THE INFLUENCE OF MAGNESIUM DEFICIENCY ON KIDNEY LYSOSOMAL ENZYME LEVELS IN THE RAT

by

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I would like to dedicate this thesis with a quote from Pike and Brown in their section on calcium-magnesium inter-relationships:

> The progression from a state of confusion to one of clarity of understanding is often tedious, but it is an essential characteristic of the development of knowledge. As Comar so aptly put it "... ....utter confusion is better than complete ignorance." [1]

Further, I would like to express my sincere appreciation and gratitude to those people who helped me in my development of knowledge:

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My Father, whose faith in me sustained me through many a weak moment.

[1] Pike, R. L. and M.L.Brown, Nutrition: An Integrated Approach, page 445, John Wiley and Sons, Inc, New York, 1967.

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ABBREVIATIONS USED

β-Gly	β-glycerophosphate
DDR	Dublin Disease Resistant - a Sprague Dawley derived strain of albino rats, purchased from Flow Research, Inc. Dublin, Virginia.
PAS	Periodic Acid - Schiff's A histochemical technique for the demonstration of carbohydrate using oxidation with periodic acid followed by reaction with Schiff's reagent (fushin ⊖ H_2SO_3).
PGA	phenolphthalein β-glucuronic acid.

INTRODUCTION

The consumption of semi-purified low magnesium diets by young rats results in the development of a deficiency condition with four major characteristics: 1) reduced growth, 2) neuromuscular and nervous system malfunction, 3) soft tissue calcification and 4) peripheral vasodilation (1).

Mechanisms have been proposed for the involvement of magnesium in the first two characteristics: reduced growth is attributed to the impairment of protein synthesis where the role for magnesium in protein synthesis has been postulated (2), and the malfunctions of the neuromuscular and central nervous system have been suggested to stem from an extracellular magnesium deficiency leading to an increase in acetylcholine action at the nerve endings which lowers the threshold of muscle membrane and inhibits the relaxing factor (3).

Mechanisms for the role of magnesium in the remaining two characteristics have not been established. The vasodilation is believed to result from the degranulation of the tissue mast cells with a consequent liberation of histamine (4); the role magnesium plays in this, however, is not known.

Through various histological and histochemical studies the process of kidney calcification occurring during the magnesium deficiency has been described (5,6,7,8,9). The earliest changes detectable by either light or electron microscopy have been swelling of the tubular epithelium and accumulation of PAS positive material (carbohydrate) in the lumen of

the proximal convoluted tubules. The first calcified foci were seen located intraluminally at the cortico medullar junction; they were primarily apatite (10). Intracellular and interstitial calcium deposits were often associated with lysosome-like bodies but the mitochondria were free of calcification at all stages of the deficiency condition. Neither total kidney magnesium nor mitochondrial magnesium have been shown to be measurably reduced during the period of these profound pathological changes.

Of the several hypothesis advanced to explain the initiation of nephrocalcinosis in magnesium deprived young rats, none seem to account for all the experimental evidence. Gitelman et al. (11) have proposed that a state of hyperparathyroidism is induced by the magnesium deprivation. They have observed increases in serum ionic calcium in magnesium deprived rats and have found that removal of the parathyroid from rats prevents kidney calcification in the deprived animal. Two characteristics of the magnesium deprivation syndrome, however are contrary to the usual picture of hyperparathyroidism; one, the absence of mitochondrial calcification and two, the fact that calcification is restricted to the cortico-medullary junction. Simple hypercalcemia has been proposed as the initiating factor, but serum levels of calcium have been shown to be normal or depressed as well as elevated, dependent upon the experimental conditions (12,13,14). Attempts to explain calcium phosphate precipitation on the basis of altered urinary ratios of calcium, magnesium and inorganic phosphorus concentrations have also failed. It would appear that magnesium deprivation in young rats induces precipitation of calcium phosphate in the renal tubules through some mechanism other than simple changes in urinary or serum levels of calcium

or phosphate. Several authors have concluded that calcium accumulation in the kidneys of the magnesium deficient rat mimics, in its essential features, the process of urolithiasis. Oliver et al. believes that in magnesium deficiency in the rat the whole pathological syndrome of clinical calculosis is reproduced in miniature within the nephron (10). Oral magnesium supplements have been found to be beneficial in the treatment of human idiopathic recurrent kidney stone disease (15,16). Adoption of this point of view has required us to examine current views on the pathogenesis of kidney stones in the effort to explain more precisely the biochemical events leading to kidney calcification in magnesium deficiency.

The problem of the pathogenesis of kidney stones may be viewed in two ways: 1) stones may be initiated by changes which increase the urinary concentration of constituent crystalloids such as a reduction in urine volume or by increased excretion of calcium oxalate, crysteine, uric acid, ammonia or xanthine; or 2) they may be considered to be caused by physiochemical changes conducive to stone formation at normal concentrations of crystalloids. These might include changes in pH, formation of stone matrix, presence of foreign bodies, or the abscence of normally protective substances such as Mg^{++}, pyrophosphate, citrate or inhibitor peptides. The factors in Group I have been more extensively investigated than those in Group II but the latter are undoubtedly of more importance to the particular problem of magnesium deficiency (17).

The "active matrix" theory has been mainly supported by the work of Boyce and King who have shown that stones develop around a glycoprotein matrix. They have identified a substance termed "matrix

substance A" which they have consistently isolated as a component of urinary calculi in humans (18). As recovered from the urine this is a glycoprotein of molecular weight about 30,000 to 40,000. It is not present in normal urine or normal kidney tissue, but is found in the urine and renal parenchyma of stone formers. Boyce and King propose that this glycoprotein may be produced as a response to a variety of renal metabolic disorders and that it is capable of acting as the nucleus for subsequent spontaneous crystallization of calcium oxalate, uric acid or other crystallizable urinary compounds. While this theory has not been proven, as its authors are quick to note, it does answer a number of questions relative to the structure and composition of the stones. This work is also noteworthy in light of the fact that the first histologically detectable abnormality of magnesium deficiency is the accumulation of a highly PAS positive material (presumably glycoprotein) in the lumen of the tubule followed by the deposition or accumulation of calcium phosphate in this same area (10).

While the "active matrix" theory is attractive, the basic cellular defect which might trigger the production of this calcium binding substance has remained elusive. Bunce and Price (19) have suggested that a defect in the ability of the renal proximal tubules to remove glycoproteins from the glomerular filtrate might lead to the accumulation of glycoproteins. The reabsorption of glomerular filtrate proteins depends upon the reabsorption of these materials by pinocytosis and their consequent digestion in the vacuolar apparatus. The goal of this thesis has been to investigate further this hypothesis. We thought it necessary therefore, to review the history and physiology of the lysosomes and their role in the digestive processes of the cellular vacuolar apparatus.

De Duve first described a new set of cytoplasmic organelles which he called lysosomes in 1955. Since that time research on these organelles has led them to be assigned a central role in many cellular processes, both normal and pathological, particularly those involving intracellular digestion, autolysis and tissue injury.

Lysosomes are a heterogeneous class of organelles, the ultrastructure of which varies depending on their origin and their function (20). Further work since 1955 has led de Duve to describe an entire digestive system involving the lysosomes which he calls the "vacuolar apparatus" (21). Its components are lysosomes and prelysosomes. Among the lysosomes de Duve distinguishes primary lysosomes as being those which have not as yet entered into a digestive act, and secondary lysosomes as those which are present or past sites of digestive activity. Prelysosomes are vacuoles containing unattacked material generally destined for future digestion within lysosomes. For prelysosomes containing phagocytized or pinocytized material, de Duve first adopted the term proposed by Straus (22) of phagosome, he later modifies this to give autophagosomes and heterophagosomes, thus being able to account for prelysosomesof the autophagic line.

Shortly after the formation of a phagosome, lysosomal hydrolases are introduced either by merger of the phagosome with a primary lysosome (23) or by contribution from the vesicles of the Golgi couple (24,25,26) toward which the phagosomes appear to stream. The resulting structure is called a "phagolysosome" (27), a "digestive vacuole" or a "secondary lysosome" (21). Depending on the origin of the material being digested, i.e. depending on whether the phagosome is an autophagosome or a heterophagosome, de Duve has suggested that secondary lysosomes be called autolysosomes or heterolysosomes.

Autolysosomes arise in tissues subject to anoxia, ischemia, acute metabolic need, or morphogenic stimuli (21,24). They are bound by the normal single or double unit membranes and frequently enclose degenerating mitachondria, endoplasmic reticulum, microbodies, etc. Frequently it is possible to observe both the products of autophagy and of heterophagy in a single vacuole (21). Vacuoles in which the digestive function has been carried to a point where there is little discrete material which remains intact are terms residual bodies (21). When these arise in cells which abut onto excretory canals such as are present in liver and kidney, the residue may be excreted into the bile or urine by a process termed exocytosis (28,29,30) which is very similar in mechanism to that of secretion.

The functional role of lysosomes in normal cell processes has been well covered in a number of reviews (21,21,24,28,31). Since this thesis is primarily concerned with the pathological situation of kidney calcification occurring during magnesium deficiency, this review will limit itself to the possible interaction of lysosomes in pathological cellular processes.

The importance of lysosomes in cellular pathology lies in the fact that they are intimately involved in almost every response of the cell to challenging agents, and invariably contribute to the pathology and symptomology of the challenge-response syndrome. Generally speaking lysosomes may effect pathological changes at the cellular level in one of two ways: by failing to perform their digestive or lytic functions adequately or by performing them in an injurious fashion. The primary fault, however, may usually be traced back to some outside stimulus rather than to the lysosome itself (21).

The following table, taken from a review article by de Duve and Wattiaux, presents their tentative classification of these pathogenic mechanisms (21).

Cellular Disorders Associated with Lysosomel Malfunction

I. Disorders associated with inadequate lytic activity

A. Infections

B. Intoxications

C. Congestive enlargements of lysosomes

1. Abnormality of enzymic equipment of the lysosomes
2. Overloading of lysosomes with digestible material
3. Overloading of lysosomes with indigestible material

II. Disorders associated with an injurious lytic activity

A. Hydrolytic function of injurious entities

B. 1. Viral infections
2. Intoxications

B. Excessive heterophagy (?)

C. Injurious self-digestion of cells

1. Autophagy
2. Autolysis following lysosome rupture

D. Damage to extracellular structure

1. Enzyme leakage
2. Excessive enzyme extrusion

Among the disorders associated with an inadequate lytic activity, infections and intoxications have very little pertainance to this review. The conditions described as congestive enlargement of the lysosomes, however may play an important part in the etiology of magnesium deficiency and so will be considered further.

Lysosomal enlargement occurs when there is some qualitative or quantitative incompatibility between the lysosomal enzymes and the

material be digested. The fault may lie in the enzymes, in the material or in both. One good example of a true inborn lysosomal enlargement disease is that identified by Hers and his coworkers (32) in glycogen storage disease type II, where the lack of an α-glycosidase capable of degrading glycogen results in the formation of membrane bound vacuoles containing glycogen. Presumably these are secondary lysosomes which, lacking the proper enzyme, only hold the glycogen rather than digesting it. Hers and van Hoof have recently reviewed the list of genetic abnormalities presumed to be lysosomal in origin (31). In this review, they conclude that since the enzymic defect is the primary detectable event, it is the only safe basis for etiological classification.

It is possible that other types of lysosomal related diseases will be discovered. There could be conditions where the primary defect is not the lack of a lysosomal enzyme, but the production of an abnormal molecule, one with which the lysosomal hydrolases could not cope, or one which, because of its altered structure, could be released at sites where it did not belong. In these conditions we would be dealing with congestive enlargement of the lysosomes, types 2 and 3, rather than type 1. Were such a disease described, one would be unable to base a classification on an enzymic defect and so would have to disagree with the conclusions of Hers and van Hoof.

It is also possible that lysosomes suffer from an acquired enzymic defect, for instance through the uptake of an inhibitor, through the lack of a cofactor, or perhaps through the derangement of enzyme biosynthesis. These could perhaps explain the events occurring in magnesium deficiency. Perhaps the deficiency is resulting in an abnormal glycoprotein with which the normal lysosomes present in the renal tubule

cells cannot cope. This is somewhat the case which occurs in many kidney nephroses where the presence of hyalin droplets have been reported. Or perhaps the magnesium is a co-factor for a lysosomal enzyme, thereby, in its absence, causing an acquired enzyme deficiency. The third possibility is that magnesium, by interfering in protein synthesis as noted earlier in this review, could result in the production of an abnormal enzyme or enzymes incapable of hydrolysing a normally present glycoprotein.

No matter what the primary cause, congestive lysosomal enlargement invariably has a detrimental effect at the cellular level. Not only does it impair normal functioning of the lysosome (33), but it may disrupt cellular organization through compressive damage or through lytic injury resulting from rupture of the enlarged lysosomes. Even when temporary and reversible, it may cause premature or accelerated aging due to abnormal residues and excessive membrane fragments (20).

Of the disorders associated with injurious lytic activity those which have been dealth with most completely (and are of most importance to this review) are those involving cellular and tissue lesions. It has been shown that there is an increase in cellular autophagy with a variety of pathological conditions (20), and that this probably contributes greatly to cellular injury. Also important in this area is the possibility of cell damage through intracellular release of enzymes from damaged lysosomes. Thus during ischemia, anoxia and necrosis the membranes of the lysosome become more permeable and the resulting escape of lytic enzymes into the cell cytoplasm leads to the digestion of the susceptible substrates present there: proteins, and polysaccharides. This could be the problem in magnesium

deficiency where a lack of magnesium could result in a premature release of lysosomal enzymes.

