)	0.44 ± 0.04 (11)	0.63 ± 0.04 (16)	0.52 ± 0.05 (16)	0.13 ± 0.02 (16)	20.6	4.15:1.00
	8 days	0.29 ± 0.02*** (10)	0.77 ± 0.07*** (8)	0.54 ± 0.03 (8)	0.40 ± 0.03** (8)	0.14 ± 0.01 (8)	25.9	1.98:1.00
	12 days	0.23 ± 0.03*** (11)		0.38 ± 0.05 (4)	0.28 ± 0.05** (4)	0.10 ± 0.01 (4)	26.4	
	16 days	0.37 ± 0.03 (15)	0.42 ± 0.04 (14)	0.60 ± 0.05 (17)	0.48 ± 0.04 (17)	0.12 ± 0.02 (17)	20.0	
10wks								
	106 days	0.41 (1)	0.70 (1)	1.37 (1)	1.07 (1)	0.30 (1)	21.8	5.52:1.00

Values are the Mean ± S.E.M.

Numbers in parentheses indicate number of animals in each group.

P (Student's t test)
*<0.05
**<0.01
***<0.001

dropped from 4:2 / 1.0 to 2.0 / 1.0 (Table VIII). Norepinephrine showed a slightly increase in concentration that was not significant, rising from 20 percent of total catecholamines for control rats to 26 percent for the eight and 12 day groups, and then falling back to 22 percent for the 30 day group. While norepinephrine appeared to increase slightly in the eight day group, epinephrine levels fell by 23 percent ($p < 0.01$). Norepinephrine showed a slight decrease in the 12 day group, about 23 percent, while epinephrine showed a 46 percent decline over control rats ($p < 0.01$).

The adrenal magnesium concentration in the group on the low magnesium diet for 12 days fell 42 percent to a low of 0.23 mg/g. This was accompanied by a decline in total catecholamines, to 0.38 mg/g (40 percent less than controls). Here again, a decline in epinephrine content accounted for most of the change.

Some recovery was apparent in the rats maintained on a low magnesium diet for 16 days (the adrenal magnesium concentration had risen to 0.37 mg/g, not significantly below normal). The adrenal ATP and catecholamine control returned to the control level. The ratio of norepinephrine to total catecholamines, which had been 26 percent at eight and 12 days, had fallen to 20 percent, approximately the same value as for the controls. The molar ratio of catecholamines ATP rose from 2:1 at eight days to the normal figure of 4:1.

One test rat maintained on a low magnesium diet for 106 days had adrenals with an ATP concentration 59 percent above normal. Total

catecholamine levels had risen to 1.37 mg/g or 311 percent above the control figures, while the ratio of norepinephrine to epinephrine was normal. The molar ratio of catecholamines to ATP had risen to 5.52:1.00.

In rats on a low magnesium diet for eight days the heart showed a slight but not significant increase in the concentration of ATP and catecholamines (Table IX). The molar ratio of catecholamines to ATP remained unchanged at 0.27 to 1.00. Since there are often wide differences in susceptibility to magnesium deficiency in each group of test animals, the rats with low ATP values were selected for study out of the test group and compared to the controls. The average serum magnesium of this pair was 0.76 mg percent, and the magnesium concentration of the heart was depressed 13 percent below that of the controls. The ATP concentration was 61 percent of that of the control rats ($p < 0.05$). The total catecholamine content was slightly elevated, giving a molar ratio of catecholamines to ATP of 0.42 to 1.00 or a ratio 50 percent to 60 percent, greater than that for the controls. However, more animals should be studied to give significance to these figures. The concentration of norepinephrine as well as the ratio of epinephrine to norepinephrine were higher than previously reported (14). This may be related to the deficiency state, but more studies are clearly indicated.

