THE ISOLATION AND PARTIAL CHARACTERIZATION OF RAT KIDNEY STONE MATRIX INDUCED BY MAGNESIUM DEFICIENCY

by

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INTRODUCTION

A normal level of magnesium in the body is of importance for many enzymatic reactions involing ATP, for muscular contraction, for neural transmission, and for the maintenance of ionic balance. A specific example of the latter is the role of magnesium in controlling the solubility of calcium phosphate in the urine (1).

Magnesium deficiency in experimental animals has been studied in some detail. Some of the more prominent effects of reduced magnesium intake in the diet are a decline in urine and plasma magnesium levels, alterations in heart mitochondrial enzymatic activities, an increase in calcium absorption from the gut (2), and calcification in the aorta (3). Perhaps the most dramatic effect, however, is the rise in kidney calcium levels in the form of calcium deposits in the renal tubules (urolithiasis). This rise in kidney calcium can be stopped by restoring the dietary magnesium to normal levels, or, in the magnesium deficient rat, by parathyroidectomy (4) or by administering thyroxine injections (5).

Experimental magnesium deficiency is one of several models that are commonly used to study renal calcification. Other methods include high phosphate diets, excess vitamin D, parathyroid hormone injections (6), and high calcium diets (7). Much of what is currently known regarding renal stone genesis has been determined using one of these methods of stone genesis with experimental animals. Valuable information has also been gained from analysis of actual stones that have been surgically removed from humans.

Human kidney stones are generally composed of a crystalline portion of calcium oxalate, calcium phosphate (as hydroxyapatite), uric acid, magnesium ammonium phosphate, and/or cystine, depending upon the ionic composition of the urine. By far the most common crystalline composition is a mixture of calcium oxalate and calcium phosphate. In addition, a matrix of protein and mucoprotein material is always present, accounting for about 3% of the gross weight of the calculus. It is not certain whether or not this matrix material functions in stone genesis, but much current thinking favors this concept over that of spontaneous precipitation of the crystalline phase (8). The biochemical composition of human renal stone matrix has been determined as well as histological studies of the structural features of matrix and of its structural relationship to the intact stone. Yet, many unanswered questions remain as the cause(s) of kidney stones, the mechanism of stone genesis, and even as to the origin(s) of the matrix material within the stone.

Clearly, a good experimental model offers the advantage of studying, under controlled conditions, the progression of events leading to the formation of a renal calculus, and provides a means of generating calculi for study. The efforts of this research project have been to determine the nature of the renal calculi induced by magnesium deficiency in the rat. Therefore, a technique has been developed to isolate rat kidney stones that have been induced by magnesium deficiency, since they have indeed been observed histologically. Secondly, efforts have been made to show that an organic matrix exists inside the stones, as it does in human stones. Finally, the biochemical composition of the matrix has

been partially characterized. This information will hopefully provide a background so that two important questions can eventually be answered: (1) What is the origin of matrix, and (2) Does it have a definitive role in stone genesis and development?

LITERATURE REVIEW

The first published studies of magnesium deficiency in rats were by Kruse et al. (9) who employed a low magnesium diet which contained only 1.8 parts per million (ppm). An adequate level of dietary magnesium for the rat is about 400 ppm, according to a 1962 report by the National Academy of Sciences. Weanling rats (strain not given) of initial weight 35-45 g were fed this diet and developed a rather spectacular symptomatology. Within 3-5 days all the exposed skin areas become vividly red from vasodilatation. The color intensified until about the 11th-14th day, after which it slowly faded. During the hyperemic period the animals were hyperexcitable, and this phenomenon became progressively more pronounced until about the 18th day when any sudden noise threw the animal into fright that was followed by a convulsive seizure and death.

Watchorn and McCance (10) fed a diet containing 40 ppm of magnesium to piebald rats of initial weight 55-83 g. Hyperemia of the skin was apparent after a few days on the diet. It was noted that these rats never had convulsions over a 3 month period on the diet; however, younger rats of the same strain (initial weight 35-40 g) showed increased excitability with some mild tremors. In certain cases albino rats (65 g initial weight) had convulsions (10). After 3 months on the low magnesium diet the total calcium in the kidney was elevated 15 fold while other tissues of the body had normal calcium levels. Upon histological examination calcareous deposits were observed in the kidney, scattered from the cortex to the medulla. The magnesium level in various tissues

including the kidney were normal in all cases. It was found that serum magnesium levels were reduced to about half of normal, but that serum calcium was not elevated in the magnesium deficient rats.

Greenberg et al. (11) also reported calcium concretions in the tubules of kidneys from magnesium deficient rats. They observed the greatest degree of calcification at the cortico-medullary junction, stating that in initial stages the tubules contained much granular debris and that in later stages of deficiency the large concretions of calcium were observed. These authors speculated that tissue damage contributed to, but might not be responsible for, the calcification that took place in the kidneys of the magnesium deficient rats.

Further efforts to explain the calcification phenomenon were presented by Hess et al. (12) who fed 95 g rats a diet containing 6 ppm of magnesium. After 3 days of magnesium deprivation, they observed mitochondrial swelling in cells of the distal part of the proximal convoluted tubules in the inner cortex. After 9 days there was apparent necrosis of the epithelia in limited segments of the distal part of the proximal convolution and a marked decrease in mitochondrial enzymatic activities. Intracellular fine calcium deposits were seen at this same location, but there was no apparent damage in other areas of the nephron. By 12 days the calcium deposits were larger and became detached into the tubular lumen forming calcareous casts. Chemical analysis of the serum and kidney tissue confirmed the results of Watchorn and McCance (10) with respect to the low serum levels of magnesium, the normal levels of kidney tissue magnesium, and the high levels of calcium in the kidney. Hess et al. proposed that the visible increase in intracellular calcium

in the proximal tubular cells might be caused by a selective loss of magnesium from those cells, not detectable when the whole kidney was analyzed for magnesium levels. This selective loss of magnesium could result in mitochondrial disfunction with subsequent cell death. Calcium levels could then rise in the damaged cells due to calcium binding to intermediates of the tricarboxylic acid cycle, especially citrate, which might possibly accumulate after a decrease in the mitochrondrial enzyme activities.