Highly important to the autolytic activity of lysosomes are the various substances and treatments shown to have an effect on the integrity of the lysosomal membrane, both *in vivo* and *in vitro*. Most of the substances investigated so far have been labilizers, but a few stabilizers have been discovered as well. Among the most important stabilizers have been the gluco-corticoids: hydrocortisone (34), chlorpromazine (35), and chloroquin (20) as well as the salicylates (30,37,38) though there has been some disagreement on the latter group.

Lucy has presented evidence (39) using his micellar model for membrane structure, that the gluco-corticoids act to stabilize the lysosomal membrane solely by virtue of their interaction with the lipid components of the lysosomal membrane. In Lucy's model, stabilization of the membrane results also in a lack of fusion, fusion arising when the membranes of the primary lysosome and either the phagosome, or the plasma membrane, have assumed a heavily micellar configuration which allows merger to occur. Fusion is prevented by the assumption of a more heavily bimolecular leaflet configuration. Lucy's hypothesis is then that the action of the gluco-corticoids is to force the lysosomal membrane to assume the heavily bimolecular leaflet membrane configuration.

How the salicylates fit into this hypothesis is poorly understood, nor is it known why, in our lab, aspirin offers the best protection against calcification (40). Our data is not sufficiently extensive to allow construction of a precise hypothesis. We have found that the several drugs which tend to stabilize lysosomal membranes *in vitro* significantly reduce kidney calcification, with aspirin being the most

effective. This coupled with the fact that several characteristics of magnesium deficiency which are poorly understood (mast cell degranulation and enhanced resistance to delayed hypersensitivity (54)) could be associated with the untimely release of lysosomal constitutents, lead us to postulate a lysosomal involvement in the etiology of magnesium deficiency.

The objectives of this thesis have been to determine the effect of magnesium deficiency on the total activity and on the distribution of activity, of lysosomal enzymes using both histopathological procedures and enzyme assays following cellular fractionation. It is hoped that the information gained can provide additional data concerning the lability or malfunction of kidney lysosomes in the magnesium diet.

MATERIALS AND METHODS

β-glycerophosphate, the cinchonidine derivative of phenolphthalein β-D glucuronic acid, napthol AS-BI phosphate, napthol AS-BI β-D glucuronic acid and pararosaniline hydrochloride was purchased from Sigma Chemical Company, St. Louis, Missouri. The Triton X-100 used was Packard's scintillation grade. The Alizarin Red S was from batch 15798 of the National Aniline Division of the Allied Chemical and Dye Corporation, New York, New York; it was the gift of Dr. H. R. Steeves, III. All rats were purchased from Flow Research Animals, Dublin, Virginia. They were young weanling males, 30-50 grams, of a Sprague-Dawley derived strain, so called: Dublin Disease Resistant (DDR).

The cinchonidine salt of phenolphthalein β-D glucuronic acid was purified by the method of Fishman (41); .493 g was dissolved in 10 ml of .5 M sodium hydroxide, stirred, then allowed to stand for 30 minutes. The mixture was then filtered and the precipitate washed with an additional 5 ml of sodium hydroxide solution. The filtrate was then diluted to 50 ml with distilled water and the pH adjusted to 5.2 with concentrated HCl.

The pararosaniline hydrochloride was hexazotized after the method of Davis and Ornstein as reported in Pearse (42). One gram of the pararosaniline hydrochloride was dissolved in 20 ml of distilled water to which was then added 5 ml of concentrated HCl. The solution was then warmed gently, allowed to cool, and filtered. This was considered solution A. Solution B was a 4% solution of sodium nitrite. For use equal parts of A and B were mixed and left to stand a minute or so until the mixture became amber. All other reagents were used without further purification or modification.

The biochemical assays for both β-glucuronidase and acid phosphatase were fashioned after de Duve and Gianetto (43) and Bowers and de Duve (44). The final volume of all assay mixtures was 2 ml; they were .05 M in acetate buffer, pH 5.0, and either .00125 M in phenolphthalein β-D glucuronic acid (PGA) or 0.5 M in β-glycerophosphate (β-gly). All assays were incubated at 37°C, for times varying from 20 minutes to two hours. β-glucuronidase activity was measured in μm phenophthalein released, the reaction being stopped with glycine buffer, pH 10.4 and the phenolphthalein measured colorimetrically using a Spectronic 20 set at 540 nm. Acid phosphatase activity was measured as the μm of phosphate released, using 10% TCA to stop the reaction and using the Fiske-Subarrow (46) method for colorimetric determination of inorganic phosphate at 660 nm.

The histochemical procedure for acid phosphatase was fashioned after Barka and Anderson and Lodja as seen in Pearse (42). The substrate, napthol AS-BI phosphate (sodium salt), was dissolved in .2 M acetate buffer pH 5.0, to give a concentration of 2mg/ml. Five ml of the substrate solution were combined with 1.6 ml of the hexazotized pararosaniline and then diluted to 20 ml with distilled water and the pH adjusted to 5.0 with 1 N NaOH. The solution was then filtered.

The procedure for the histochemical demonstration of β-glucuronidase was taken from Hayashi *et al*. (45); 28 mg of the napthol AS-BI β-D glucuronic acid was dissolved in 1.2 ml of .05 M sodium bicarbonate and made up to 100 ml with .2 M acetate buffer, pH 5.0. Ten ml of the substrate mixture were then combined with .6 ml of the hexazotized pararosaniline, diluted to 20 ml with distilled water and the pH adjusted to 5.2 with 1 N NaOH. The solution was then filtered.

A standard PAS procedure was employed for carbohydrates using 5 minutes in periodic acid and 20 minutes in the Schiff's reagent.

Calcium deposits were demonstrated initially using a .5% solution of Alizarin Red S buffered with glycine buffer to pH 9.0 as suggested by Puchtler et al. (46) using hematoxylin as a counterstain. With this procedure it was found that hematoxylin alone stained calcium and since it provided much better background cell staining than the alizarin it was chosen as the method of choice for demonstration of calcium deposits.

Protein was determined using a modified Biuret reaction, using approximately .001 ml of homogenate, and measuring the response at 263 nm on a Hitachi Double Beam Spectophotometer.

EXPERIMENTAL DATA

Experiment I
Preliminary Trials with Small Numbers of Animals

Work done in our lab has shown certain compounds to be efficacious in preventing the renal calcification occurring in growing rats fed a magnesium deficient diet. These compounds have in common the characteristic of being lysosomal membrane stabilizers in vitro. In light of this and other data, it was decided to measure the levels of lysosomal enzymes in kidney homogenate and in the serum, of rats fed on a magnesium deficient diet. The hypothesis here was that some malfunction or breakdown of the lysosomal membrane resulted in the calcification occurring in magnesium deficiency, this malfunction being alleviated or corrected by the stabilizing compounds. It was felt that a possible measure of this hypothesized malfunction could be found in a change in the quantity or distribution of the lysosomal enzymes.

Generally speaking lysosomal enzymes are hydrolytic in nature and display an acid pH optimum. They also display the quality de Duve has described as latency. In other words these enzymes do not display full activity until released from the lysosome either by physical means such as freezing and thawing or sonication, or by the use of a detergent such as Triton X-100, which serve to break open the lysosomal membranes or at least to make them more permeable so that the enzyme may be exposed to substrate.

Because of this latency, the majority of studies on lysosomal enzymes measure a free and a total activity; the latter being where the homogenate has been treated in some way so as to open up the lysosomal membrane. If we were to see a change in the ratio of free to total

activity in the magnesium deficient rat then this would be a fairly good indication that the integrity of the lysosomal membrane has been altered. Changes in the totals would indicate that some other mechanism is occurring.

A pilot run of the experiment was initiated to enable the investigator to coordinate procedures previously used separately to establish the various parameters of the experiment (assay concentrations, length of assay, etc.). Previously it was determined that enzyme activity was linear with time and concentration, that Triton X-100 in 1% concentration did not inhibit and that the homogenate data was reproducable within ±7%. In this experiment, young (30-50 gm) male rats of the DDR strain were divided into 12 groups of 3 animals each. They were individually housed in stainless steel cages and given water ad libitum. Two diets were prepared. The composition of the complete (control) diet is given in Table I. The magnesium deficient diet contained the same ingredients as the control diet, except that cellulose was substituted for $MgSO_4 \cdot 7H_2O$. All animals were fed the control diet for 5 days prior to the start of the experiment. At day zero of the experiment half of the animals (GROUP I) began to receive the magnesium deficient diet ad libitum, the other half (GROUP II) remained on the control diet. On days 5, 6, 8, 9, 10, 12 three animals from each diet were sacrificed. They were first stunned by a blow to the head followed by decapitation and bleeding. The kidneys were then removed and immersed in .25 M sucrose at $4^{o}C$. Each kidney was cut longitudinally and in cross section to give 4 approximately equal quarters. Two quarters from each kidney were homogenized, the other two were fixed for future histochemical examination.

Homogenization was performed in a Dounce homogenizer in from 5 to 7 ml of .25 M sucrose using 5 strokes with the loose fitting pestle and keeping the homogenizer in an ice bath for the entire procedure. The crude homogenate was spun at 1000 g for 10 minutes to remove nuclear debris and unbroken cells. The pellet was discarded and a .2 ml aliquot was added to a test tube containing all the reagents for the enzyme assay except the substrate and left for an hour and a half, at 4°C. The reagents for the assay and the quantities in which they appear in the assay mixture are in Table II. The supernatant from the low speed centrifugation was assayed in the presence and in the absence of .1% Triton X-100. The low speed supernatant assayed in the presence of Triton was designated the T fraction, assayed without Triton it was designated the S fraction. All assays were run at 37°C for two hours. Table III gives the individual activities, and their mean for each of the two groups of animals for days 6, 8, 10, and 12; days 5 and 9 were aborted. Time was too limited to permit the histological examination originally intended for this first experiment.

TABLE I

CONTROL DIET
Magnesium Deficiency Experiments

DIET	gm/100 gm diet
Casein	20
Sucrose	61
Cottonseed Oil	8 + 10 drops Oleum Percomorph/100 gm oil
Cellulose	5
Mineral Mix	5
Vitamin Mix	1
Choline Chloride	5 ml/kilo (20% solution)

MINERAL MIX	gm
NaCl	66.6
Ki	0.79
NaH_2PO	100.00
KH_2PO_4	289.00
$CaCO_3$	381.40
$FeSO_4 \cdot 7H_2O$	27.00
$MnSO_4 \cdot H_2O$	4.00
$ZnSO \cdot 5H_2O$	0.548
$CuSO_4 \cdot 5H_2O$	0.477
$CoCl_2 \cdot 6H_2O$	0.023
$MgSO_4 . 7H_2O$	130.00

VITAMIN MIX	gm
Thiamine HCl	.5
Pantothenate	2.0
Niacin	1.0
Inositol	1.0
Menadione	0.4
Riboflavin	0.3
Pyridoxine HCl	0.3
Folic Acid	0.02
Biotin	0.01
Vitamin B_{12}	0.001
Sucrose	994.5

TABLE II

REACTION MIXTURE

	Total	Free	Final concentration
Buffer	1.5 ml	1.5 ml	.05 M
Substrate solution	0.2 ml	0.2 ml	.00125 M PGA .05 M B-Gly
Triton	0.1 ml	---	1%
Water	---	0.1 ml	---
Homogenate	0.2 ml	0.2 ml	---
Total	2 ml	2 ml	

TABLE III

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment I
Acid Phosphatase Activity*
(μmoles/minute/mg protein)
X 10^3

		T		S	
		GROUP		GROUP	
	ANIMAL	I	II	I	II
Day 6	1	0.31	0.20	0.18	0.14
	2	0.19	0.21	0.09	0.10
	3	0.17	0.18	0.17	0.15
	Average	0.29	0.19	0.15	0.13
Day 8	1	0.93	0.97	0.64	0.65
	2	0.58	1.10	0.64	0.16
	3	0.81	0.74	0.64	0.61
	Average	0.77	0.94	0.64	0.47
Day 9	1	0.52	0.70	0.41	0.43
	2	0.68	0.42	0.45	0.21
	3	0.56	0.54	0.39	0.43
	Average	0.59	0.55	0.42	0.51
Day 10	1	0.62	0.61	0.32	0.34
	2	0.79	0.66	0.36	0.21
	3	0.74	0.75	0.60	0.45
	Average	0.72	0.67	0.43	0.33
Day 12	1	0.55	0.42	0.45	0.16
	2	0.63	0.40	0.39	0.36
	3	0.39	0.42	0.33	0.37
	Average	0.52	0.41	0.39	0.29

* T: Total activity (Triton treated) of low speed supernatant
S: Free activity of low speed supernatant

TABLE IV

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment I
β -Glucuronidase Activity*
(μmoles/minute/mg protein)

X 10^4

		T GROUP		S GROUP	
	ANIMAL	I	II	I	II
Day 6	1	0.038	0.025	0.030	0.045
	2	0.035	0.023	0.032	0.023
	3	0.050	0.030	0.042	0.018
	Average	0.041	0.026	0.035	0.029
Day 8	1	0.092	0.110	0.087	0.093
	2	0.090	0.098	0.073	0.092
	3	0.120	0.057	0.097	0.055
	Average	0.100	0.088	0.085	0.080
Day 9	1	0.062	0.083	0.048	0.073
	2	0.078	0.043	0.073	0.040
	3	0.059	0.067	0.053	0.065
	Average	0.066	0.064	0.063	0.059
Day 10	1	0.052	0.058	0.043	0.052
	2	0.058	0.065	0.063	0.087
	3	0.059	0.067	0.053	0.065
	Average	0.056	0.063	0.052	0.058
Day 12	1	0.050	0.050	0.038	0.043
	2	0.075	0.035	0.060	0.038
	3	0.035	0.042	0.030	0.043
	Average	0.053	0.047	0.042	0.041

*
T: Total activity (Triton treated) of low speed supernatant.
S: Free activity of low speed supernatant

Experiment II
A Full Scale Experiment

A second experiment was run using 70 animals and modifying experiment I in the following ways:

1) Both kidneys were homogenized; it was realized from Experiment I that histochemistry would have to be done on a separate experiment; time was too limited to attempt simultaneous examination.