The concentrations of adenosine triphosphate (ATP) in the myocardium was significantly depressed at eight days, - about 27 percent below that of the control group ($p < 0.05$) despite only a small change

TABLE IX

Effect Of A Low Magnesium Diet On The Concentration Of ATP And Catecholamines In The Hearts Of Five Week Old Female Rats

Group	Heart Mg mg/100 g	Heart ATP mg/g	Total Catechol. mg/g	Epin. mg/g	Norep. mg/g	%Norep. T. Cat.	Molar Ratio Cat./ATP
Control	18.0 ± 1.1 (4)	0.264 ± 0.029 (4)	0.022 ± 0.002 (4)	0.014 ± 0.001 (4)	0.008 ± 0.003 (4)	36	0.27:1.00
Test (16 days)	16.3 ± 0.4 (8)	0.274 ± 0.028 (8)	0.026 ± 0.002 (8)	0.017 ± 0.002 (8)	0.009 ± 0.001 (8)	35	0.27:1.00
Susceptible	15.6 ± 0.2* (2)	0.162 ± 0.01 (2)	0.024 ± 0.006 (2)	0.016 ± 0.005 (2)	0.008 ± 0.000 (2)	33	0.42:1.00

Values are the Mean ± S.E.M.

Numbers in parentheses indicate the number of animals in each group.

P (Students' t test)

* <0.05

in the concentration of magnesium (Table X). At 16 days, however, the ATP level had returned to normal levels, while the magnesium concentrations apparently dropped to about 24 percent below that of the controls ($p < 0.01$). The rat surviving to 106 days was found to have a concentration of ATP in the myocardium of only 0.10 mg per g, or 38 percent of normal. The heart of this rat was considerably heavier than that of controls, when compared on the basis of body weight.

The kidney showed the most dramatic change in animals on a low magnesium diet (Table X). The ATP level fell 27 percent by the eighth day, exactly paralleling a drop of 27 percent in the magnesium concentration in the kidneys ($p < 0.05$). The depletion of these two continued; at 16 days the ATP level was 45 percent of normal ($p < 0.05$) while the magnesium concentration was 60 percent of normal ($p < 0.01$). In one rat surviving to 106 days on the low magnesium diet, the ATP concentration was 27 percent of normal; the concentration of magnesium in the kidneys ofthis animal was 75 percent of the controls.

Since lesions were found in the brain and peripheral nerves, the brain was assayed for ATP in rats that had been on the test diet for 16 days. While the ATP level in the test animals was depressed by slightly more than 10 percent, the number studied did not permit a test for significance (Table X).

TABLE X

Tissue Concentration Of Adenosine Triphosphate In Rats On A Low Magnesium Diet

Age Group	Serum Mg mg/100 ml	Heart mg/g	Muscle mg/g	Kidney mg/g	Brain mg/g
5wks					
Controls	2.1 ± 0.1 (30)	0.26 ± 0.01 (9)	0.34 ± 0.02 (5)	0.11 ± 0.1 (4)	0.054 ± 0.04 (2)
8	0.93 ± 0.1*** (14)	0.19 ± 0.03* (4)	0.32 ± 0.03 (4)	0.08 ± 0.01 (4)	
16	0.94 ± 0.1*** (21)	0.27 ± 0.02 (10)	0.26 ± 0.02* (2)	0.05 ± 0.01* (2)	0.048 ± 0.01 (2)
10wks					
106	0.86 (1)	0.10 (1)	0.14 (1)	0.03 (1)	

Values are the Mean ± S.E.M.

Numbers in parentheses indicate number of animals in each group.

P (Students' t test)
* <0.05
** <0.01
*** <0.001

CHAPTER IV

DISCUSSION

Young rats (five weeks old) responded more acutely to magnesium deficiency than older rats (ten weeks old). This may be due to a greater metabolic demand made of the younger rats by their more rapid rate of growth. In addition, although growth hormone is reported to increase the intestinal absorption of magnesium, it also increases urinary excretion of magnesium (16). When dietary magnesium is present in insufficient amounts, growth hormone may then contribute to a magnesium deficient state by its effect on urinary magnesium excretion.

In both the five week-old and ten week-old rats, growth was arrested by the eleventh or twelfth day (Figures 2 and 3). A rat surviving 106 days weighed less than at the start of the test, despite the fact that it was able to maintain a serum magnesium level of 0.86 mg percent. One seven week-old rat lost weight steadily over a period of 18 days (about 20 percent of its body weight). When magnesium chloride was added to the drinking water, it had a remarkable renewal of growth, having a 42 percent increase in body weight within five days (Table III). Comparable test rats with a serum magnesium level of about 1.0 mg percent during this period

continued to lose weight. In rats, growth appears to be arrested when serum magnesium levels fall to about 1.0 to 1.2 mg percent.