Calcified casts were seen by Ko et al. (13) in the inner cortex in the distal part of the proximal convoluted tubules of 75 g rats fed a diet containing 16 ppm of magnesium. The casts were described as being usually solid with concentric arrangements suggesting the slow accretion of material. In contrast to previous work, Ko et al. observed calcification only in the tubular lumen, not in the cells. In addition no mitochondrial swelling was seen, although the histological sections were taken from animals that had been deprived of magnesium for longer periods of time than in the work of Hess et al. (12).

Sabour et al. (14) also did not observe morphological changes in the mitochondria during the calcification process. These workers fed a 24 ppm magnesium diet to rats weighing initially 120-150 g. After 11 days the typical symptoms were observed, vasodilatation in the nose, ears, and paws, signs of hyperirritability, the drop in serum magnesium, and the elevation of kidney calcium. Tubular abnormalities were evident by histological sectioning after 3 days of deficiency, but these effects were more pronounced after 13 days, affecting tubules in all areas of the cortex and outer zone of the medulla. After 3 days the cells

surrounding the dilated tubules were swollen and freqently vacuolated. No calcified casts were observed at 13 days deficiency. However, dense granules were seen in the cytoplasm which the authors postulated as being deposits derived from the tubular lumen. Their observations led them to propose that in deficient rats two adjacent microvilli could come in contact with each other at their luminal ends enclosing a droplet of tubular fluid, shown to be low in magnesium in deficient rats. Thus, the proximal tubular cells may recover excess calcium from the glomerular filtrate. The excess calcium ions may be largely deposited locally in these cells, appearing as dense granules in micrographs. This could account for nephrocalcinosis, or calcium deposition within the tubular space, at later stages of deficiency. Unfortunately, it was not determined in the paper whether these intracellular dense granules were indeed rich in calcium.

The particular area in the kidney exhibiting morphological changes in magnesium deficiency is not agreed upon by all workers. Schneeberger and Morrison (15) observed, by electron microscopy, calcific deposits in the nephrons of 50-75 g rats in the thin limb of Henle and less frequently in the thick ascending limb. Deposits were both intracellular and intraluminal. In addition, they observed calcified lysosomes and swollen mitochondria. In most cases calcium deposits completely destroyed the epithelial cells, leaving the basement membrane to surround the calcareous mass.

Heaton and Anderson (4) found that in histological sections from kidneys of 100 g rats fed a diet containing 3 ppm of magnesium that there were lesions in two areas. Swelling, vacuolation, and focal

necrosis were observed in epthelial cells lining the proximal convoluted tubules of the inner cortex and the thin limb of Henle at the cortico-medullary junction. This change was accompanied by precipitation of calcium phosphate in the necrotic cells and the formation of calcium casts in the affected tubules. The casts appeared to be composed equally of granular calcified debris and of laminated bodies. They postulated that the granular component was from necrotic tissue and the laminated component of lumenal origin.

In addition to the effects observed by simple magnesium deficiency, Heaton and Anderson observed that parathyroidectomized rats fed a low magnesium diet did not develop renal calculi and exhibited a normal level of total kidney calcium. Serum magnesium was low in these animals, as in simple magnesium deficiency, but serum calcium was normal in the parathyroidectomized rats fed the low magnesium diet. This is in contrast to simple magnesium deficiency where it has been observed by MacIntyre and Davidsson (16) that serum calcium was slightly elevated in 100 g rats fed a diet containing 24 ppm of magnesium. Since parathyroidectomy prevented renal lesions and any increase in plasma calcium during magnesium deficiency, and since injection of parathyroid extracts into normal animals produces renal lesions and hypercalcemia (6), it appeared to Heaton and Anderson that renal lesions and hypercalcemia in simple magnesium deficiency are due to increased activity of the parathyroid glands.

Battifora et al. (17), however, noted differences in the histological effects upon the kidney of simple excess parathyroid hormone contrasted to histological changes due to simple magnesium deficiency in

100-150 g rats fed a diet containing 5-7 ppm of magnesium. In magnesium deficiency these workers found that the calcium deposits were restricted to the area of the cortico-medullary junction and that there was an absence of mitochondrial calcification. Control rats given injections of a parathyroid extract, however, had more widespread kidney calcification and exhibited calcium accumulation in the mitochondria (18).

In a recent report Parker and Forbes (19) found a significant positive correlation in parathyroid injected male rats (weighing about 100 g) between amount of the hormone injected and the amount of cyclic adenosine monophosphate (cAMP) excreted in the urine. However, by feeding varying suboptimal levels of magnesium, it was observed that the lower the level of dietary magnesium consumed, the less cAMP excreted. From these and other data the authors concluded that renal calcification in magnesium deficient rats can not be explained by increased levels or activity of parathyroid hormone since in magnesium deficiency there is an apparent decrease in parathyroid activity.

Oliver et al. (20) have utilized a microdissection technique which allowed them to remove and examine the entire nephron. They assert that this technique is particularly suited to the description of the location of the progressive lesions leading to intranephronic calculus formation. Rats of initial weight 30-60 g were fed a diet containing 69.6 ppm of magnesium. During the first week of deficiency no abnormalities were observed in histological sections of the kidney. During the second week, however, minute spherical microliths from 13-23 microns in diameter were seen within the thin limb of Henle's loop. From their first appearance, the microliths reacted positively with the Periodic Acid Schiff (PAS)

procedure and with the von Kossa and alizarin stains. The PAS stain is a nonspecific indication of carbohydrate, usually taken as indirect evidence for glycogen, mucopolysaccharide and/or glycoprotein. The von Kossa and alizarin stains are indicative of the presence of calcium. Thus, the microliths appeared as precipitated calcium within a matrix of PAS positive substances. These microliths were observed to reach a limiting size of about 25 microns in diameter above which they increase in length by repetition of the process of formation and accretion of new microliths until the lumen is filled by a composite mass of fused concretions.

Oliver et al. (20) have further observed that there are both local effects in the nephron containing the calculi and in adjacent nephrons. Their interpretation of these and other results have led them to conclude that upon formation of a single spherolithic calculus in the first descending portion of the thin limb of Henle, there is subsequent movement down the tubule, causing part of the epithelium of the tubule to be stripped off. The calculus lodges in the hair-pin bend of Henle's loop, while the cellular debris is distributed beyond the calculus reaching into the ascending limb of Henle. The lodged spherolith then grows as previously described. The cellular debris, is initially PAS positive and negative for calcium, but eventually does form a calcific deposit. Upon closer examination of these secondary calcium deposits it was observed that there is no internal organized structure, but rather they appear as amorphous aggregates of PAS and calcium positive material. In contrast, the lodged spheroliths do show a laminated structural pattern. Thus it was concluded that two types of calcium deposits,

differing in their pathogenesis, exist in the nephron. These authors explain the renal damage seen by workers in other areas of the nephron as being the result of hydronephrotic effects caused by the lodged calculi.