2) A second, high speed, centrifugation was employed to give a "soluble" fraction designated S'. It was possible that there was a difference between the amount of enzyme which was free and that which was soluble, i.e. that there could be enzyme which was still somewhat membrane bound but available to substrate. The difference between this enzyme and that which was free in solution would not be detectable though assaying only the S fraction. Consequently, an aliquot of S was spun for 1 hour at 100,000 g to give an S' activity.

3) Data was taken only on days 2, 5, and 8. The animals were entered on the diet in split shifts so that ¼ of the animals were on day 2 at two separate times. This allowed for easier processing.

4) The kidneys were homogenized in 10-20 ml (20:1 v/w) .25 M sucrose.

5) The rats were fasted twelve hours prior to killing.

Tables V - X given individual activities and their mean for the two groups of animals.

TABLE V

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment II
Acid Phosphatase Activity*
(μmoles/minute/mg protein)

X 10^3

		T		S		S'	
		GROUP		GROUP		GROUP	
	ANIMAL	I	II	I	II	I	II
	1	0.32	0.35	0.30	0.25	0.13	0.17
	2	0.36	0.29	0.23	0.47	0.15	0.18
	3	0.36	0.35	0.29	0.25	0.13	0.12
Day 2	4	0.34	0.39	0.29	0.29	0.17	0.14
	5	0.32	0.24	0.25	0.22	0.15	0.13
	6	0.20	0.22	0.17	0.19	0.12	0.12
	7	0.33	0.32	0.30	0.28	0.22	0.12
	8	0.30	0.28	0.24	0.22	0.14	0.12
	Average	0.32	0.31	0.26	0.27	0.23	0.20
	1	0.46	0.40	0.37	0.33	0.23	0.20
	2	0.48	0.52	0.37	0.40	0.23	0.23
	3	0.52	0.56	0.42	0.47	0.21	0.27
Day 5	4	0.33	0.43	0.28	0.38	0.20	0.16
	5	0.38	0.26	0.32	0.21	0.16	0.20
	6	0.37	0.42	0.24	0.33	0.27	0.16
	7	0.36	0.44	0.33	0.33	0.22	0.22
	8	0.48	0.37	0.33	0.28	0.21	0.18
	Average	0.47	0.43	0.33	0.34	0.22	0.20
	1	0.50	0.42	0.30	0.37	0.16	0.14
	2	0.41	0.45	0.35	0.37	0.22	0.15
	3	0.47	0.51	0.41	0.45	0.17	0.19
Day 8	4	0.50	0.41	0.41	0.34	0.19	0.17
	5	0.39	0.46	0.31	0.42	0.17	0.31
	6	0.38	0.39	0.28	0.36	0.19	0.20
	7	0.32	0.36	0.31	0.30	0.19	0.16
	8	0.47	0.37	0.32	0.34	0.18	0.17
	Average	0.43	0.42	0.34	0.37	0.18	0.19

* T: Total activity (Triton treated) of low speed supernatant
S,S': Free activity of low speed and high speed supernatants

TABLE VI

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment II
β-Glucuronidase Activity*
(μmoles/minute/mg protein)
X 10^4

		T GROUP		S GROUP		S' GROUP	
	ANIMAL	I	II	I	II	I	II
Day 2	1	0.117	0.122	0.094	0.071	0.071	0.061
	2	0.083	0.137	0.043	0.132	0.044	0.071
	3	0.084	0.106	0.076	0.086	0.035	0.056
	4	0.173	0.112	0.104	0.098	0.077	0.085
	5	0.101	0.069	0.069	0.026	0.077	0.031
	6	0.058	0.065	0.027	0.027	0.029	0.041
	7	0.090	0.085	0.045	0.039	0.037	0.044
	8	0.086	0.074	0.037	0.027	0.038	0.029
	Average	0.099	0.096	0.061	0.063	0.051	0.052
Day 5	1	0.153	0.105	0.060	0.052	0.058	0.051
	2	0.133	0.147	0.049	0.080	0.047	0.079
	3	0.137	0.143	0.063	0.064	0.067	0.077
	4	0.157	0.138	0.760	0.056	0.055	0.051
	5	0.106	0.112	0.042	0.051	0.043	0.059
	6	0.115	0.109	0.063	0.032	0.058	0.046
	7	0.107	0.142	0.060	0.069	0.041	0.061
	8	0.152	0.106	0.069	0.044	0.055	0.042
	Average	0.132	0.125	0.060	0.056	0.053	0.058
Day 8	1	0.143	0.139	0.111	0.085	0.047	0.048
	2	0.159	0.131	0.125	0.088	0.056	0.047
	3	0.150	0.158	0.140	0.134	0.042	0.059
	4	0.180	0.122	0.129	0.078	0.057	0.049
	5	0.129	0.159	0.114	0.159	0.039	0.072
	6	0.130	0.148	0.124	0.117	0.046	0.045
	7	0.130	0.123	0.115	0.123	0.057	0.034
	8	0.177	0.125	0.144	0.100	0.048	0.067
	Average	0.149	0.138	0.125	0.110	0.049	0.052

* T: Total activity (Triton treated) of low speed supernatant
S, S': Free activity of low speed and high speed supernatants.

TABLE VII

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment II
Acid Phosphatase Activity*
(μmoles/minute/gm kidney)

		T GROUP		S GROUP		S' GROUP	
	ANIMAL	I	II	I	II	I	II
	1	1.54	1.71	1.42	1.24	0.54	0.62
	2	1.70	1.84	1.12	1.32	0.56	0.68
	3	1.58	1.68	1.28	----	0.52	0.46
Day 2	4	1.86	----	1.54	1.36	0.72	0.56
	5	2.00	1.40	1.62	1.36	0.82	0.58
	6	1.35	1.62	1.14	1.40	0.60	0.56
	7	1.54	1.62	1.40	1.50	0.68	0.52
	8	1.42	1.42	1.38	1.36	0.64	0.56
	Average	1.62	1.61	1.36	1.36	0.64	0.57
	1	1.66	2.00	1.34	1.70	0.74	0.66
	2	1.86	2.00	1.44	1.54	0.64	0.60
	3	1.88	2.06	1.50	1.74	0.64	0.70
Day 5	4	1.98	1.84	1.66	1.60	0.64	0.46
	5	1.62	1.58	1.34	1.24	0.50	0.60
	6	1.58	1.58	1.00	1.26	0.60	0.56
	7	1.58	1.66	1.22	1.20	0.60	0.70
	8	1.70	1.58	1.18	1.18	0.62	0.52
	Average	1.73	1.60	1.33	1.64	0.62	0.60
	1	2.14	1.80	1.28	1.58	0.60	0.56
	2	1.66	1.82	1.40	1.48	0.86	0.52
	3	1.84	2.00	1.58	2.20	0.64	0.76
Day 8	4	1.92	1.64	1.46	1.36	0.68	0.
	5	1.60	1.64	1.28	1.48	0.60	0.92
	6	1.60	1.36	1.16	1.26	0.62	0.56
	7	1.32	1.32	1.26	1.10	0.60	0.51
	8	1.74	1.48	1.22	1.40	0.61	0.60
	Average	1.72	1.63	1.33	1.63	0.65	0.63

* T: Total activity (Triton treated) of low speed supernatant
S,S': Free activity of low speed and high speed supernatants

TABLE VIII

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment II
β-Glucuronidase Activity*
(μmoles/minute/gm kidney)

		T		S		S'	
		GROUP		GROUP		GROUP	
	ANIMAL	I	II	I	II	I	II
Day 2	1	0.099	0.105	0.079	0.062	0.053	0.041
	2	0.071	0.106	0.036	0.103	0.031	0.047
	3	0.089	0.088	0.060	0.072	0.026	0.035
	4	0.135	0.094	0.100	0.082	0.055	0.057
	5	0.114	0.072	0.077	0.029	0.075	0.024
	6	0.069	0.084	0.031	0.028	0.028	0.031
	7	0.074	0.075	0.037	0.031	0.027	0.033
	8	0.082	0.065	0.036	0.024	0.030	0.023
	Average	0.092	0.086	0.057	0.054	0.041	0.036
Day 5	1	0.099	0.096	0.039	0.048	0.034	0.028
	2	0.092	0.101	0.034	0.055	0.026	0.037
	3	0.089	0.093	0.041	0.042	0.036	0.036
	4	0.101	0.103	0.049	0.042	0.031	0.036
	5	0.080	0.088	0.032	0.041	0.024	0.031
	6	0.075	0.073	0.041	0.022	0.023	0.029
	7	0.034	0.094	0.040	0.045	0.020	0.034
	8	0.096	0.081	0.043	0.034	0.029	0.022
	Average	0.083	0.091	0.040	0.041	0.028	0.032
Day 8	1	0.109	0.106	0.109	0.065	0.031	0.034
	2	0.113	0.093	0.089	0.062	0.036	0.030
	3	0.103	0.111	0.079	0.094	0.027	0.042
	4	0.123	0.087	0.088	0.055	0.037	0.032
	5	0.094	0.102	0.083	0.101	0.027	0.041
	6	0.096	0.091	0.091	0.072	0.026	0.026
	7	0.094	0.080	0.083	0.079	0.033	0.019
	8	0.118	0.090	0.097	0.072	0.030	0.041
	Average	0.106	0.095	0.089	0.075	0.031	0.033

* T: Total activity (Triton treated) of low speed supernatant
S,S': Free activity of low speed and high speed supernatants

TABLE IX

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment II
Total β-Glucuronidase Activity*
(μmoles/minute/2 kidneys)

		GROUP	GROUP
	ANIMAL	I	II
Day 2	1	0.041	0.050
	2	0.041	0.058
	3	0.032	0.036
	4	0.057	0.037
	5	0.035	0.042
	6	0.050	0.047
	7	-----	0.035
	8	0.035	0.054
	Average	0.041	0.045
Day 5	1	0.047	0.029
	2	0.044	0.058
	3	0.034	0.033
	4	0.035	0.039
	5	0.049	0.025
	6	0.049	0.029
	7	0.038	0.038
	8	0.050	-----
	Average	0.043	0.036
Day 8	1	0.032	0.007
	2	0.020	0.013
	3	0.017	0.020
	4	0.017	0.018
	5	0.018	0.008
	6	0.014	0.012
	7	0.011	0.021
	8	0.017	0.021
	Average	0.018	0.015

* Total activity (Triton treated) on the basis of low speed supernatant

TABLE X

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment II
Total Acid Phosphatase Activity*
(μmoles/minute/2 kidneys)

		GROUP	GROUP
	ANIMAL	I	II
	1	0.967	1.101
	2	1.079	1.161
	3	1.040	1.005
Day 2	4	1.242	-----
	5	1.390	1.007
	6	0.979	1.090
	7	1.087	0.857
	8	1.112	1.014
	Average	1.110	1.170
	1	1.335	1.344
	2	1.828	1.548
	3	1.470	1.366
Day 5	4	1.678	1.612
	5	1.379	1.080
	6	1.234	1.345
	7	1.337	1.358
	8	1.348	1.368
	Average	1.450	1.380
	1	2.532	1.680
	2	1.818	1.625
	3	1.776	1.528
Day 8	4	1.724	1.566
	5	1.312	1.310
	6	1.643	1.168
	7	1.348	1.428
	8	1.929	1.304
	Average	1.760	1.450

* Total activity (Triton treated) on the basis of low speed supernatant

Experiment III

A Full Scale Experiment Using Shorter Incubation Times

Experiment III was run using shorter incubation times because it was feared that the long incubation time of two hours for both assays could be causing total rupture of the lysosomes thus giving uninterpretable results. The procedures remained the same as in Experiment II with the following exceptions:

1) The concentrations of the reagents remained unchanged but the incubation time was cut to 20 minutes for the T and S fractions while remaining at two hours for the S' and serum assays.
2) .5 ml of homogenate was used for T, S and S' fractions.
3) The kidneys were homogenized in approximately 10 ml of .25 M sucrose.
4) Serum levels of the enzymes were desired as a further possible measure of lysosomally linked changes. This required further modifications: the rats were etherized and blood drawn from the renal artery. The blood was allowed to clot and the clot spun down, then the serum was drawn off and a .2 ml aliquot was assayed using standard concentrations and a two hour incubation period. (Author's note: while other researchers have shown that chloroform acts immediately in its effects on lysosomal enzymes levels, no information was available for ether. Consequently, while having some reservations about using anaesthesia, it was decided that this was the best way to obtain the required .5-1.0 ml of serum from rats weighing as little as 60 gm.).