Although female rats were used primarily in this study, one group of five-week old male rats was studied to note sex differences in response to a low magnesium diet. Seelig has commented on the greater resistance of women to magnesium depletion, but no one has noted this in other species. Seelig cites a number of magnesium balance studies in humans, clearly demonstrating that women absorb magnesium more completely from the gut and are able to reduce urinary magnesium loss much more efficiently than men when dietary magnesium is insufficient (77). Seelig uses this evidence as part of the support for her hypothesis that chronic magnesium depletion states occur more commonly in men than in women. The young male rats studied here showed little or no differences in clinical signs, as compared to the females of comparable age. However, at necropsy there were severe lesions in the kidneys of all the male rats, whereas few renal changes were observed during dissection of the female rats of the same age. No one has presented an explanation for this difference in resistance. Some indication was found in this study that the female rats normally have higher magnesium levels than the males (Table VI).

The first signs of deficiency (at four days) of hyper-irritability, may actually have been present earlier, in view of the fact that the serum magnesium levels drop as much as 36 percent in the first 24 hours on a low magnesium diet. If, however, the hyper-

irritability is more dependent on metabolic changes, this would account for the appearance of signs at four days, since histological and biochemical changes are first detected on the fourth day. The rapid drop in serum magnesium suggests that serum magnesium levels may fluctuate widely according to variation of the dietary intake of magnesium. Perhaps the portion of the serum magnesium that is so quickly depleted is the ionic form (probably as a hydrate) with the protein-bound portion (about 45 percent) being more resistant to loss in the face of a decreased magnesium intake for a short period of time (16). The serum magnesium remained fairly constant at the lower level (about 1.2 mg percent) until the eighth day, at which time it dropped precipitously again to 0.8 mg percent, a level only 32 percent of that of the controls (Table V). This sudden change is hard to explain. Ko et al. have demonstrated marked changes in the kidney tubules at eight days, and injury to the mitochondria, and dissociation of oxidative phosphorylation occurs even earlier than this (45). Perhaps magnesium bound to serum protein is also lost at this time, due to the renal tubular injury.

The clinical signs appearing at about this time are well-documented, except for piloerection, which was observed as early as eight days, and seemed to be present in almost all test rats (Figure 1). Bois describes the release of histamine at this time -- perhaps catecholamines are also released, and could in part account for the hyper-reactivity of these rats, along with the ionic imbalance and the effect at the neuromuscular junction (10). As the magnesium level

falls, the serum calcium level has been found to rise, and this will, in itself, result in enhanced contractions of vascular smooth muscle, and presumably increased vascular resistance and elevated blood pressure (30).

A period of apparent compensation for the low magnesium diet has been observed between 12 and 16 days in many of the rats. This is probably the result of reabsorption from the bone which is a major source of magnesium in the young animal in magnesium deficiency (27). Some failed to compensate, and died in convulsions. It would seem that the kidney changes in some were so severe that what little serum magnesium was present could not be conserved. That this is true is suggested by changes in the rate of the urinary magnesium excretion (Table IV). After two days, the 24 hour urinary loss of magnesium had dropped to only 21 percent of normal. Either most of the unbound serum magnesium had been lost, or the kidney tubules adapt very quickly to magnesium depletion states. Magnesium excretion remained at a very low figure until the 12th day when it began a rapid rise, reaching control levels at 23 days. Kidney tubular lesions resulting in decreased reabsorption of magnesium at this point may explain this apparent decompensation. Susceptible rats died in this period -- as many as two in every four in some groups. A "cycle" of increasingly severe renal tubular changes and falling serum magnesium values may bring these susceptible rats to the point where death in convulsions occurs from the low serum magnesium (about 0.6 mg percent). Rats

surviving this critical stage are again able to conserve magnesium to some degree, despite severe renal tubular injury, as evidenced by a fall in 24 hour urine magnesium values in a 30 day group (i.e. more effective tubular resorption).