The factors that lead to the formation of calculi in the kidney of the magnesium deficient rat are not clear. In an attempt to explain the phenomenon, Bunce and Bloomer (21) utilized calcium and divalent cation-selective electrodes to measure serum and urinary ions in magnesium deficient rats. Rats weighing about 80 g were fed a moderately deficient diet containing 70 ppm magnesium. The authors found that the serum calcium levels did not change in 7 days deficiency, and that the proportion of free to total serum calcium remained at normal levels as well. Total serum magnesium, however, significantly declined in the first 24 hours following consumption of the first low magnesium meal. The serum magnesium continued to decline until day 4 or 5 when it reached a level of 40-50% of normal. Excretion of total calcium in the urine dropped within 15 hours of the first low magnesium meal with an increase in the proportion of free to total calcium after two days deficiency. Urine magnesium dropped to about 1% of normal within 15 hours of the first low magnesium meal. Urine inorganic phosphate remained at normal levels throughout the experiment of 14 days as did the average volume and pH of the urine. Total kidney magnesium was normal, as well, throughout the two week study, but total kidney calcium was elevated in magnesium deficiency. The rate of accumulation of calcium in the kidney was noted to be variable, some animals displaying only slight increases after 6 or 7 days while others had 2-4 fold increases in total kidney calcium

at this time.

The authors noted that octocalcium phosphate has been proposed to be the salt that governs the solubility of calcium and phosphate in the urine. According to their results rat urine is normally on the verge of spontaneous precipitation of calcium phosphate, based on the fact that the ion product is typically in the metastable region. It is interesting that the ion product in the magnesium deficient rat urine was slightly but not significantly reduced. Spontaneous precipitation may occur in the metastable region only when there is an appropriate nucleating material. Bunce and Bloomer proposed that a calcium avid matrix could be derived from the glycocalyx (cell coat) of the proximal tubules or possibly from the lysosomes. Both of these materials have been shown to stain strongly PAS positive which is consistent with the observations of Oliver et al. (20) concerning the PAS positive nature of stone matrix. The involvement of the glycocalyx and the lysosomes, the authors believe, could be explained by their activity in the vacuolar apparatus system. By endocytosis of glomerular filtrate in the proximal tubules, a vacuole with a high proportion of calcium to magnesium, contained in a membrane derived from the PAS positive glycocalyx, could be brought into the cell. Upon merger with a primary lysosome containing acid phosphatase, it is conceivable that conditions might favor formation of insoluble crystals of calcium phosphate. By a process of exocytosis these crystals could then be regurgitated back into the lumen where they could grow into calculi in the metastable calcium phosphate medium of the glomerular fluid.

In a later report Bunce et al. (22) fed 70-85 g male rats a 10-20 ppm magnesium diet. Calcium accumulation in the kidney was significant after 2 days on the diet. By differential centrifugation it was shown that the majority of the increased calcium was in the heavy nuclear fraction. Histological sections of kidney were only taken from 8 day deficient rats, and microliths were found in sections taken from the nuclear fraction. This indirectly suggests that the large increase in the kidney calcium of magnesium deficient rats could be due to calculi formation.

In addition to studies involving experimentally induced renal calculogensis, efforts have been made to determine the nature of kidney stones from humans. Boyce and Sulkin (23) have surgically removed kidney stones from humans and have decalcified the stones in order to determine the chemical composition of the matrix material. They have also sectioned stones without decalcifying them to study the morphological relationship of the matrix and crystalline components. Intact human kidney stones range in weight from 60 milligrams to 28 grams per stone. The matrix comprises approximately 2.5% of the weight of each calculus, and is PAS positive. The crystalline part of the stone is usually either calcium phosphate, calcium oxalate, or a mixture of the two. The elemental analysis (23, 24), amino acid, and carbohydrate compositions of the matrix material have been determined.

Boyce and King (25) have also put forth a hypothesis for initiation and growth of renal calculi in humans. They have proposed that some as yet undetermined event causes normal cellular metabolism to be disturbed by altered mitochondrial enzyme activities which results in intracellular

accumulation of mucoprotein that is the precursor of stone matrix. The rupture of these mucoprotein laden cells fills the tubular lumen with this mucoprotein which soon accretes calcium. The growth and composition of the mature calculus, the authors believe, reflects the ambient composition of the urine. This hypothesis is strongly rooted in the results of experimental stone genesis in animals.

Boyce (26) describes the matrix material as being present from center to surface in the stone, and as being a laminated structure consisting of compact parallel fibrils between successive laminations of a more amorphous material. The histochemical similarity between human kidney stone matrix and that seen by Oliver et al. (20) in the magnesium deficient rat is encouraging, but the true origin of the matrix remains unknown.

In an earlier report, King and Boyce (27) compared the chemical composition of a variety of calcifiable matrices. These included urinary calculi, bone, and salivary calculi, which all have in common an organic matrix and hydroxyapatite as the most common crystal structure. Furthermore, the various matrices all had a protein-carbohydrate component. However, hexuronic acid, which would suggest the presence of acid mucopolysaccharides, was consistently present only in bone matrix. Glucose, galactose, and mannose were present in all the matrices, but in varying concentrations. It was determined that gall stones, which are predominantly composed of cholesterol, do not contain any of these neutral carbohydrates. Finally, it was noted that the absolute concentrations of protein bound glucides and hexosamine were quite variable. These data led the authors to conclude that a calcifiable matrix is

probably a prerequisite of calcification in biological systems, but that it is not dependent upon the presence of any one molecular structure.

The isolation and partial characterization of the calcifiable matrix from the kidney stones of magnesium deficient rats has been the goal of the present study. An understanding of the matrix composition from magnesium deficient rats may lead to the ultimate goal of understanding stone genesis in these animals and provide useful information for understanding stone genesis in man.

MATERIALS AND METHODS

The procedures used in this research will be presented as follows: (1) Treatment of animals and preparation of kidney homogenates, (2) Isolation of rat kidney stones, (3) Decalcification and fractionation of kidney stones into a nondialyzable (matrix) fraction and a dialyzable fraction, and (4) Analytical techniques.