5) Due to analytical difficulties data was reported only as μmoles/minute/gm kidney and μmoles/minute/2 kidneys. Serum values are given as μmoles/minute/ml serum.

Individual activities and their mean are given in Tables XI - XVI.

TABLE XI

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment III
Acid Phosphatase Activity*
(μmoles/minute/gram)

		T		S		S'	
		GROUP		GROUP		GROUP	
	ANIMAL	I	II	I	II	I	II
	1	2.46	2.49	1.68	2.49	0.42	0.34
	2	2.41	2.20	1.37	1.25	0.43	0.44
	3	3.70	1.50	1.48	1.24	0.38	0.28
Day 2	4	2.22	2.02	1.20	1.39	0.35	0.42
	5	----	1.48	----	1.20	0.34	0.42
	6	1.34	1.57	0.76	1.34	0.14	0.30
	7	1.67	1.56	0.79	0.81	0.30	0.40
	8	1.40	1.31	0.92	1.00	0.37	0.41
	Average	2.17	1.77	1.17	1.34	0.34	0.38
	1	1.68	1.09	0.91	0.51	----	0.53
	2	0.85	1.22	0.64	0.56	0.40	0.67
	3	0.96	1.34	0.45	0.64	0.59	0.43
Day 5	4	0.88	1.65	0.40	0.67	0.69	0.73
	5	1.09	0.90	0.66	0.52	0.58	0.41
	6	1.73	0.96	0.94	0.65	0.46	0.47
	7	1.70	----	0.71	----	0.71	----
	8	1.64	1.19	0.93	0.62	0.59	0.38
	Average	1.31	1.19	0.72	0.59	0.57	0.52
	1	1.20	0.67	1.14	0.19	0.58	0.24
	2	1.52	1.30	0.64	0.60	0.33	0.26
	3	1.55	0.97	0.58	0.16	0.25	0.30
Day 8	4	0.89	1.35	0.35	0.58	0.27	0.34
	5	1.49	0.91	0.99	0.43	0.35	0.35
	6	0.50	0.28	1.07	0.61	0.36	0.35
	7	1.07	1.28	1.85	0.63	0.43	0.39
	8	1.28	1.47	0.51	0.73	----	0.47
	Average	1.19	1.03	0.89	0.49	0.36	0.33

* T: Total activity (Triton treated) of low speed supernatant
S,S': Free activity of low speed and high speed supernatants

TABLE XII

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment III
β-Glucuronidase Activity*
(μmoles/minute/gram)

		T GROUP		S GROUP		S' GROUP	
	ANIMAL	I	II	I	II	I	II
Day 2	1	0.063	0.072	0.042	0.042	0.024	0.026
	2	0.059	0.077	0.032	0.055	0.024	0.037
	3	0.048	0.062	0.031	0.035	0.023	0.024
	4	0.080	0.064	0.051	0.036	0.028	0.021
	5	0.057	0.075	0.015	0.051	0.015	0.035
	6	0.071	0.071	-----	0.044	0.008	0.034
	7	-----	0.056	0.025	0.035	0.013	0.019
	8	0.063	0.078	0.031	0.047	0.024	0.031
	Average	0.063	0.069	0.032	0.043	0.020	0.028
Day 5	1	0.069	0.043	0.028	0.032	0.032	0.031
	2	0.054	0.088	0.039	0.040	0.032	0.045
	3	0.055	0.051	0.023	0.022	0.033	0.034
	4	0.050	0.060	0.020	0.028	0.024	0.028
	5	0.068	0.036	0.027	0.024	0.041	0.026
	6	0.063	0.041	0.040	0.044	0.029	0.034
	7	0.066	0.051	0.050	0.027	0.049	0.035
	8	0.071	-----	0.040	-----	-----	-----
	Average	0.062	0.053	0.033	0.031	0.034	0.033
Day 8	1	0.045	0.009	0.022	0.004	0.027	0.016
	2	0.027	0.016	0.020	0.011	0.026	0.018
	3	0.021	0.026	0.014	0.009	0.016	0.011
	4	0.020	0.021	0.014	0.014	0.022	0.018
	5	0.026	0.012	0.015	0.006	0.024	0.032
	6	0.017	0.018	0.014	0.011	0.025	0.026
	7	0.017	0.022	0.013	0.022	0.034	0.023
	8	0.022	0.027	0.020	0.012	0.027	0.026
	Average	0.024	0.018	0.016	0.011	0.025	0.021

* T: Total activity (Triton treated) of low speed supernatant
S,S': Free activity of low speed and high speed supernatants

TABLE XIII

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment III
Total Acid Phosphatase Activity*
(μmoles/minute/2 kidneys)

	ANIMAL	GROUP I	GROUP II
Day 2	1	1.582	1.716
	2	1.665	1.650
	3	2.453	0.863
	4	1.591	1.176
	5	-----	0.821
	6	0.951	1.031
	7	1.325	0.967
	8	1.787	0.899
	Average	1.290	1.140
Day 5	1	1.134	0.736
	2	0.691	0.802
	3	0.593	0.872
	4	0.612	1.073
	5	0.791	0.637
	6	1.346	0.676
	7	0.978	-----
	8	1.164	0.879
	Average	0.913	0.810
Day 8	1	0.850	0.490
	2	1.099	1.074
	3	1.229	0.738
	4	0.765	1.138
	5	1.013	0.641
	6	0.414	0.181
	7	0.709	1.043
	8	0.007	1.119
	Average	0.880	0.680

* Total activity (Triton treated) on the basis of low speed supernatant

TABLE XIV

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment III
Total β-Glucuronidase Activity*
(μmoles/minute/2 kidneys)

		GROUP	GROUP
	ANIMAL	I	II
	1	0.062	0.068
	2	0.045	0.067
	3	0.059	0.053
Day 2	4	0.090	0.062
	5	0.079	0.052
	6	0.050	0.057
	7	0.052	0.040
	8	0.064	0.046
	Average	0.063	0.056
	1	0.079	0.056
	2	0.090	0.078
	3	0.070	0.062
Day 5	4	0.086	0.090
	5	0.068	0.060
	6	0.059	0.062
	7	0.029	0.076
	8	0.076	0.070
	Average	0.063	0.070
	1	0.129	0.099
	2	0.124	0.083
	3	0.099	0.085
Day 8	4	0.110	0.083
	5	0.078	0.081
	6	0.099	0.081
	7	0.096	0.086
	8	0.131	0.079
	Average	0.108	0.074

* Total activity (Triton treated) on the basis of low speed supernatant

TABLE XV

Enzyme Activities in Blood Taken from Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment III
Serum Acid Phosphatase Activity
(μmoles/minute/ml of serum)

X 10^2

	ANIMAL	GROUP I	GROUP II
Day 5	1	0.62	0.81
	2	0.87	1.00
	3	1.00	0.70
	4	0.70	0.90
	5	0.80	0.70
	6	0.80	1.20
	7	0.40	----
	8	1.00	1.50
	Average	0.78	1.00
Day 8	1	0.80	0.90
	2	0.80	0.20
	3	0.70	0.80
	4	0.60	0.80
	5	0.80	0.70
	6	1.00	0.70
	7	0.40	0.50
	8	0.75	0.90
	Average	0.67	0.69

TABLE XVI

Enzyme Activities in Blood Taken from Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment III
Serum β-Glucuronidase Activity
(μmoles/minute/ml of serum)

X 10^2

	ANIMAL	GROUP I	GROUP II
Day 5	1	0.14	0.09
	2	0.16	0.16
	3	0.16	0.13
	4	0.13	0.15
	5	0.15	0.14
	6	0.16	0.14
	7	0.14	----
	8	0.13	----
	Average	0.15	0.13
Day 8	1	0.07	0.09
	2	0.10	0.06
	3	0.08	0.06
	4	0.10	0.07
	5	----	0.09
	6	0.08	0.09
	7	0.09	0.09
	8	0.05	0.10
	Average	0.07	0.08

Experiment IV

Histochemical Investigation of Magnesium Deficiency

Experiment IV was the culminating histochemical experiment. Previous trial experiments led to the adoption of the following procedures.

Young, male, DDR rats were used. They were divided into three groups and fed either the low magnesium diet, the control diet or rat pellets ad libitum. The third group was included as a check to be sure that the high sucrose diet had no effect. Two rats were killed from each group on days 2, 5, and 8. They were first stunned followed by decapitation and bleeding. The rats had been fasted 12 hours prior to killing. The kidneys were removed, stripped of their outer membrane, cut in half and placed in phosphate-buffered formalin, pH 7.0, for 18-24 hours at 4°C. After fixation they were removed, blotted and transferred to Holt's gum sucrose solution (.88 M sucrose with 1% gum acacia) for from 7-14 days. For sectioning each half was cut in half and each quarter frozen on the quick freeze attachment of a International Equipment Company (IEC) cryostat. Sections were cut at -26°C and were 8 microns thick. Serial sections were obtained and stained for one of the following substances:

1. β-glucuronidase
2. Acid phosphatase
3. Carbohydrate
4. Calcium

Staining procedures for β-glucuronidase and acid phosphatase used simultaneous coupling methods with napthol AS-BI substrates and incubating floating sections for 50 and 15 minutes respectively at 37°C.

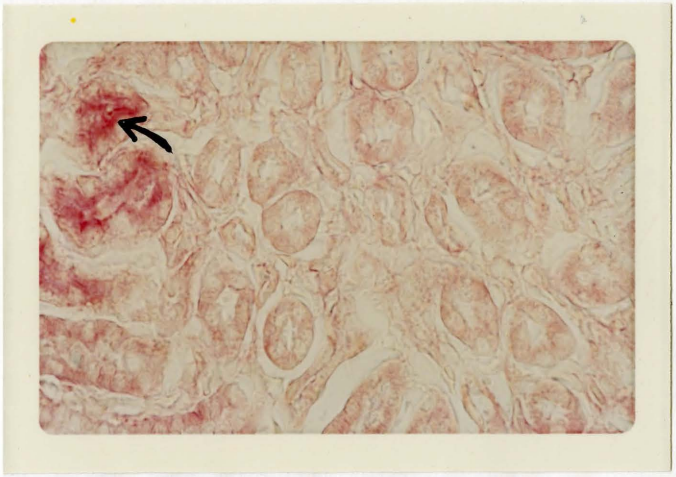
Staining for carbohydrate used the Periodic Acid Schiff's (PAS) procedure using floating sections for 10 minutes in the acid and 5 minutes in Schiff's reagent at room temperature.

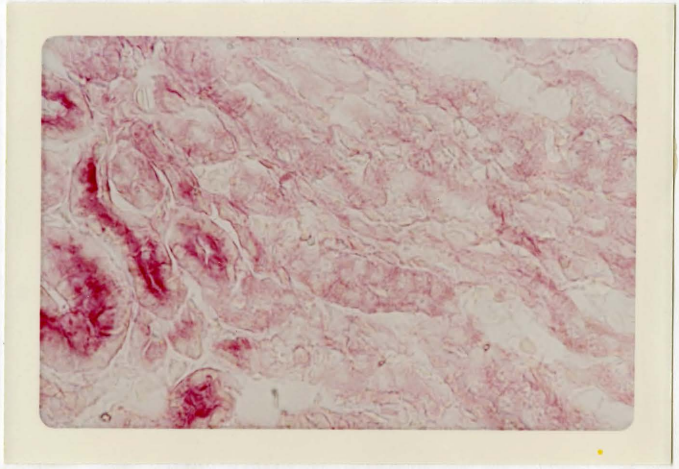
Hematoxylin was used to stain for calcium using floating sections at room temperature.

FIGURE 1 Photomicrograph of cryostat section.
PAS stain, rat kidney, rat pellet diet, day 5.
X 1600
Note lumen width and PAS positive staining (arrow) at the basement membrane of the proximal epithelial cells.

FIGURE 2 Photomicrograph of cryostat section.
PAS stain, rat kidney, control diet, day 5.
X 1600
Lumen has shrunk, probably due to swollen tubular cells; PAS staining more intense at the basement membrane of the cells.

FIGURE 3 Photomicrograph of cryostat section.
PAS stain, rat kidney, low magnesium diet, day 5.
X 1600
Staining is more intense throughout tubular cells still appear swollen.





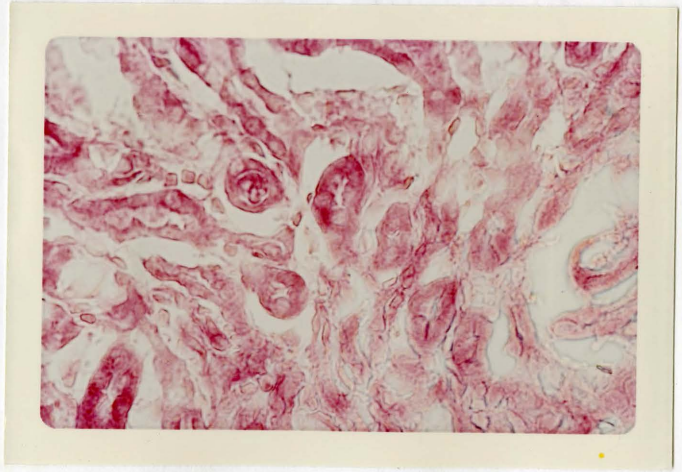


FIGURE 4 Enlargement of Figure 3. X 4000
Note PAS positive material in lumen of the tubule (arrow). Tubule has been distended by material.