The most obvious clinical sign of magnesium deficiency, the cutaneous hyperemia and edema appearing at six to eight days, could be related to the release of histamine by the mast cells (serum levels of histamine rise to four times the normal level) as Bois has suggested (10). However, severe vascular lesions occur also, and the hyperemia may represent vasodilatation accompanying degenerative changes in the media of the small arteries and arterioles. The edema may accompany the degenerative changes in the intima. Some reports describe a pallor that precedes the hyperemia (47). This was not noted in these rats. The hyperemia at times was a fairly bright red, at other times was a dark purplish-red, a marked local passive congestion. Edema has been reported also in magnesium deficiency in the dog and in man (96,79). Purpuric areas were noted in one case of experimental magnesium deficiency in man; these proved to be due to a "perivasculitis", but this may be analogous to the lesions in the rat (79). Patchy loss of hair, noted in some of these test rats, has been previously reported in magnesium deficiency states in the rat and the dog. This may be due to poor nutrition due to the "polyarteritis" or to growth failure. The same may be said for the skin lesions seen in this or other studies. Interestingly, the skin lesions in the ten-week old rats, which never were as severe as in

the younger rats, appeared to fade after the fourth week. The only signs of deficiency after this in these older rats were the arrest of growth and slowly increasing debility, a scaly dermatitis, weakness, tremors, irritability and incoordination. The edema, however, returned in the final weeks before death. None of the ten week-old rats survived to six months, and none of the five week-old rats survived to eleven weeks.

The convulsions of magnesium deficiency have been previously described, but not in great detail for the rat. As in man, confusion, apprehension, agitation, aimless or wild behavior often preceded the convulsions. Tremors, weakness, incoordination were present in nearly all rats with severe deficiency, but did not seem to be associated with the convulsions. The convulsions often occurred ten to twenty minutes after handling. Piloerection was noted to be prominent in all of these rats. Handling or other stress in these rats undoubtedly causes a release of catecholamines. The ability of the heart, brain, blood vessels, etc., to store catecholamines is probably diminished, due to decreased levels of magnesium and ATP. This appeared to be the case in one group of test rats in which ATP and catecholamine levels were determined in the myocardium. The two most susceptible rats in the group showed an excess of catecholamine as compared to the amount of ATP present, the ratio of the two being 0.42 : 1.00 as compared to 0.27 : 1.00 for the controls. In addition, the inactivation of catecholamines may be diminished. As a result,

in a state of magnesium depletion the body may be unable to cope with increased levels of catecholamines, and convulsions may result due to a combination of this and other factors.

Pulmonary edema is a prominent feature of hypomagnesemic convulsions -- this is most likely due to congestive failure, which was found in all rats dying of magnesium deficiency. The congestive failure may be due to sino-atrial block, which has been reported to occur by Greenberg and Tufts (28). In addition, a dissociation in oxidative phosphorylation occurring in myocardial mitochondria as early as four days (Vitale) probably leads to decreased levels of ATP and phosphate reserves and a marked drop in cardiac reserve. Evidence that this is true was found in this study, - the ATP level in the myocardium was depressed by 27 percent at eight days ($p < 0.05$). Heart weights of the test rats were increased as compared to control rats on the basis of body weight. Histologic and electron microscopic changes in the heart are well documented (34,96). The edema observed in many of these animals may represent chronic congestive failure as well as a generalized vasculitis. Myocardial lesions observed in dogs and cattle were certainly severe enough to result in congestive failure, and may explain the pedal edema observed in dogs (34,16). One case of experimental magnesium deficiency in man also resulted in dependent edema and this was relieved by administration of $MgSO_4$ (79).

Another likely factor in the edema is the retention of sodium. Magnesium depletion has been shown to result in an elevation of serum

sodium (1). Intracellular levels of potassium fall in magnesium deficiency despite adequate dietary intake of potassium (55). This may represent a decreased ability of the cell to actively transport potassium into the cell from the extracellular fluid, and to expel sodium. The rats in this study were not observed to have partial flexing of the hocks or the "knuckling" from marked weakness as observed in dogs, though progressive muscular weakness was evident, as has also been reported in experimental magnesium deficiency in man (96,92,77). Surprisingly, muscle magnesium content in this study showed a slight increase in the female rats (on the basis of fresh muscle weight) in contrast to work by McIntyre and others who performed their determinations on the basis of dried, fat-free weight and demonstrated a decrease in muscle magnesium (55). This apparent increase in muscle magnesium content may actually represent dehydration. The muscle of the male test rats did show a significant fall in muscle magnesium, on the basis of dry weight. Many of the test rats appeared severely dehydrated, with "tenting" of the skin. Dehydration would appear to make the determinations showing an absolute change in serum and tissue levels of magnesium, ATP and catecholamines doubly significant. Also, the arrest of growth may represent in part a dehydration of the animal. The dehydration may be due to loss of potassium and magnesium from the cell, and also to decreased renal tubular resorption of water because of the severe injury to the renal tubules occurring in magnesium deficiency. These rats accepted the

distilled water as well as tap water, and had an adequate intake. Urine volumes were noted to be markedly increased in some rats, however, from a normal amount of 5 to 10 ml to as much as 20 to 30 ml.