Treatment of animals and preparation of kidney homogenates--Groups of of 20 male Sprague-Dawley rats having an initial weight of 45-55 g were purchased from Flow Laboratories (Dublin, Virginia). The animals were housed in stainless steel wire cages (two animals per cage) and were fed a low magnesium diet (Table 1) and given double distilled water *ad libitum* for a period of 9 days. At this time the animals were sacrificed by a blow to the skull followed by dislocation of the vertebrae. The kidneys were then removed, the kidney capsules were discarded, the tissue minced with scissors, and homogenized in a Potter-Elvehjem homogenizer (fitted with a teflon pestle with a 2 mm clearance) in a medium of cold 0.3*M* sucrose, 1.0*mM* ethylenediamine tetraacetic acid (EDTA), adjusted to pH 7.0 with NaOH (volume of homogenizing medium=2 times the weight of the original tissue), using 10 up and 10 down strokes.

Procedure for isolating kidney stones--The kidney homogenate was diluted to 10 times the weight (w/v) of the original tissue withtthe 0.3*M* sucrose reagent described above, mixed well, and centrifuged on a Sorvall centrifuge using an SS-34 rotor head at a speed of 1250 rpm (120xg) for 10 minutes at 5^{o}. The pellets were combined and frozen until the following day. Then the pellet was diluted with the 0.3*M* sucrose

TABLE I

Composition of the Low Magnesium Diet[a,b]

Dietary Component	Percentage of Diet
Casein (vitamin free)[c]	20.0
Sucrose[d]	26.6
Starch[e]	33.2
Cellulose[e]	5.0
Vitamin mix[c]	2.2
Cottonseed oil[d]	8.0
Mineral mix[f]	5.0

[a] Wet ashed sample of diet contained 17.5 ppm magnesium as analyzed by atomic absorption spectroscopy.
[b] Bunce et al. (22).
[c] Nutritional Biochemicals Corporation
[d] Commercial retailers
[e] ICN Pharmaceuticals, Inc.
Percent composition of mineral mix (w/w%): NaCl 2.54; KH_2PO_4 12.52; $CaHPO_4$ 24.2; $CaCO_3$ 18.20; $MnSO_4 \cdot H_2O$ 0.037; $FeSO_4 \cdot 7H_2O$ 0.3; $CuSO_4 \cdot 5H_2O$ 0.005; $ZnSO_4 \cdot 7H_2O$ 0.127; KI 0.00047; $CoCl_2 \cdot 6H_2O$ 0.00008; Cellulose 42.07.

reagent to 2 times the weight (w/v) of the original tissue and homogenized again with the Potter-Elvehjem homogenizer using 3 up and 3 down strokes. This homogenate was then layered on top of a solution containing 1.8M sucrose, 1.0mM EDTA, pH 7.0 contained in cellulose nitrate tubes of 30 ml capacity, such that the homogenate in each tube represented approximately 3-4 g of original tissue. The tubes were then put in SW 27 swinging buckets, attached to the rotor head, and centrifuged for 3 hours on a Beckman L5-65 preparative ultracentrifuge at a temperature of 5^{o} and at a speed of 20,000 rpm (approximately 60,000xg). Following the centrifugation, the supernatants from each tube were discarded, the pellets washed with distilled water, centrifuged at 1000xg and at ambient temperature in an IEC model UV centrifuge, equipped with a #279 rotor, to pellet the tissue, and the wash was discarded. The pellets were then combined and transferred into 15 ml conical centrifuge tubes, washed with water again, centrifuged in a clinical centrifuge at approximately 4000 rpm, and the washings were discarded. To the pellets were added about 5 ml of 1.0 M KOH, and the tubes were placed in an incubator for 24 hours at 38^{o}. Then, the tubes were centrifuged on a clinical centrifuge at 4000 rpm (setting 4), the supernatants discarded, a fresh 5 ml of KOH added, and the tubes incubated for another 24 hours. At this point the kidney stones were visible, since the tissue had been solubolized by the alkaline treatments. Next, the stones were centrifuged at 4000 rpm, the supernatants discarded, and the pellets washed twice with 10 ml of distilled water, centrifuging at 4000 rpm after each wash and discarding the washes. The stones were white and visually appeared to be free of any debris except for a few fibrous materials.

The fibrous contamination was removed by passing the stones through a fine mesh screen, such that the stones passed through but the fibrous materials did not. The stones were collected in a small plastic vessel and frozen. This was then lyophilized, put in a vacuum desiccator containing P_2O_5, and left for at least 24 hours before weighing. Twenty rats had kidneys totalling about 18 g wet weight. This procedure yielded about 9-18 mg of kidney stones.

Isolation of kidney stone matrix--A known weight of dry kidney stones (usually 50-100 mg of pooled stones) were placed in a 20 ml beaker containing 10 ml of 0.5M formic acid. The stones were gently stirred in the acid, by means of a magnetic stirrer, at a temperature of 4^{o} for 24 hours in order to decalcify the stones. Even after the 24 hour period, the solution appeared cloudy. This suspension was quantitatively washed with water into a cellulose dialysis bag (Arthur H. Thomas Co.) having a 12,000 molecular weight exclusion limit. The bag had been previously soaked in several changes of hot water to remove the glycerine. The bag was tied such that it was turgid and placed in a graduated cylinder containing about 300 ml of distilled water (previously passed through filter paper) and a stirring bar magnet. The calcium, phosphate, formate ions and other small molecules in the dialysis bag were allowed to equilibrate with the distilled water on the outside for a period of 6 hours at 4^{o} while being continuously stirred on a magnetic stirrer. At the end of 6 hours, the dialyzate was removed and saved for later analysis, and a fresh 300 ml of filtered distilled water was added to the graduated cylinder. The contents of the dialysis bag were again allowed to equilibrate for 6 hours, as before. Then, the dialyzate was pooled

with the previous dialyzate, replaced by a fresh 300 ml volume, and the bag was allowed to equilibrate overnight. The next morning, the dialyzate was added to the previous two volumes of dialyzate, the total volume was measured and the solution saved for later analysis. The contents of the bag, which still appeared cloudy, were quantitatively washed with water into a plastic container, frozen, lyophilized, and placed in a desiccator containing P_2O_5 for at least 24 hours before weighing. 100 mg of decalcified stones yielded approximately 5 mg (5% of the stone weight) of nondialyzable matrix. This quantity of matrix represented that amount obtainable from 100-200 rats.