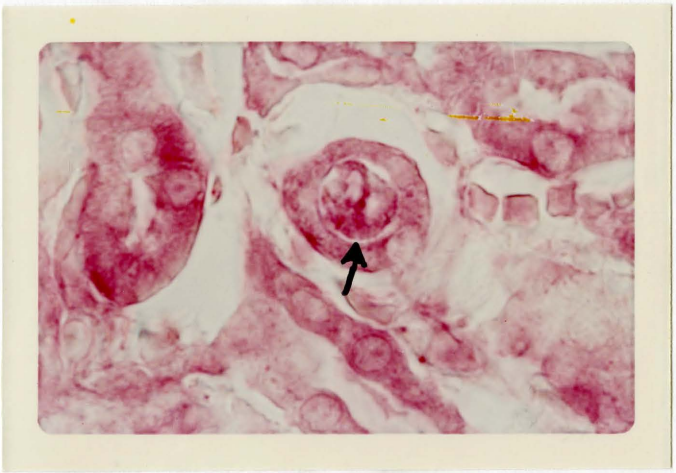
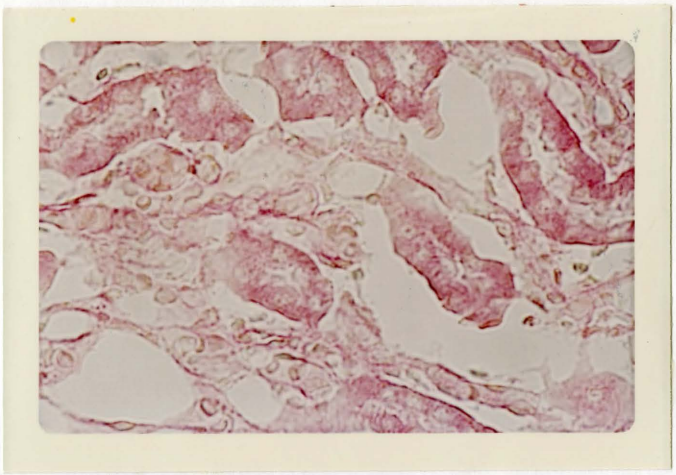
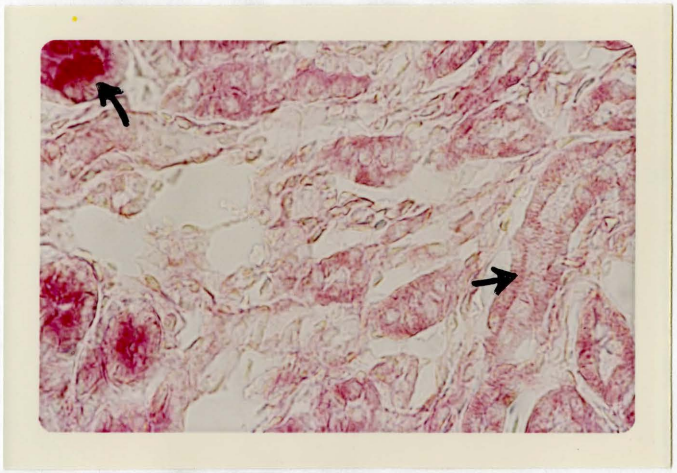


FIGURE 5 Photomicrograph of cryostat section.
PAS stain, rat kidney, rat pellet diet, day 8.
X 1600
Some shrinkage has occurred due to poor fixation.
The lumen is still larger however than in the
subsequent plates.

FIGURE 6 Photomicrograph of cryostat section.
PAS stain, rat kidney, control diet, day 8.
X 1600 (left)
Note heavier PAS staining and swollen cells (arrow).
Lightly stained tubules show larger lumens.

FIGURE 7 Photomicrograph of cryostat section.
PAS stain, rat kidney, low magnesium diet, day 5.
X 1600
Calcification has begun. Note PAS positive material
on outer edges of calcified casts (arrows).





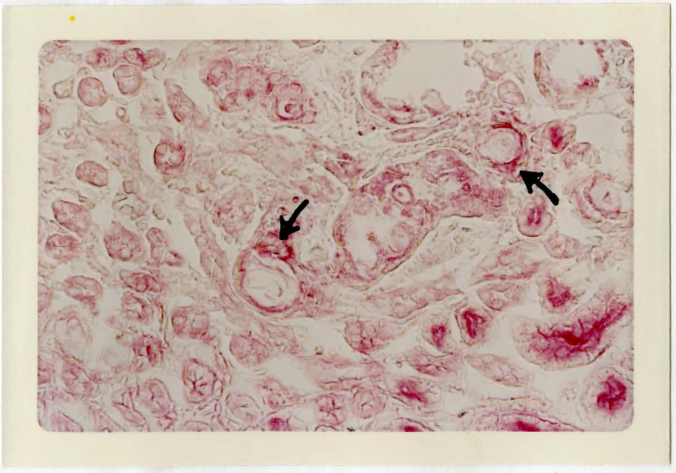


FIGURE 8 Enlargement of Figure 7. X 4000
Note layered appearance of cast (arrow).

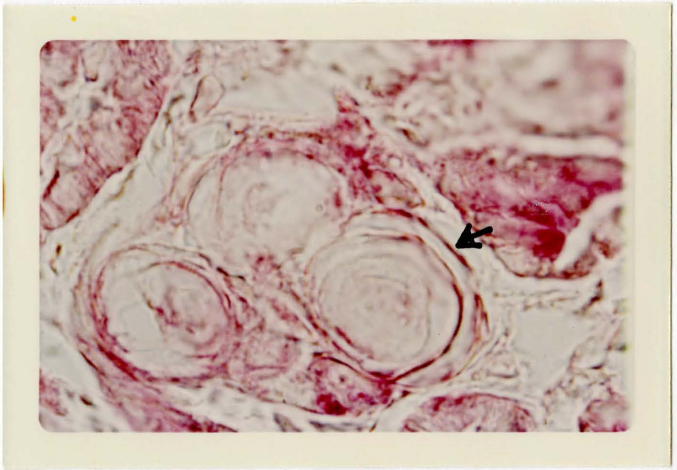
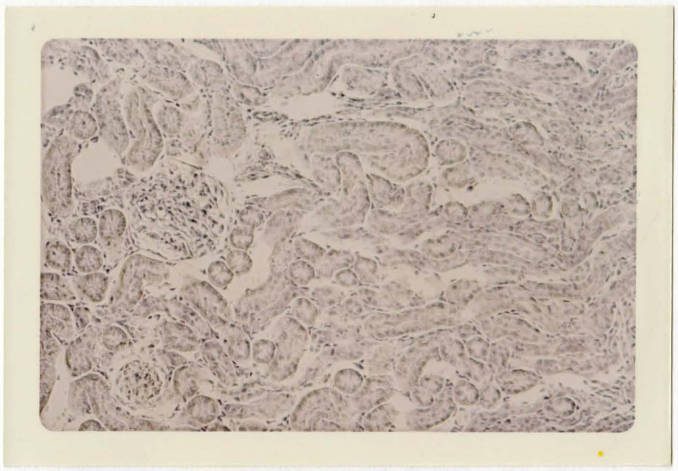
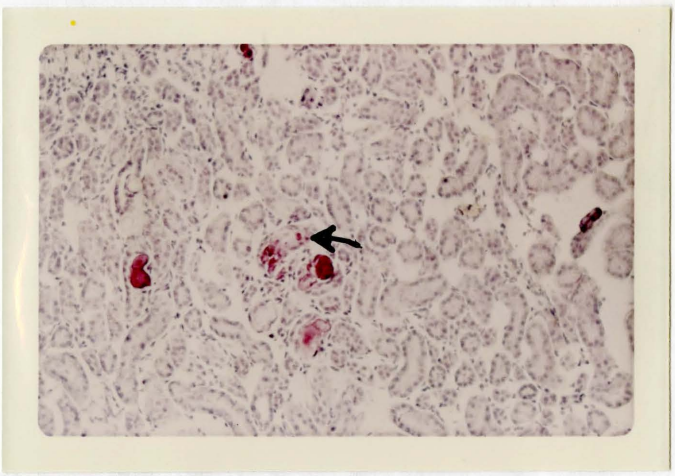


FIGURE 9 Photomicrograph of cryostat section.
Hematoxylin, rat kidney, control diet, day 8.
X 1600

FIGURE 10 Photomicrograph of cryostat section.
Hematoxylin, rat kidney, low magnesium diet, day 8.
X 1600
Calcified casts stain red. Nuclei stain blue.

FIGURE 11 Enlargement of Figure 10. X 4000
Calcified cast has begun to destroy tubule. Note early calcification appears to be in the lumen (arrow).





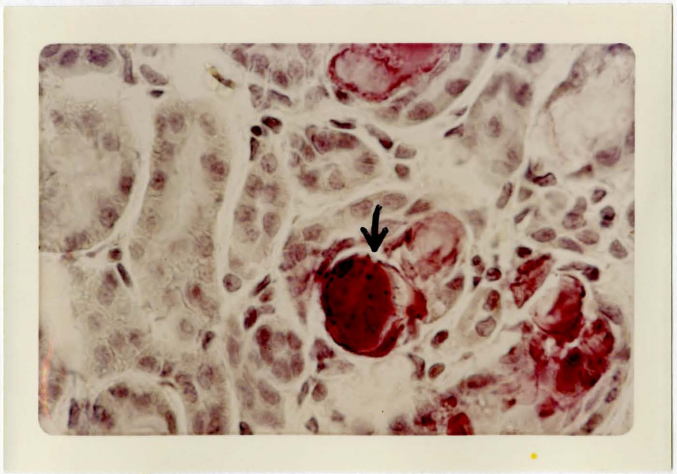
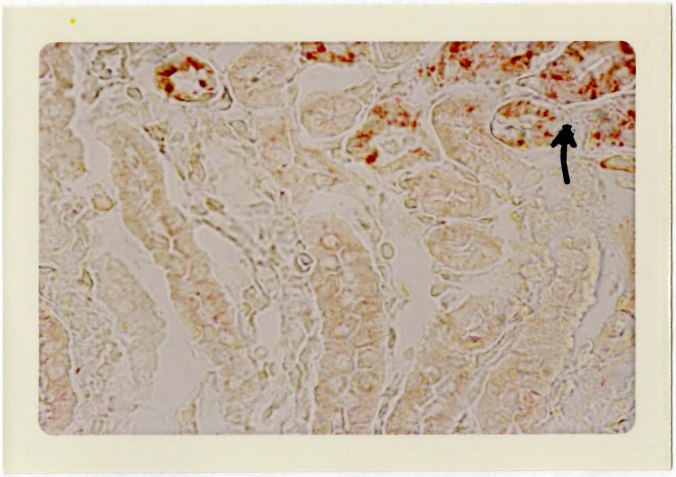


FIGURE 12 Photomicrograph of cryostat section.
Stain for acid phosphatase, rat kidney, control diet, day 8. X 1000
Sites of acid phosphatase activity lie in proximal tubules (arrow).

FIGURE 13 Photomicrograph of cryostat section.
Stain for acid phosphatase, rat kidney, low magnesium diet, day 8. X 1600
Note calcified cast and heavy areas of acid phosphatase activity (arrows).