A loose, dark stool was observed in some of the deficient rats. Others have reported diarrhea and melena (34). Atrophy of gastrointestinal mucosa and gastrointestinal lesions are found in some of the deficient rats. These changes, and injury to epithelium of the skin (and renal tubules) may be due to depression of reproduction of the basal epithelial cells. Microscopic examination of the basal cell layer of the duodenum of one severely deficient rat disclosed fewer mitotic figures than in stock rats.

Rats selected for study of recovery from magnesium deficiency made a remarkable recovery, as previously noted. Clinical signs disappeared in two days, similar to reports of experimental magnesium deficiency in man. The remarkable spurt of growth following the test period is hard to explain; perhaps part of this "growth" is due to recovery from dehydration and part is due to the effect of an increase in rate of secretion of growth hormone.

Skeletal muscle changes were noted during necropsy of the test rats and were observed in the tissue sections as well. These have been thoroughly investigated by Heggtviet et al. (35). The effect on skeletal muscle may be related to the fall in muscle potassium and the increase in muscle calcium that occurs in magnesium deficiency. Calcium is deposited in muscle fibers, perhaps as calcium phosphate. Ko et al. suggest that magnesium ion is necessary to keep calcium and

phosphate in solution (45). In connection with this, it is known that administration of calcium salts will aggravate magnesium deficiency (54).

Many of the clinical signs indicate that the central nervous system is injured in magnesium deficiency -- irritability, vascular changes, progressive incoordination, weakness, tremors, convulsions. Greenberg and Tufts have previously reported that there is irritability or injury to the pons or midbrain in magnesium deficiency, resulting in convulsions (28). Brain weights were heavier, in general, than those of the controls (compared on the basis of body weight) and appeared swollen. The histologic changes in the cerebellum were as described by others. In addition, degenerative changes were found in the hippocampus. No change was observed in the cortical cells.

These lesions may be the result of anoxia suffered during convulsions or may be due to a metabolic derangement associated with magnesium deficiency. While a decline of 10 percent in the concentration of ATP was found in the deficient rats after 16 days on a low magnesium diet, the number studied was too small to make this difference significant. It is known that the pyruvic oxidase system of the brain is magnesium dependent (54). Elevated levels of catecholamines may also be a factor in these changes, as well as the imbalance between the calcium and magnesium ions, and the loss of potassium from the cell. Krnjević has observed in his micropipette studies that extracellular Mg^{++} is involved with the suppression of

spontaneous firing of cortical neurons (46). Apparently a fall in extracellular brain magnesium concentration (shown to occur in cerebrospinal fluid in magnesium deficiency) will result in an increase in the irritability of cortical neurons, and perhaps other neurons as well (91).

Peripheral nerves were swollen, dull in appearance, and soft, suggesting changes in dorsal root ganglia and spinal cord. Cranial nerves were similarly affected. The magnesium concentration in these nerves was slightly increased in concentration in the deficient rats.

The clinical appearance (including piloerection), growth failure, and mortality rate suggested that magnesium deficiency produces stress as early as six to eight days, and adrenal changes were found at necropsy. Changes that might have been due specifically to magnesium deficiency were difficult to dissociate from those due to stress. In general, the adrenal medulla of the test rats appeared shrunken at eight days, as compared to the controls. In addition, the serum magnesium was at its lowest level at this point (Figure 8). Many of the rats compensated for the rapid depletion of magnesium (12 to 16 days), and the adrenal medulla appeared larger and more like the controls. This change in size of the medulla was approximately paralleled by changes in the adrenal magnesium concentration, which fell significantly at eight and 12 days, then rose to normal levels at 23 days. Decompensation then occurred, and adrenal magnesium levels fell to one-half the normal value at 30 days. No five week-old rats survived beyond 40 days on the low magnesium diet.

The ten-week old rats were better able to compensate, - one rat surviving to 106 days had normal adrenal magnesium values. ATP values were about 60 percent above normal, and total catecholamine levels were over 300 percent above normal (Table VIII). However, other organs in this rat showed severe changes.