Prior to the development of the formic acid procedure, outlined above, another method was employed using ethylenediamine tetraacetic acid (EDTA) to decalcify kidney stones. However, it was felt that the EDTA method was not as efficient due to long dialysis step. Since a portion of the matrix isolated for this research was obtained utilizing the EDTA method, the procedure has been included. An 8% EDTA solution was prepared by mixing solutions of the di- and tetra- sodium salts of EDTA such that the resulting solution had a pH of 8.0. To 40 or 45 mg of pooled dry calculi contained in a 20 ml beaker were added 9.5 ml of the EDTA solution. This was stirred gently at 4° for a period of 24 hours on a magnetic stirrer. This cloudy suspension was transferred with a syringe into an Amicon ultrafilter model MMC fitted with a UM-10 membrane (10,000 molecular weight exclusion limit). The ultrafiltration step was also carried out at 4°. The matrix material was dialyzed against distilled water for a period of about 3 days to remove EDTA and other small molecules. The flow rate from the Amicon was extremely slow

under these conditions which was believed to be due to insoluble matrix particles clogging the surface of the membrane. The dialysis was judged complete when samples of the effluent had a conductance approaching that of distilled water and when absorption spectra of effluent samples showed only slight absorbances at 215 nm, where EDTA absorbs strongly. At the end of the dialysis step, the contents of the Amicon were quantitatively removed and put in a plastic container. This suspension was then frozen, lyophilized, and put in a desiccator containing P_2O_5 for at least 24 hours before weighing. With this method, the recovery was such that 7.5-8.0% of the original stone weight could be accounted for as non-dialyzable matrix.

Elemental analysis of matrix--The carbon, hydrogen, and nitrogen content of the matrix isolated by both procedures were determined with a Perkin Elmer model 240 Elemental Analyzer. The analysis involved pyrolysis of a known weight of sample and conversion of the carbon, hydrogen, and nitrogen to carbon dioxide, water, and molecular nitrogen, respectively, and measurement of these compounds by means of a series of thermal conductivity detectors and traps.

Calcium determinations--The calcium content of the dialyzates from both isolation procedures was determined by removing a small aliquot of the total dialyzate, adding an appropriate amount of lanthanum oxide, diluting to a determined volume, and measuring the calcium concentration with a Perkin Elmer model 303 atomic absorption spectrophotometer, utilizing an air-acetylene flame. The calcium concentration in the sample was determined by taking readings of a series of known standards.

It was found that wet ashing samples of dialyzate did not increase the quantity of measureable calcium; therefore, the wet ashing step was omitted for dialyzate samples. However, for the determinations of calcium in the matrix and in the whole kidney stones a wet ashing procedure was used, consisting of a series of steps involving the addition of concentrated nitric and perchloric acids to the sample contained in a beaker and heated on a steam bath. To the dry sample, contained in a 150 ml beaker, was added 2-3 ml of concentrated HNO_3. This was heated on a steam bath, under a hood, to dryness. Next, about 1 ml of 30% hydrogen peroxide was added, and this was taken to dryness on the steam bath. About 3 ml of concentrated HNO_3 was then added; this was heated and taken to dryness as before. Next, 8 ml of concentrated perchloric acid and 10 ml of concentrated HNO_3 was added to the beaker. A watch glass was placed on top of the beaker, and the beaker was heated on an electric hot plate until dense white fumes appeared in the beaker. Then, the watch glass was removed, and the acids were allowed to evaporate to dryness. Following wet ashing, the calcium concentration in the sample was measured as previously described.

Measurements of phosphate--The inorganic phosphate content of the dialyzate was measured directly by the method of Fiske and SubbaRow (28). It was also determined that the total phosphate content of the dialyzate equalled the inorganice phosphate content. This was found by carefully wet ashing a sample of the dialyzate in a test tube with a small volume of sulfuric acid. The heat required for the wet ashing was provided by a Bunsen burner. The sample was then measured for free phosphate, as before, by comparison to a standard curve. The total

phosphate of the matrix (nondialyzable fraction) isolated by the formic acid procedure was also determined using the same procedure of wet ashing and subsequent measurement of the free phosphate.

Total nitrogen content of the dialyzate--The micro-Kjeldahl procedure (29) was used to determine the nitrogen content in the dialyzate from the formic acid isolation procedure.

Determination of neutral sugars--Neutral sugars were measured in the matrix isolated by the EDTA method and in the dialyzate by the phenol-sulfuric acid technique (30); matrix isolated by the formic acid treatment was analyzed for neutral sugars by the orcinol reaction (31). In both assays glucose was used as the standard. Pentose was measured in the matrix isolated by the formic acid treatment by a second version of the orcinol reaction (32) that is more specific for pentoses. In that assay ribose was used as the standard. Neutral sugars in the matrix isolated by the EDTA method were further characterized by gas chromatrography, according to the procedure of Metz et al. (33) with several minor modifications; the hydrolysis step was done in 5 ml Reactivials (Pierce Chemical Co.), and Amberlite MB-3 (Mallincrodt Chemical Works), a mixed cation and anion exchange resin, was substituted for Dowex I acetate to neutralize the hydrolyzate (34). The neutral sugars, as the volatile alditol acetate derivatives, were dissolved in chloroform and separated using a Varian model 2740 gas chromatrograph with dual flame ionization detectors. A 10 foot glass column (2 mm inside diameter) packed with 1% OV-225 on Chromosorb G-HP was used with nitrogen as the carrier gas at a flow rate of 13 ml/min. The flow rates for air and hydrogen were 225 ml/min and 25 ml/min, respectively. A temperature

program was used to achieve the desired separation of sugars; the temperature was held at 205° for 7 minutes, increased to 230° at 20°/min, and held at 230° for 11 minutes. Arabinose was added as an internal standard before hydrolysis of the matrix sample. After computing relative response factors, the quantity of each sugar was determined by measuring relative peak areas. The gas chromatographic procedure, as outlined, gave satisfactory results with the commercial glycoprotein, transferrin (Sigma Chemical Co.). The reported neutral sugar content of transferrin ranges from 2.4-2.8%, consisting of mannose and galactose. Upon analysis only these sugars were detected, totalling 2.4% by weight of transferrin.