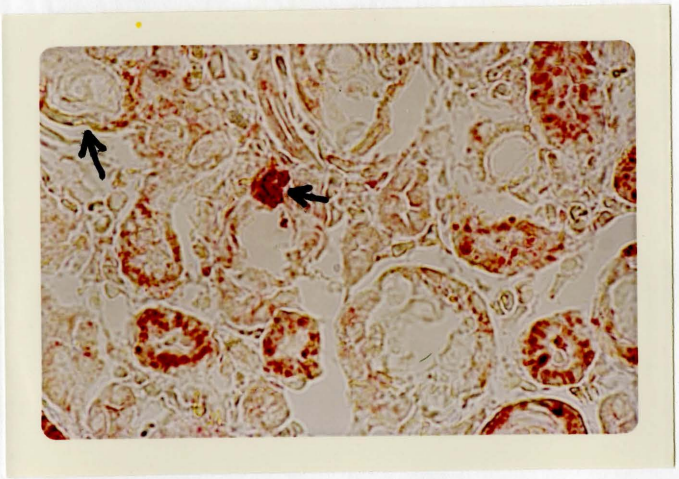
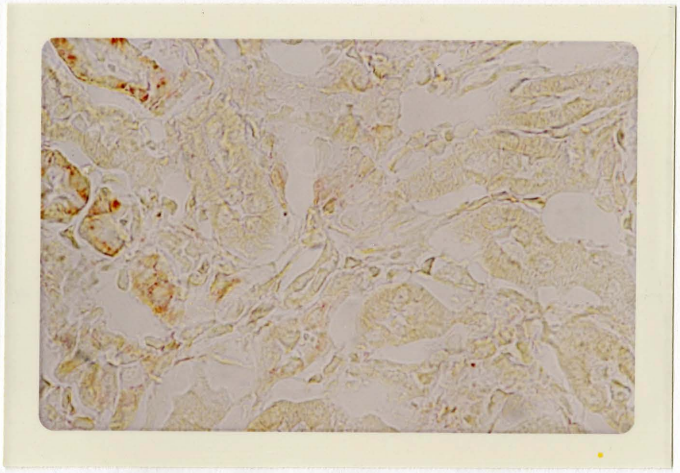


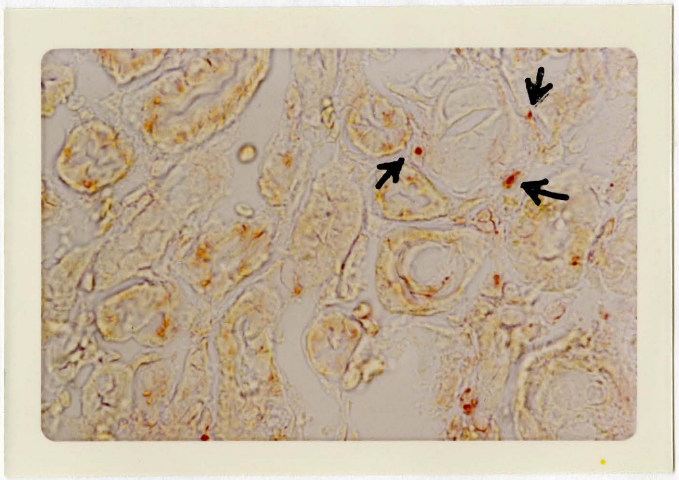
FIGURE 14 Photomicrograph of cryostat section.
Stain for β-glucuronidase, rat kidney, control diet, day 8. X 1600

β-glucuronidase activity lies mainly in the proximal tubular cells.

FIGURE 15 Photomicrograph of cryostat section.
Stain for β-glucuronidase, rat kidney, low magnesium diet, day 8. X 1600

Note β-glucuronidase activity surrounding calcified cast (arrows).





Experiment V
The Final Experiment

A final experiment was felt necessary for several reasons. Primarily, we wanted statistically interpretable data, but we also wanted to see if we could show a better display of latency. To achieve these ends Experiment III was modified in the following manner:

1) Young, male, DDR rats were weighed and divided into 12 groups of 6 rats each, taking the weight distribution from low to high. Within each group of 6 the rats were assigned a number at random of 1-6, then the 12 groups were assigned numbers at random from 1-12. The batteries of cages were treated as 12 ranks of 6 cages each, the cages in each rank being assigned numbers at random from 1-6 and the 12 ranks being assigned random numbers of 1-12. The rats were then placed in their correspondingly numbered cages. Rats numbered 1-3 received the control diet and were called Group II. Rats numbered 4-6 were fed the low magnesium diet and were called Group I. The purpose of the random arrangement was to limit interference from outside variables such as heighth from the floor or side of the battery.
2) Animals on day 2 were acclimated to the control for 6 days, those on days 5 and 8 were acclimated for 7 days.
3) In order to obtain a better idea of the degree of latency of the lysosomes in these experiments, it was decided to

to measure the activity of the nuclear fraction which had hitherto been discarded. Thus instead of discarding the pellet from the first low speed centrifugation it is resuspended twice in 4 ml of .25 M sucrose with 5 strokes of the loose fitting pestle and recentrifuged at 500 g for 10 minutes. The three supernatants are pooled and total approximately 13 ml. The final pellet is resuspended in 8 ml with 8 strokes of the tight-fitting pestle so to give a uniform nuclear fraction. This is then assayed in the presence of 1% Triton to ascertain to total activity in the nuclear fraction of T_N. T_S then becomes the total activity in the first supernatant — that obtained from pooling the two washings and the first supernatant.

4) Assay times were cut to 10 minutes for all fractions except the serum which was incubated for 90 minutes.

5) All aliquots were .5 ml except the serum where .2 ml aliquots were used.

Individual activities and their means are given in Tables XVII - XXIV.

TABLE XVII

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment V
Acid Phosphatase Activity*
(μmoles/minute/mg protein)
X 10^3

		T_N GROUP		T_S GROUP		S GROUP		S' GROUP	
	ANIMAL	I	II	I	II	I	II	I	II
	1	0.831	0.634	0.486	0.447	0.513	0.322	0.486	0.260
	2	1.370	0.710	0.624	0.567	0.479	0.437	0.414	0.463
	3	0.860	0.928	0.554	0.432	0.482	0.403	0.480	0.419
Day 2	4	0.850	0.740	0.810	0.789	0.472	0.441	-----	0.370
	5	0.868	0.943	0.933	1.030	0.393	0.523	0.324	0.356
	6	0.713	0.718	0.753	0.732	0.465	0.461	0.284	0.422
	7	1.450	1.250	1.110	1.190	-----	0.568	-----	-----
	8	1.730	1.030	1.011	1.080	1.030	1.010	-----	-----
	9	1.560	1.060	1.030	1.190	0.939	1.040	-----	-----
	10	1.120	0.956	0.893	0.980	0.445	0.537	0.470	1.240
	11	0.925	1.130	1.000	1.070	0.598	0.934	0.423	0.433
	12	0.934	1.060	0.781	-----	0.365	-----	0.727	0.515
	Average	1.100	0.931	0.832	0.864	0.562	0.607	0.451	0.498
	1	1.180	0.805	0.479	0.170	0.354	-----	0.593	-----
	2	1.050	0.656	0.336	-----	1.170	0.497	0.599	0.350
	3	0.440	1.040	0.367	-----	0.493	0.245	0.542	0.438
Day 5	4	-----	-----	0.680	0.301	0.191	0.172	0.209	0.140
	5	0.710	0.467	0.484	0.860	0.319	0.264	0.302	0.230
	6	0.318	0.375	0.500	0.558	0.284	0.323	0.280	1.040
	7	0.776	1.280	0.544	0.479	0.420	0.418	-----	0.331
	8	0.379	1.070	0.395	0.537	0.358	0.570	0.250	0.299
	9	-----	0.650	-----	0.504	-----	0.420	-----	0.209
	10	0.243	0.269	0.237	0.258	0.157	0.189	0.111	0.119
	11	-----	0.325	-----	0.268	-----	0.448	-----	0.163
	12	0.218	-----	0.238	0.151	0.239	-----	0.142	0.140
	Average	0.590	0.694	0.426	0.409	0.399	0.355	0.336	0.314
	1	0.794	-----	0.562	0.430	0.443	0.324	0.320	0.230
	2	0.514	0.435	0.593	0.413	0.458	0.306	0.322	0.209
	3	0.743	0.487	0.573	0.471	0.346	0.329	0.271	0.221
Day 8	4	1.010	1.890	1.530	1.890	0.690	0.629	0.540	0.586
	5	1.340	2.040	1.460	0.956	0.759	0.481	0.561	0.447
	6	4.260	0.524	-----	-----	-----	0.705	0.651	0.570
	7	1.670	0.703	0.885	0.747	0.425	0.694	0.405	0.342
	8	1.090	0.756	0.864	0.903	0.645	0.548	0.385	0.272
	9	1.450	0.981	0.786	0.894	0.569	0.533	0.305	0.307
	10	0.989	0.518	0.657	0.746	0.502	0.527	0.424	0.256
	11	1.180	1.070	0.584	0.665	0.650	0.465	0.248	0.273
	12	1.350	0.974	0.857	0.857	0.479	0.594	-----	-----
	Average	1.370	0.943	0.779	0.816	0.542	0.511	0.403	0.338

* T_N, T_S: Total activity (Triton treated) of nuclear pellet and low speed supernatant
S,S': Free activity of low speed and high speed supernatants

TABLE XVIII

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment V
β-Glucuronidase Activity*
(μmoles/minute/mg protein)
X 10^4

		T_N		T_S		S		S'	
		GROUP		GROUP		GROUP		GROUP	
	ANIMAL	I	II	I	II	I	II	I	II
	1	0.233	0.285	0.134	0.163	0.054	0.029	0.073	0.035
	2	0.195	0.274	0.119	0.127	0.088	0.043	0.045	0.081
	3	0.192	0.221	0.148	0.148	0.073	0.045	0.072	0.042
Day 2	4	0.282	0.251	0.192	0.174	0.117	0.087	0.083	0.052
	5	0.303	0.346	0.209	0.207	0.103	0.157	0.058	0.053
	6	0.189	0.132	0.227	0.144	0.112	0.058	0.056	0.061
	7	0.179	0.186	0.117	0.128	0.058	0.114	-----	-----
	8	0.203	0.168	0.080	0.141	0.035	0.114	-----	-----
	9	0.171	0.141	0.129	0.115	0.052	0.092	-----	-----
	10	0.160	0.168	0.090	0.096	0.046	0.036	0.018	0.060
	11	0.163	0.188	-----	0.129	0.055	0.029	-----	0.016
	12	0.101	0.155	0.055	0.115	0.040	0.042	0.094	0.011
	Average	0.198	0.210	0.136	0.141	0.074	0.071	0.062	0.046
	1	0.114	0.117	0.079	0.057	0.033	0.047	0.054	0.048
	2	0.154	0.193	0.082	0.050	0.076	0.016	0.066	0.049
	3	0.094	0.140	0.048	0.118	0.023	0.039	-----	0.067
Day 5	4	0.430	-----	0.077	0.062	0.040	0.012	0.028	-----
	5	0.157	0.086	0.381	0.077	0.054	0.026	0.034	0.016
	6	0.203	0.151	0.115	-----	0.057	0.043	0.021	0.115
	7	0.198	0.148	0.215	0.245	0.075	0.076	0.045	-----
	8	0.184	0.162	0.210	0.222	0.109	0.077	0.021	0.020
	9	-----	0.196	-----	0.229	-----	0.073	-----	0.040
	10	0.202	0.148	0.130	0.121	0.055	0.058	0.033	0.021
	11	-----	0.140	-----	0.089	-----	0.028	-----	0.008
	12	0.167	0.148	0.131	0.144	0.040	0.064	0.016	0.045
	Average	0.172	0.148	0.147	0.129	0.056	0.047	0.035	0.043
	1	0.150	-----	0.144	0.112	0.086	0.060	0.040	0.038
	2	0.101	0.173	0.157	0.092	0.067	0.052	0.022	0.032
	3	0.240	0.207	0.130	0.122	0.092	0.053	0.042	0.036
Day 8	4	0.353	0.931	0.384	0.427	0.147	0.147	0.050	0.081
	5	0.048	0.391	0.374	0.262	0.171	0.097	0.074	0.099
	6	0.114	0.368	-----	0.249	-----	0.140	0.062	0.116
	7	0.170	0.134	0.130	0.076	0.045	0.053	0.019	0.031
	8	0.203	0.172	0.110	0.143	0.085	0.035	0.020	0.025
	9	0.165	0.257	0.086	0.092	0.066	0.064	0.019	0.023
	10	0.181	0.158	0.101	0.124	0.093	0.078	0.070	0.021
	11	0.132	0.172	0.102	0.140	0.058	0.076	0.032	-----
	12	0.163	0.150	0.080	0.102	0.063	0.084	-----	0.039
	Average	0.168	0.274	0.163	0.162	0.081	0.078	0.041	0.049

* T_N, T_S: Total activity (Triton treated) of nuclear pellet and low speed supernatant
S, S': Free activity of low speed and high speed supernatants

TABLE XIX

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment V
Acid Phosphatase Activity*
(μmoles/minute/gm kidney)

		T_N		T_S		S		S'	
		GROUP		GROUP		GROUP		GROUP	
	ANIMAL	I	II	I	II	I	II	I	II
Day 2	1	4.20	3.69	2.45	2.60	3.06	3.05	1.40	2.24
	2	6.67	3.67	3.05	2.90	2.90	2.73	1.99	2.22
	3	4.76	5.55	3.10	2.59	3.08	3.01	2.16	2.30
	4	5.00	4.54	4.76	4.84	2.67	2.14	----	1.70
	5	4.59	4.76	4.93	5.20	2.19	2.22	1.58	1.44
	6	5.77	4.96	6.10	5.06	2.73	2.47	1.25	1.58
	7	7.13	5.91	5.47	5.63	----	3.62	----	----
	8	8.53	5.70	5.02	5.96	4.17	3.74	----	----
	9	7.84	6.14	5.17	6.88	4.13	4.69	----	----
	10	8.38	7.61	6.66	7.80	2.73	5.53	3.32	2.71
	11	5.94	6.97	6.44	6.59	3.52	4.89	2.62	2.73
	12	5.14	7.32	3.19	----	3.18	----	7.43	3.50
	Average	6.16	5.56	4.26	5.09	3.03	3.12	2.72	2.26
Day 5	1	8.45	5.07	3.42	1.07	3.58	----	3.00	----
	2	6.20	5.06	1.99	----	2.77	2.98	2.77	2.90
	3	2.21	5.91	1.81	----	2.92	1.51	2.74	2.38
	4	----	----	2.46	3.02	1.63	1.67	1.93	4.48
	5	5.17	1.67	3.53	3.07	2.23	2.20	2.03	1.92
	6	2.54	2.74	3.97	4.08	2.47	2.26	1.81	1.79
	7	4.99	6.56	3.50	2.46	2.50	2.42	1.44	1.77
	8	2.67	4.31	2.79	2.15	2.33	1.73	1.45	1.31
	9	----	2.50	----	2.90	----	2.16	----	1.68
	10	2.34	2.55	2.28	2.39	1.47	1.60	1.15	1.28
	11	----	1.75	----	2.11	----	1.48	----	1.30
	12	1.75	----	1.92	1.06	1.77	----	1.13	0.94
	Average	4.04	3.81	2.77	2.43	2.37	2.00	1.95	1.98
Day 8	1	4.09	----	2.89	3.45	2.28	2.67	1.65	1.46
	2	3.57	3.61	4.12	3.42	3.14	2.51	1.46	1.60
	3	3.70	3.51	2.85	3.40	1.79	2.57	1.29	1.63
	4	2.32	3.57	3.50	3.57	2.46	2.43	1.64	1.82
	5	2.20	8.23	3.51	3,92	2.34	2.63	1.65	1.91
	6	5.73	2.08	----	3.79	----	2.56	1.51	2.35
	7	6.90	4.01	3.66	3.78	2.53	3.46	1.84	1.83
	8	3.50	3.50	2.76	4.18	2.36	3.79	1.41	1.54
	9	6.73	3.96	2.65	3.61	2.94	2.68	2.00	1.58
	10	4.55	3.74	3.02	3.43	2.60	2.73	1.37	1.57
	11	5.41	4.97	4.33	3.06	3.36	2.41	2.04	1.38
	12	6.21	4.48	2.71	3.94	2.48	3.07	1.21	2.13
	Average	4.58	4.15	3.28	3.28	2.57	2.79	1.59	1.73