In the five-week old rats, adrenal ATP concentrations rose significantly above normal after eight days. Total catecholamines fell significantly (due primarily to changes in epinephrine concentrations) and the molar ratio of catecholamine to ATP fell from 4:1 for the controls to 2:1 for the rats depleted of magnesium for eight days (Table VIII). This change might conceivably have been due to stress alone. However, the concentration of magnesium in the adrenal did fall significantly. The depletion of magnesium in the cytoplasm may have resulted in an accumulation of ATP, since reactions involving ATP require Mg^{++}. If cytoplasm is depleted of magnesium much more quickly than mitochondria (as is suggested by studies with Mg^{28}), then the mitochondria could continue to synthesize ATP for a time at a faster rate than it could be utilized.

Along with decreased storage of adrenal medullary catecholamines (because of low levels of Mg^{++}), the release of catecholamines due to stress must be considered. At eight days the effects of circulating catecholamines or vascular smooth muscle would be potentiated by the imbalance between calcium and magnesium, and may account for the marked vascular changes at this time (although histamine may be a factor here, also). Elevated catecholamine levels may also be a factor

in the early changes in the kidneys, along with the direct effects of low serum magnesium on the enzyme activity of tubular epithelium.

Further depletion of magnesium and catecholamine was found in the group of rats on a low magnesium diet for 12 days (Table VIII). This was the point in this study in which most of the early deaths occurred. Those rats surviving to 16 days showed evidence of compensation, - the adrenal medulla was near normal size again; magnesium, ATP and catecholamine levels were again approximately normal, and the molar ratio of catecholamine to ATP was again about 4:1. This occurred despite severe changes in the kidney, heart and other organs. Although the scope of this study did not allow studies of catecholamine and ATP concentration over a longer period of time, it is probable that these values fell in those animals that failed to compensate, or remained high in those that were able to adapt to magnesium deficiency for a longer time. This was found to be the case for the rat that survived to 106 days (Table VIII).

In one rat of a group of four kept for a study of the effects of chronic magnesium deficiency, an 8.6 gram malignant thymoma was found. This brings the total number of cases reported to seven, among a total of 142 rats in which chronic magnesium deficiency was induced, or an incidence of about 5 percent. Spontaneous tumors of the thymus in the rat are rare, and this incidence would seem to indicate more than a spurious association. The suggestion has been made that in chronic magnesium deficiency there is a mobilization of magnesium from the chromatin material in the nucleus (where Mg^{++} is in higher concentration

and somewhat less freely exchangeable than in the cytoplasm) resulting in mutations and neoplasms (39). No one has advanced an explanation for the thymus being the only organ in which the neoplastic change is produced. In this study, there was an infiltration of nearby tissues, involvement of lymph nodes, and an infiltration of the spleen (with massive splenomegally), liver, bone marrow and heart. In addition, there was an area of neuroblastoma-like tissue near the abdominal aorta.

The neoplasm seemed very similar to thymic lymphosarcoma which has been produced experimentally in the rat with mouse leukemia virus (passage A) by Gross. Leukemia extracts from mice were injected intra-peritoneally in newborn rats. Spontaneous leukemia in rats is very rare, and then occurs in old animals. Perhaps the rat harbors this virus, but does not develop the disease unless it is infected at a very early age.

As demonstrated by ionizing radiation, chromosomal changes are more easily induced in the lymphocyte and this may apply for the "thymocyte" as well. If prolonged magnesium deficiency results in mobilization of magnesium from the nucleus and chromatin material, this may induce mutations similar to those induced by peroxides and other radicals created by ionizing radiation (40). This type of tumor is probably produced by a combination of factors. A third factor in pathogenesis may be the depression and arrest of growth due to magnesium deficiency despite the presence of growth hormone. A more

primitive mutant cell type with less need for magnesium may then be able to respond to growth hormone without competition from normal tissues, and with diminished resistance of the body toward the neoplastic tissue, as is suggested by the work of Petrov and Fidler (66). Finally, there may be a general impairment of resistance so that any mutogenic virus that is harbored in the animal may be able to produce disease.

In connection with this finding, the test rats in general were found to have large thymi, - on an absolute weight basis in some cases, and when compared to the control rats on the basis of body weight for almost all cases. If one considers that these animals are stressed, it is remarkable that the thymus did not atrophy.