Determination of amino sugars--Amino sugars were assayed in the matrix isolated by the EDTA treatment by gas chromatography by a modification of the procedure of Metz et al. (33). The sample was hydrolyzed with 4N HCl for 4 hours at 100° under nitrogen; Dowex I HCO_3^- was used in a batch method to adjust the pH to 4.1 after hydrolysis, instead of neutralizing the sample with Dowex I acetate. For the analysis a 6 foot (2 mm inside diameter) column was employed, packed with 1% OV-225 on Chromosorb G-HP. The chromoatographic conditions were a 240° isothermal column temperature and a carrier gas flow rate of 13 ml/min. This method gave satisfactory results with transferrin, which is reported to contain 1.8-2.0% glucosamine. Upon analysis 1.98% of transferrin was found to be glucosamine. Amino sugars were further assayed in the matrix with a Beckman model 120B amino acid analyzer utilizing an amino sugar program and an Autolab System AA integrator. To prepare the sample for analysis, a previously developed technique (used to measure

hexosamines in chitin) (35) was used involving hydrolysis with 8 N HCl for 2 hours at 95°. Still a further modification of hydrolysis conditions was to hydrolyze the matrix, isolated by either the EDTA or formic acid treatments, in 6 N HCl for 24 hours at 110°, evaporating the acid in a vacuum, and analysis of the amino sugars with the amino acid analyzer, as before. With this latter technique two commercial mucopolysaccharides, heparin and chondroitin sulphate, were analyzed for their hexosamine content. Fully sulphated heparin and chondroitin sulphate contain about 38% glucosamine and galactosamine, respectively. Upon analysis 8.8% glucosamine was found in heparin and 19.2% galactosamine was found in chondroitin sulphate. This would indicate 50-75% destruction of hexosamines by this method of analysis.

Assay for hexuronic acids--The carbazole reaction (36) was employed to assay for hexuronic acids in the matrix prepared by the EDTA decalcification procedure. The Amicon effluents from the dialysis step were also monitored from these preparations for hexuronic acid content, following concentration of the effluents by lyophilization. Glucuronic acid was used as the standard for these determinations. Using this technique commercial heparin was subjected to analysis. Fully sulphated heparin contains about 37% glucuronic acid by weight. Upon analysis 29% was found.

Measurement of sialic acids--The thiobarbituric acid assay (37) for measurement of total sialic acids was used to assay for their presence in matrix isolated by the formic acid treatment and also in the dialyzate, following concentration by lyophilization. N-Acetylneuraminic acid was used as a standard.

Cholesterol determination--Total cholesterol in the formic acid treated matrix was assayed by gas chromatography (38). The matrix sample was saponified to free cholesterol, extracted with heptane, and injected into a Bendix model 2600 gas chromatograph fitted with a 2 foot 4 inch (4 mm outside diameter) glass column packed with 5% SE-30 on 80/100 mesh Chromosorb W-HP (Supelco, Inc.). The concentration under the peak was computed on the basis of an external commercial standard (cholestane-cholesterol standard, Supelco, Inc.) of known concentration. The cholesterol was detected with a flame ionization detector. Chromatographic conditions were a temperature program of 240-250^{o} at 4^{o}/min, hydrogen at a flow rate of 40 ml/min, air at 550 ml/min, and nitrogen at 60 ml/min.

Mass spectra--The identification of neutral sugars in matrix and the confirmation of cholesterol, both separated by gas chromatographic techniques, were done on a Varian MAT model 112 gas chromatography-mass spectroscopy interface unit.

Amino acid analyses--The amino acid content in matrix isolated by both the EDTA and formic acid techniques and the dialyzates from the latter technique were determined by first hydrolyzing samples in a sealed tube containing 6 N HCl for 24 hours at 110^{o}. Samples were then taken to dryness under a vacuum, diluted up to an appropriate volume with 0.5 N sodium citrate buffer, pH 2.2 and analyzed on the automatic amino acid analyzer. Free amino acids were analyzed in a nonhydrolyzed sample of the dialyzate, from the formic acid isolation procedure, by precipitation of any protein amino acids with 5% sulfosalicylic acid and amino acid analysis of the nonprecipitated portion.

Fatty acid determination--Gas chromatography was used to separate fatty acids from the formic acid treated matrix. The fatty acids were derivatized to the volatile methyl esters by prior methanolic saponification to free the fatty acids and were subsequently treated with boron trifluoride in methanol to methylate the fatty acids released (39). The methyl esters were then extracted in hexane and injected into an F&M model 400 gas chromatograph fitted with a 6 foot glass column packed with 10% SP-2330 on 100/120 mesh Chromosorb W-AW (Supelco, Inc.). The column was at a isothermal temperature of 230°, hydrogen had a flow rate of 40 ml/min, nitrogen 30 ml/min, and air 250 ml/min. The instrument was equipped with a flame ionization detector. Methyl heptadecanoate was used as an internal standard, and peaks were identified on the basis of retention times, compared to a commercial standard (fatty acid standard RM-6, Supelco, Inc.).

Assay for carbohydrate sulphate--A modification of the procedure of Lloyd et al. (40) was developed to assay for carbohydrate sulphates in kidney stone matrix isolated by formic acid treatment. A known weight of matrix was quantitatively transferred with a syringe into a capillary tube (sealed on one end by heating in a flame) with about 40 microliters of concentrated HNO_3. The tube was then sealed on the other end in a similar fashion, and put in an oven at 100° for 24 hours. Then the tube was cooled, one end was broken, and the tube was put back in the oven until the HNO_3 had completely evaporated (about 2 hours). Next, 40 microliters of $BaCl_2$ reagent (0.85% w/v aqueous $BaCl_2$) were added to the tube to precipitate the sulphate as barium sulphate. Approximately 2 microliters of concentrated HCl were then added to the

tube, and this was centrifuged at 6000 rpm (setting 6) in a clinical centrifuge, utilizing an IEC centrifuge rotor head #930 (Arthur H. Thomas) especially made to fit capillary tubes, for 5 minutes. The supernatant was drawn off with a syringe and the pellet washed two times with approximately 40 microliters of distilled water, centrifuging after each wash. The pelleted barium sulphate was then quantitatively transferred with an EDTA reagent (prepared by dissolving 5.0 g of EDTA, in the free acid form, and 0.75 g NaCl in 50 ml of concentrated ammonium hydroxide, and diluting to a final volume of 500 ml with water) to a test tube, such that the final volume in the tube was 2.0 ml. The barium concentration was then measured by atomic absorption spectrophotometry, utilizing a nitrous oxide-acetylene flame. The barium content in the sample was determined by reference to a standard curve in which varying concentrations of sodium sulphate were precipitated with barium chloride, just as the samples were treated. The assay as outlined was shown to work well with commercial heparin which was found to contain 30.0% sulphate. Fully sulphated heparin may contain up to 40.4% sulphate by weight. The assay is linear up to about 17 ppm sulphate.