* T_N, T_S: Total activity (Triton treated) of nuclear pellet and low speed supernatant
S, S': Free activity of low speed and high speed supernatants

TABLE XX

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment V

β-Glucuronidase Activity*

(μmoles/minute/gm kidney)

		T_N		T_S		S		S'	
		GROUP		GROUP		GROUP		GROUP	
	ANIMAL	I	II	I	II	I	II	I	II
Day 2	1	0.139	0.166	0.068	0.095	0.032	0.028	0.028	0.030
	2	0.118	0.141	0.056	0.066	0.053	0.027	0.022	0.039
	3	0.123	0.123	0.083	0.088	0.047	0.034	0.032	0.023
	4	0.166	0.154	0.113	0.107	0.070	0.042	0.040	0.025
	5	0.160	0.175	0.111	0.105	0.057	0.049	0.029	0.022
	6	0.153	0.091	0.184	0.099	0.070	0.052	0.025	0.023
	7	0.088	0.089	0.058	0.061	0.027	0.040	-----	-----
	8	0.100	0.093	0.040	0.078	0.035	0.041	-----	-----
	9	0.086	0.081	0.065	0.067	0.023	0.040	-----	-----
	10	0.118	0.133	0.067	0.077	0.028	0.037	0.011	0.013
	11	0.105	0.116	-----	0.080	0.032	0.015	0.006	0.010
	12	0.115	0.113	0.062	0.084	0.019	0.029	0.012	0.007
	Average	0.123	0.123	0.083	0.084	0.041	0.036	0.023	0.021
Day 5	1	0.079	0.090	0.056	0.044	0.033	0.028	0.027	0.040
	2	0.091	0.109	0.048	0.028	0.018	0.099	0.030	0.026
	3	0.059	0.069	0.031	0.058	0.012	0.023	-----	0.034
	4	0.153	-----	0.137	0.062	0.033	0.012	0.023	-----
	5	0.114	0.076	0.084	0.067	0.038	0.022	0.022	0.015
	6	0.161	0.111	0.092	0.120	0.050	0.030	0.021	0.019
	7	0.102	0.095	0.137	0.158	0.044	0.045	0.024	0.024
	8	0.078	0.114	0.084	0.157	0.033	0.050	0.009	0.013
	9	-----	0.113	-----	0.132	-----	0.038	-----	0.033
	10	0.159	0.137	0.102	0.113	0.050	0.049	0.026	0.023
	11	-----	0.135	-----	0.085	-----	0.026	-----	0.008
	12	0.116	0.119	0.091	0.110	0.026	0.047	0.010	0.037
	Average	0.111	0.106	0.086	0.094	0.034	0.029	0.021	0.025
Day 8	1	0.077	-----	0.074	0.090	0.044	0.051	0.021	0.024
	2	0.070	0.144	0.108	0.076	0.046	0.048	0.014	0.024
	3	0.119	0.149	0.064	0.088	0.048	0.041	0.020	0.027
	4	0.081	0.176	0.088	0.081	0.057	0.057	0.016	0.027
	5	0.114	0.158	0.115	0.143	0.053	0.053	0.022	0.040
	6	0.140	0.146	-----	0.091	-----	0.051	0.012	0.035
	7	0.068	0.077	0.054	0.043	0.029	0.027	0.010	0.017
	8	0.065	0.080	0.046	0.057	0.031	0.018	0.008	0.015
	9	0.077	0.104	0.043	0.044	0.034	0.032	0.012	0.012
	10	0.083	0.073	0.047	0.057	0.048	0.040	0.023	0.012
	11	0.061	0.079	0.047	0.064	0.040	0.040	0.026	0.017
	12	0.075	0.069	0.047	0.047	0.044	0.044	0.017	0.030
	Average	0.086	0.114	0.067	0.073	0.043	0.041	0.017	0.021

* T_N, T_S: Total activity (Triton treated) of nuclear pellet and low speed supernatant

S, S': Free activity of low speed and high speed supernatants

TABLE XXI

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment V
Total Acid Phosphatase Activity*
(μ moles/minute/2 kidneys)

		T_N		T_S		S		S'	
		GROUP		GROUP		GROUP		GROUP	
	ANIMAL	I	II	I	II	I	II	I	II
Day 2	1	2.83	2.24	1.66	1.58	2.06	1.86	1.28	1.34
	2	4.84	2.62	2.21	2.06	2.10	1.94	1.44	1.50
	3	3.17	3.19	2.06	1.48	2.05	1.72	1.44	1.31
	4	3.04	3.10	2.89	3.31	1.62	1.46	----	1.16
	5	2.89	3.17	3.11	3.46	1.38	1.47	1.00	0.96
	6	3.66	3.66	3.86	3.73	1.73	1.82	0.79	1.16
	7	5.04	4.56	3.86	4.33	----	2.79	----	----
	8	6.22	4.62	3.66	4.84	3.04	3.04	----	----
	9	5.24	4.07	3.46	4.56	2.76	3.11	----	----
	10	5.39	4.17	4.28	4.28	1.75	3.04	2.13	1.49
	11	4.84	5.11	5.24	4.84	2.87	3.58	2.14	2.00
	12	5.52	4.90	1.66	----	1.66	----	3.86	2.34
	Average	4.39	3.78	3.16	3.49	2.09	2.34	1.76	1.47
Day 5	1	5.24	3.89	2.13	0.82	2.24	----	1.87	----
	2	5.84	3.29	1.87	----	2.61	1.94	2.61	1.89
	3	1.87	4.64	1.53	----	2.47	1.18	2.31	1.87
	4	----	----	1.90	2.02	1.26	1.12	1.49	1.30
	5	4.94	1.30	3.37	2.40	2.14	1.72	1.94	1.50
	6	2.18	2.61	3.43	3.89	2.13	2.16	1.56	1.64
	7	3.74	5.68	2.63	2.13	1.87	2.09	1.08	1.53
	8	1.79	5.38	1.87	2.69	1.56	2.17	0.98	1.64
	9	----	2.83	----	2.20	----	1.64	----	1.24
	10	1.53	1.87	1.79	1.79	0.96	1.20	0.75	0.96
	11	----	2.17	----	1.79	----	1.26	----	1.11
	12	1.51	----	1.64	1.04	1.51	----	0.96	0.92
	Average	3.51	3.36	2.21	2.08	1.88	1.66	1.55	1.41
Day 8	1	5.38	----	3.81	2.99	3.00	2.31	2.17	1.26
	2	3.63	3.07	4.19	2.91	3.19	2.13	2.09	1.37
	3	5.24	3.21	4.04	3.11	2.54	2.35	1.83	1.50
	4	2.54	3.51	3.82	3.51	2.69	2.39	1.79	1.79
	5	3.47	6.44	3.81	3.07	2.54	2.05	1.79	1.50
	6	7.11	1.70	----	3.11	----	2.09	2.05	1.92
	7	6.36	3.13	3.37	2.91	2.33	2.70	1.69	1.43
	8	4.26	2.81	3.37	3.35	2.87	2.24	1.72	1.24
	9	5.92	3.37	3.21	3.07	2.59	2.27	1.76	1.34
	10	4.19	3.74	2.78	3.43	2.39	2.73	1.26	1.57
	11	4.04	5.24	3.24	3.22	2.51	2.54	1.53	1.46
	12	6.26	3.47	2.77	3.94	2.54	2.38	1.24	1.65
	Average	4.87	3.61	3.23	3.37	2.65	2.34	1.74	1.50

* T_N, T_S: Total activity (Triton treated) of nuclear pellet and low speed supernatant
S,S': Free activity of low speed and high speed supernatants

TABLE XXII

Enzyme Activity in Fractions of Rat Kidney Homogenates of Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment V
Total β-Glucuronidase Activity*
(μmoles/minute/2 kidneys)

		T_N		T_S		S		S'	
		GROUP		GROUP		GROUP		GROUP	
	ANIMAL	I	II	I	II	I	II	I	II
	1	0.094	0.101	0.046	0.058	0.022	0.017	0.019	0.018
	2	0.085	0.101	0.042	0.047	0.038	0.019	0.016	0.028
	3	0.082	0.076	0.055	0.050	0.031	0.019	0.022	0.013
Day 2	4	0.101	0.106	0.068	0.073	0.042	0.029	0.024	0.014
	5	0.101	0.116	0.070	0.070	0.036	0.032	0.018	0.014
	6	0.097	0.067	0.116	0.073	0.044	0.038	0.016	0.017
	7	0.062	0.068	0.041	0.047	0.019	0.031	-----	-----
	8	0.073	0.076	0.029	0.064	0.025	0.034	-----	-----
	9	0.058	0.054	0.043	0.044	0.016	0.026	-----	-----
	10	0.076	0.073	0.043	0.042	0.018	0.020	0.007	0.007
	11	0.085	0.085	-----	0.059	0.026	0.011	0.005	0.007
	12	0.060	0.076	0.032	0.056	0.017	0.019	0.006	0.005
	Average	0.081	0.081	0.053	0.057	0.028	0.025	0.013	0.014
	1	0.050	0.059	0.035	0.029	0.021	0.018	0.017	0.026
	2	0.086	0.089	0.045	0.022	0.017	0.008	0.029	0.021
	3	0.046	0.059	0.023	0.049	0.009	0.020	-----	0.029
Day 5	4	0.120	-----	0.107	0.042	0.026	0.078	0.018	-----
	5	0.109	0.059	0.081	0.052	0.036	0.017	0.021	0.012
	6	0.139	0.105	0.080	0.114	0.043	0.029	0.018	0.018
	7	0.088	0.072	0.118	0.118	0.038	0.034	0.021	0.017
	8	0.092	0.077	0.105	0.105	0.042	0.034	0.012	0.008
	9	-----	0.085	-----	0.100	-----	0.029	-----	0.025
	10	0.135	0.103	0.087	0.085	0.038	0.036	0.022	0.017
	11	-----	0.088	-----	0.056	-----	0.017	-----	0.005
	12	0.114	0.101	0.090	0.094	0.026	0.040	0.010	0.031
	Average	0.098	0.081	0.077	0.072	0.030	0.029	0.019	0.019
	1	0.101	-----	0.098	0.078	0.059	0.043	0.027	0.021
	2	0.072	0.122	0.111	0.065	0.047	0.036	0.014	0.021
	3	0.169	0.137	0.091	0.081	0.068	0.038	0.029	0.025
Day 8	4	0.088	0.073	0.096	0.079	0.057	0.060	0.020	0.025
	5	0.124	0.124	0.125	0.112	0.057	0.042	0.023	0.031
	6	0.190	0.120	-----	0.074	-----	0.042	0.020	0.029
	7	0.062	0.060	0.049	0.034	0.025	0.021	0.008	0.013
	8	0.079	0.064	0.056	0.049	0.038	0.014	0.009	0.012
	9	0.068	0.088	0.038	0.038	0.030	0.027	0.010	0.010
	10	0.077	0.073	0.043	0.057	0.044	0.040	0.021	0.012
	11	0.046	0.083	0.035	0.068	0.030	0.042	0.020	0.018
	12	0.077	0.053	0.038	0.036	0.034	0.034	0.017	0.023
	Average	0.096	0.100	0.071	0.064	0.043	0.037	0.018	0.020

* T_N, T_S: Total activity (Triton treated) of nuclear pellet and low speed supernatant
S, S': Free activity of low speed and high speed supernatants

TABLE XXIII

Enzyme Activities in Blood Taken from Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment V
Serum Acid Phosphatase
(μmoles/minute/ml serum)