It seems worth noting that the changes associated with magnesium deficiency are in some respects similar to those reported to be associated with hypertrophy and tumors of the thymus in man, such as muscular weakness and histological changes in the skeletal muscles and leukemia. Hypogamma-globulinomia and dysgamma-globulinemia are also found at times in persons with an enlarged thymus. It would be worthwhile to look at gamma globulin changes in the rat with magnesium deficiency.

The association between chronic deficiency of magnesium and an enlarged thymus in man (a seven-year old child) has already been noted by Miller (62). Mendel also reports that magnesium deficiency may be associated with abnormalities of the thymus, and with myasthenia gravis

as well (58). These may be only spurious associations, of course. However, it is suggested that magnesium metabolism may merit investigation where an enlarged thymus and one of these associated conditions is found. The thymus may have some relationship to magnesium metabolism, as Mendel hypothesizes (58).

As a result of these studies, which have demonstrated a definite relationship between magnesium deficiency, decreased tissue concentration of adenosine triphosphate, and a generalized impairment of metabolism (including the storage of catecholamines), it seems certain that either severe magnesium deficiency states or long-term, low-grade magnesium deficiency states can produce degenerative changes in the cardiovascular and renal systems, central nervous system, and in other organs, as Seelig suggested (77). Severe deficiency states occur infrequently, although greater numbers are being diagnosed now than previously. Some balance studies have indicated that low-grade deficiency states are common, and inspection of data on serum and blood magnesium concentrations collected by Jordan and Batsakis et al. gives additional support to these findings (42,6). Apparently, magnesium deficiency states occur more often in children than in adults, according to Jordan's data and therefore, it would be worthwhile to consider possible deficiency states in clinical disorders affecting both children and adults.

CHAPTER V

CONCLUSIONS

1. A review of current literature indicates that while magnesium deficiency may occur commonly in children and adults, and have important clinical effects, the pathogenesis of these effects is poorly understood.

2. Magnesium deficiency in rats caused a marked suppression of growth, and did arrest growth completely for long periods.

3. Younger rats (five-weeks old) were more susceptible to magnesium deficiency. Older rats (ten-weeks old) survived for long periods in a magnesium deficient state, but were chronically ill.

4. Male rats were more susceptible to magnesium deficiency than females.

5. Gross changes and microscopic lesions were as described by others. Lesions found in the central nervous system are consistent with Greenberg and Tufts' observation that the convulsions of severe magnesium deficiency are primarily of central origin rather than due to increased release of acetylcholine at the myoneural junction.

6. Serum magnesium levels fell below the normal range within 24 hours when the dietary intake of magnesium was decreased. Some fluctuations occurred later, indicating an internal mobilization of

magnesium from tissue stores. Moribund animals showed a sharp decline in serum magnesium concentrations.

7. The concentration of magnesium in the heart fell significantly after 16 days on a low magnesium diet. A significant decrease in ATP concentrations was seen in one-half of the deficient group, but little change occurred in the concentration of catecholamines in the heart.

8. After 16 days on a low magnesium diet, the concentration of magnesium in skeletal muscle decreased significantly in male rats, but not in the females.

9. Of the organs studied, the kidneys were most easily depleted of magnesium, the magnesium concentration falling 27 percent in eight days and 40 percent in sixteen days in female rats, and to less than half the normal concentration in male rats. Unlike heart, adrenal, and brain, the fall in magnesium content continued throughout the period of magnesium depletion.

10. Magnesium deficiency results in a significant decrease in the concentration of ATP in the heart, kidney, and skeletal muscle. A 10 percent decrease in the concentration of ATP in the brain was also noted.

11. The adrenal magnesium concentrations fell significantly after eight days on a low magnesium diet. The molar ratio of total catecholamines to ATP in the adrenals of the control female rats (4:1) fell to 2:1 after eight days, but returned to normal after sixteen

days. This change was due to an elevation of ATP with a decrease in epinephrine concentrations, and indicates that catecholamine storage in the adrenal medulla is affected in magnesium deficiency states.

12. Chronic magnesium deficiency was observed to produce a malignant neoplasm of the thymus in one out of a group of four rats studied. This observation is in agreement with several recent reports associating chronic magnesium deficiency with thymic tumors.

13. The results of this study further support the view that magnesium deficiency states may be involved in the pathogenesis of some of the common disorders of the cardiovascular, renal, neuromuscular and central nervous systems in man. More definitive biochemical studies are clearly indicated.

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