Sodium dodecylsulfate-disc gel electrophoresis--The techniques of Neville (41) and Weber and Osborn (42) were combined in an attempt to determine the nature of the protein in the matrix isolated by formic acid treatment. The gels consisted of 11% acrylamide, pH 8.47, according to Neville. The upper reservoir buffer (containing 0.04 M boric acid, 0.041 M Tris, and 0.1% SDS) was adjusted to pH 8.64. The lower reservoir and gel buffer (containing 0.1716 M Tris and 0.0494 M

HCl) was adjusted to pH 8.47. Prior to electrophoresis the sample was incubated with 1 ml of a solution containing 1% SDS and 5% beta mercaptoethanol in upper gel buffer (containing 0.0267 M H_2SO_4 and 0.0541 M Tris) at a pH of 6.1 for 2 hours at 37°. The sample was then centrifuged at 4000 rpm to pellet isoluble material. The supernatant was then pipetted off and dialyzed in an Amicon model MMC ultrafilter fitted with a PM-10 membrane (10,000 molecular weight exclusion limit) against the upper reservoir buffer containing in addition 2% sucrose and 0.1% beta mercaptoethanol. The pellet was incubated 2 more hours at 37° in 1 ml of a solution containing 1% SDS, 5% beta mercaptoethanol, and 8 M urea in upper gel buffer. Following the incubation, the sample was dialyzed in the same manner as the soluble portion. After dialysis was judged complete, the solutions were concentrated to about 200 microliters and subsequently applied separately to the gels. The gels were electrophoresed at 2 ma per gel employing a Bio-Rad model 400 electrophoresis unit. Following electrophoresis, gels were stained with Coomassie Blue. Prior to running the matrix sample, the validity of the technique was confirmed using commercial bovine serum albumin.

RESULTS

Observations of animals fed the low magnesium diet--Hyperemia of the nose, ears, paws, and feet was apparent in all animals within 3-4 days of the first low magnesium meal. About 5% of the animals exhibited signs of hyperexcitability after about 7 days and had convulsions when loud noises occurred.

Histochemical visualization of kidney stones in situ--Histological sections of kidney from 9 day magnesium deficient rats (initial weight 45-55 g) showed that many stones were present at the cortico-medullary junction. The calculi stained positively for calcium, using the von Kossa stain. Intense PAS positive rings were evident (Figure 1) in the interior of the stones.

Gross morphological characteristics of rat kidney stones--The appearance of kidney stones at low and higher magnification is shown in Figures 2 and 3. Many of the stones have an elongated shape composed of what appear to be fused spherical bodies. Under higher magnification some surface imperfections and cracks are evident.

Control experiment--An experiment was done with control rats to determine if kidney stones could be isolated from these animals. Twenty male Sprague Dawley rats (initial weight 45-55 g) were fed the magnesium deficient diet supplemented with 1.1 $gMgO/xg$ diet (660 ppm Mg) for 9 days. The kidneys were removed and homogenized according to the procedure previously outlined. To half the homogenate was added 50 micromoles calcium/g kidney (22) (as $CaCl_2$) and 29.7 micromoles phosphate/g kidney (as phosphate buffer, pH 7.0) in 0.3M sucrose, 1 mm EDTA,

FIGURE 1

Periodic Acid Schiff positive nature of kidney stones in situ

Kidney tissue from a 9 day magnesium deficient rat was fixed in formalin, embedded in paraffin, sectioned, and stained by the PAS procedure. The stone shown here was located in the medulla near the cortico-medullary junction. The magnification factor is 7500x.

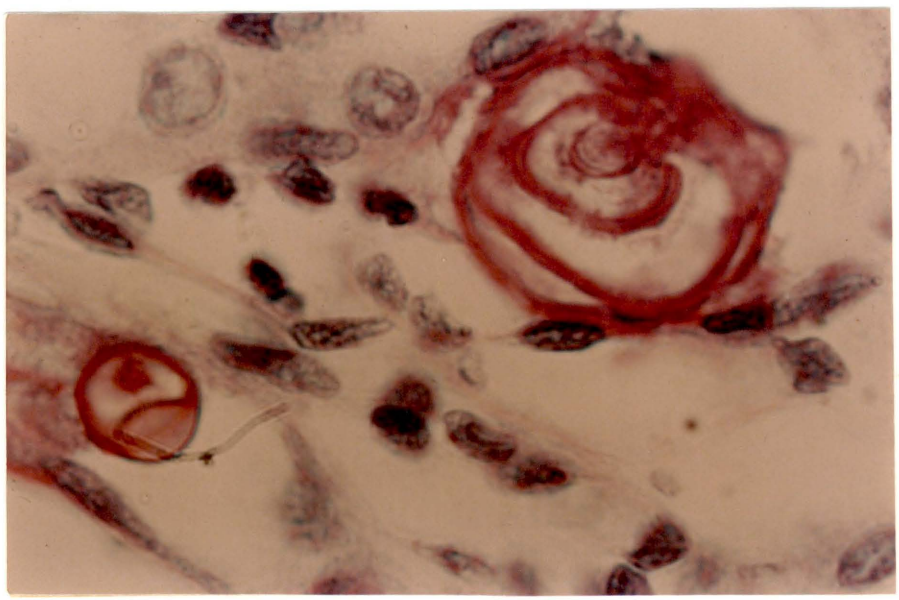
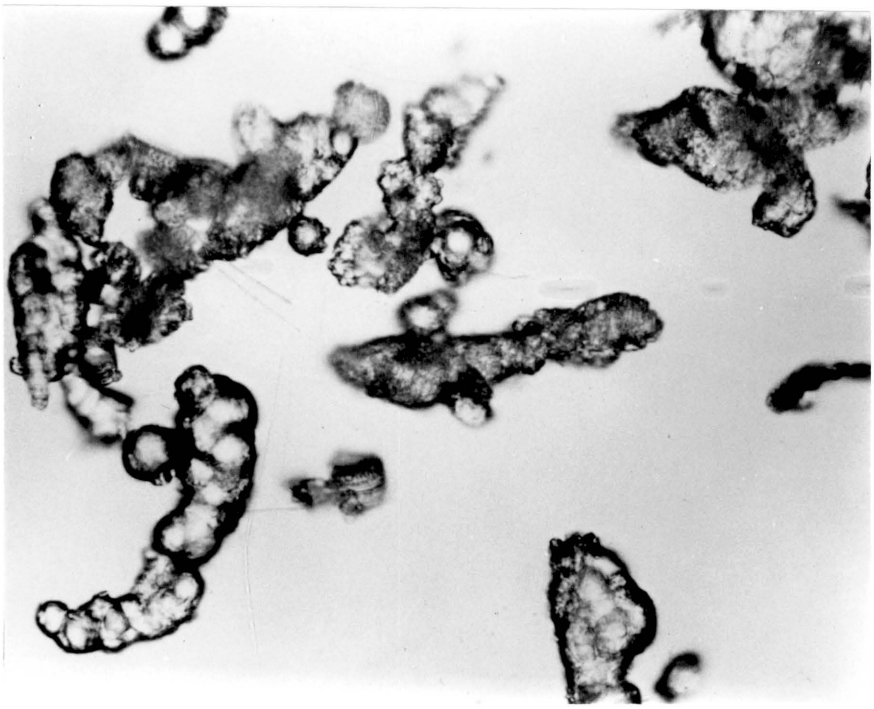