X 10^2

	ANIMAL	GROUP I	GROUP II
	1	0.39	0.44
	2	0.49	0.47
	3	----	0.40
Day 2	4	0.40	----
	5	0.30	0.36
	6	0.29	0.30
	7	----	0.84
	8	0.97	0.81
	9	0.79	0.75
	10	0.67	0.93
	11	0.87	0.79
	12	0.65	1.00
	Average	0.54	0.69
	1	0.69	0.41
	2	0.51	0.39
	3	0.53	0.91
Day 5	4	0.46	0.48
	5	0.38	0.48
	6	0.33	0.46
	7	0.47	----
	8	0.57	----
	9	----	0.39
	10	0.31	0.28
	11	----	0.26
	12	0.36	0.33
	Average	0.53	0.46
	1	0.65	0.55
	2	0.57	0.64
	3	0.49	0.64
Day 8	4	----	0.60
	5	0.50	0.63
	6	0.44	0.53
	7	0.32	0.59
	8	0.49	0.43
	9	0.45	0.58
	10	----	0.60
	11	0.44	0.57
	12	0.54	0.61
	Average	0.49	0.58

TABLE XXIV

Enzyme Activities in Blood Taken from Rats Fed a Low Magnesium (I) or a Control Diet (II)

Experiment V
Serum β-Glucuronidase Activity
(μmoles/minute/ml serum)
X 10^2

	ANIMAL	GROUP I	GROUP II
Day 2	1	0.11	0.07
	2	0.12	0.11
	3	----	0.13
	4	0.14	----
	5	0.14	0.14
	6	0.14	0.13
	7	0.07	0.04
	8	0.06	0.07
	9	0.06	0.02
	10	0.10	0.13
	11	0.09	0.04
	12	0.12	0.15
	Average	0.10	0.08
Day 5	1	0.09	0.09
	2	0.09	0.11
	3	0.10	0.07
	4	0.13	0.16
	5	0.13	0.18
	6	0.18	0.13
	7	----	0.15
	8	0.17	0.17
	9	----	0.17
	10	0.11	0.10
	11	----	0.10
	12	0.11	0.15
	Average	0.12	0.13
Day 8	1	0.14	0.13
	2	0.15	0.12
	3	0.14	0.12
	4	----	0.14
	5	0.24	0.18
	6	0.20	0.15
	7	0.07	0.08
	8	0.07	0.05
	9	0.09	0.09
	10	----	0.08
	11	0.14	0.10
	12	0.13	0.16
	Average	0.12	0.12

RESULTS AND DISCUSSION

In the introduction it was noted that lysosomes become involved in pathological processes in either one of two ways: by failing to perform their lytic function, or by performing it in an injurious fashion. Further, several possibilities were discussed as to the primary pathological event responsible for the accumulation of the PAS positive material in the lumen of the tubule, the main ones being: one, congestive enlargement of the lysosomes through the production of an abnormal glycoprotein, the absence of a co-factor, or the presence of an abnormal enzyme; and two, the premature release of the lysosomal contents resulting from instability of the lysosomal membrane due to some requirement for magnesium. The latter possibility was of primary interest due to the research in our lab with lysosomal stabilizers and the additional information that the lysosomes contain a protein known to effect release of histamine from mast cells - an event which occurs in the rats on about the sixth day of an experiment and which is a characteristic symptom of the deficiency.

Because of an interest in the second possibility, research was geared to test for abnormal levels or distributions of lysosomal enzymes. With the primary hypothesis being that of a loss of membrane integrity, the result expected was elevated levels of either soluble enzymes or free but particulate bound enzyme as well as possibly elevated serum levels. Had the result been lower levels of these enzymes one would have to surmise an increase in lysosomal stability or possibly the production of a somewhat abnormal enzyme. Conceivably this would also give lower totals as well. Had an increase in total activity been found, this hypothesis would have to be abandoned and another mechanism looked for,

perhaps an over-production of lysosomes.

Unfortunately, there were no differences between groups. Statistical evaluation of the data from Experiment V confirms the data from Experiments II and III showing no effect due to treatment. In no instance in Experiment V did the f value for any parameter examined fall into either the 1% or the 5% confidence intervals. The activity level of the S fraction in Experiment II was slightly higher than that in Experiments III and V, perhaps indicating a breakdown of the lysosomal membrane over the two hour incubation period. The values of the T and S fractions of Experiment V were slightly higher than those of III probably due to the pooling of the supernatants from the first centrifugation and the two washings. The assays of the further T_N fraction in Experiment V shows clearly that a large amount of activity was lost in the discarded nuclear pellet of previous experiments; it also brings total values into agreement with those of de Duve where he shows latency to be 50-60% of total activity. Surprisingly while the activity levels for acid phosphatase agree fairly well with literature values (44,48), values for β-glucuronidase in the earlier experiments were one-tenth of what they should be. Even in Experiment V combining the values from T_N and T_S gives values which are low when compared to the literature values for totals. This could possibly be due to the age of the animals; these experiments were with weanlings where Bowers (44) and Wattiaux (48) were working with full grown animals.

The high level of activity in the T_N fraction is probably indicative of a fairly high concentration of whole cells which survived the homogenization technique and consequently donated neither their intact lysosomes, lysosomal fragments or soluble lysosomal enzymes to

the succeeding fractions. It is even conceivable that a certain specific population of cells is more hardy to homogenization and that it is this population which might have responded to the treatment used.

Another possible explanation of the inability to show a treatment effect could be attributed to the failure to measure the right parameter. Work by Verity et al. (49) has shown a differential response of lysosomes to cations. Using homogenates of mouse liver, Verity sees enhanced release of β-glucuronidase and N-acetylglucosamidase with increasing univalent cation concentrations, while acid pyrophosphatase remains unaffected. Using increasing concentrations of Ca^{++} and Mg^{++}, however, he sees parallel release of acid pyrophosphatase and N-acetylglucosamidase where β-glucuronidase is unaffected. While admittedly this is work done in vitro, it would seem to indicate at least a duality of lysosomal response, suggesting either two or more distinct populations of lysosomes or two or more lysosomal membrane responses.

Early in his career, de Duve suggested that perhaps the acid hydrolases in the lysosomes were compartmentalized, thus perhaps different compartments might have responded to a magnesium deficiency differently and perhaps those containing β-glucuronidase and acid phosphatase responded not at all. Another viewpoint and one that is increasingly popular with de Duve is that lysosomes display a heterogeneity of enzyme complement as well as the heterogeneity of structure and function already noted. Thus it could be that particular populations of lysosomes respond differently to the cations used by Verity. This idea could dovetail very nicely into the problem of the kidney calcification occurring during magnesium deficiency in that it might serve to explain why the calcification occurs specifically in the corticomedullary region. Cells located in that area would have their own

peculiar function and consequently probably a particular population of lysosomes with a characteristic complement of enzymes. Conceivably only this population of lysosomes might respond to magnesium deficiency and if their response was to free their enzymes and they contained only certain enzymes (or even only certain compartments were affected) measuring levels of β-glucuronidase and acid phosphatase might miss the significant event.

In looking for a lysosomal role in the sequence of events in magnesium deficiency possibly the best approach would be to find one where a lysosomal enzyme is involved. The most obvious event to look at is the first one which is the accumulation of PAS positive material in the lumen of the proximal convoluted tubule. Boyce and King, in characterizing their matrix substance A have noted that it differs from normal urinary Tamm-Horsfall glycoprotein by sialic acid (50). Normal Tamm-Horsfall glycoprotein has been shown to originate at the basement membrane of the tubular epithelial cells (51). Conversion of Tamm-Horsfall protein to matrix substance A would require that it lose sialic acid, a reaction catalyzed, nicely enough, by a lysosomal enzyme: neuraminidase. Once such glycoproteins lose their sialic acid they couple readily with mucopolysaccharides and the resulting complex precipitates (52). The glycoprotein minus sialic acid is extremely reactive and has also been shown to bind calcium more readily (53). Conceivably a combination of these reactionscould lead to the formation of stone matrix in magnesium deficiency.

Though the statistical evaluation of Experiment V showed no significant change with treatment, it did show significant change with day of the diet. Both groups showed lower activity for day 5. It

could be argued that this is perhaps an error in technique, that perhaps the assay time was longer or that the reaction mixture was less than 2 ml, but this is considered to be highly unlikely. For each day four sets of animals were run, 8 in the morning and in the afternoon of two consecutive days. It does not seem reasonable that a mistake would be repeated for these four runs and not for the first or the last four. Furthermore, the standard error for day 5 was no greater than for days 2 and 8, ruling out the possibility of a single run contributing the error. A similar phenomenon had been seen in earlier experiments; however, in Experiments I and II, there is an increase in activity at day 8. This is possibly attributable to slight variations in technique: different lengths of acclimation, lack of fasting prior to killing, etc. Though the data is not consistent, it does point out an interesting fact: there are statistically significant differences in lysosomal enzyme levels from day to day. One of two conclusions can be drawn: lysosomal enzyme levels change radically with age, or the rats are undergoing some kind of an adaptive change from the rat pellet diet to one extremely high in sucrose. It seems unlikely that enzymes would drop and then rise in Experiment V if it were a change with age, yet it also seems somewhat doubtful that adaption could account for the changes either. The latter seems the more likely explanation, as frequently after the drop in day 5 the activity returns to a somewhat lower level, simulating the return of a system to a lower equilibrium.

The major change between these two diets is the carbohydrate source. Where rat pellets are mainly starch, the high sucrose diet is, of course, sugar. It is interesting to note that the staining is increased in the area of the tubular epithelial basement membrane and not throughout.

This might indicate an increase in production of Tamm-Horsfall glycoprotein although assuredly, there are probably many other carbohydrates produced here as well.

To speculate further, if the diet were to cause an increase in Tamm-Horsfall protein production or in other glycoprotein or carbohydrate production it could act, in part, like an adjuvant. The system would have more material which if cleaved of sialic acid would bind calcium or carbohydrate and precipitate out. This theory would seem to hold up in view of previous work (53) using a cornstarch carrier diet which showed a protective effect against the calcification occurring during magnesium deficiency. However, to be perfectly fair this same experiment showed increased calcification using rice as a carbohydrate and protein source.

The histochemical picture as presented in Figures 1-15 is an interesting one. No differences could be detected in any of the sections from day 2. By day 5, however, differences could be seen in the PAS stained sections. Not only did both groups of animals on the sucrose diet show increased stainability of the tubular basement membranes, but the animals on the low magnesium diet also show PAS positive material in lumen of these tubules (Figures 1-4). By day 8 the PAS sections of animals on the low magnesium diet, show the replacement of PAS positive material with typical calcified casts which present a layered appearance (Figures 5-8). Again both the control and the low magnesium diets seem to produce heavier staining in the basement membrane of the tubule cells as well as smaller lumen, probably due to swollen cells. It is quite evident that the earliest accumulation of PAS positive material is within the cell as the lumens are still well defined,

and that the accumulation of PAS positive materials in the lumen comes at a later stage. Conceivably what is occurring in the explusion of material into the lumen of the tubule when the cell or lysosome becomes too heavily engorged (Figures 5-8).

The hematoxylin stained sections show the destruction of the tubule by the calcification process as well as the swollen tubule cells. The calcification seen in Figures 10 and 11 seems to occur in the lumen of the tubule. In Figure 10 the initial calcification can be seen (arrow) to occur in the lumen of the tubule as both sides of the tubule can be seen intact. In Figure 11 the calcification has begun to destroy the tubule.

Acid phosphatase and β-glucuronidase activities show no difference up until day 8 where the areas where calcium appears, appear heavier in enzyme. Both enzymes exhibit particulate rather than diffuse activities indicative, perhaps, of their lysosomal location (Figures 12-15). The increase in intensity of staining in the area of the calcification seems to indicate a possible shift in distribution of the enzymes. Conceivably this could be the response of the system to the intrusion of the calcium and might indicate an increased phagocytic activity in this area.

In conclusion it must be said that based on the levels of acid phosphatase and β-glucuronidase measured in the kidneys of rats fed a low magnesium or a control diet, there is no reason to suggest any difference in lysosomal enzyme levels due to treatment. The histochemical picture suggests a shift in distribution of the enzymes within the tissue, probably in response to the calcification; this, however, would probably not influence the biochemical assay. An explanation of this inability to demonstrate any differences could lie in a failure to examine the proper parameter. Examination of an enzyme conceivably more

intimately involved in the mechanism of calcification such as neuraminidase might produce entirely different results.

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ABSTRACT

The Influence of Magnesium Deficiency on
Kidney Lysosomal Enzyme Levels in the Rat

by

Janice D. Longstreth

Feeding growing rats a diet deficient in magnesium results in a deficiency condition whose major characteristic, that of kidney calcification, exhibits a mechanism very similar to that of urolithiasis in humans. Work done with lysosomal stabilizers and rats fed this deficient diet shows a reduction in calcification with the administration of these drugs. Accordingly, a study was undertaken to examine the mechanism of this process more closely with regards to a possible involvement of kidney lysosomes or the vacuolar apparatus. Lysosomal enzyme levels in the kidneys of rats fed either a low magnesium or a control diet were examined and an attempt made to determine if there were any differences due to treatments. While biochemical evidence suggests no differences, histochemically we see what appears to be a shift in activity to the area where calcification occurs. At the same time, while there is no effect from treatment, there appears to be a time effect, enzyme activity decreasing or increasing significantly with day. This seems to appear histochemically in the form of increased PAS stainability in sections from rats on either low magnesium or control diet compared to rats on a rat pellet diet.