FIGURE 2

Appearance of isolated kidney stones
at low magnification

Isolated kidney stones were observed using a light microscope. The magnification factors are 890x for the upper photograph, and 2225x for the lower photograph. The dimensions of the stones varied; for larger stones the width approached 24 microns and the length approached 94 microns.



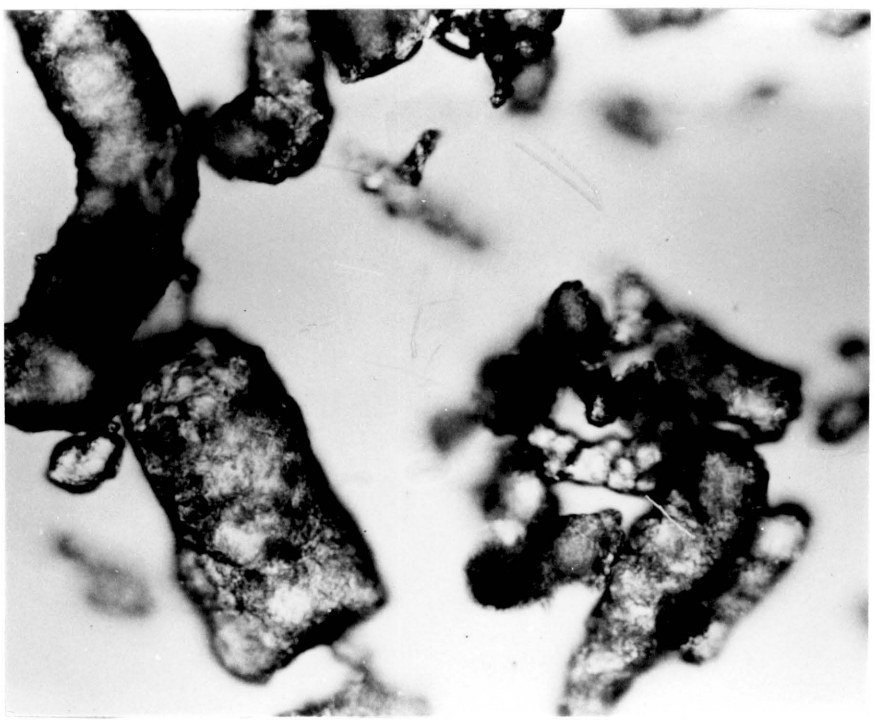
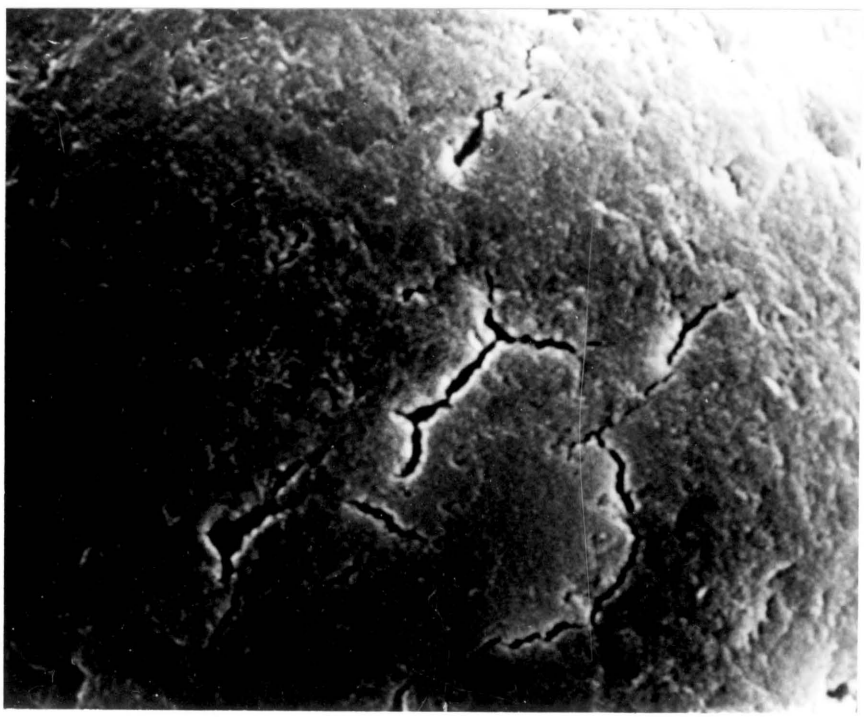


FIGURE 3

Appearance of isolated kidney stones
at higher magnification

Isolated kidney stones were observed using a scanning electron microscope. The magnification factors are 3000x for the upper photograph and 30,000x for the lower photograph.



pH 7.0, such that the final volume was 100 ml. The added calcium and phosphate was in a molar ratio of 1.67, the same ratio as in apatite. To the other half of the homogenate was added 100 ml, of 0.3M sucrose, 1 mm EDTA, pH 7.0. Both halves were then treated according to the procedure for isolating kidney stones from magnesium deficient rats. Only minute pellets were observed following the centrifugation step through 1.8M sucrose. Upon alkaline treatments, both pellets solubilized completely, and no kidney stones were observed to be present.

Analysis of the crystalline portion of rat kidney stones--X-ray emission analysis, done by Dr. David E. Pettry (Agronomy Department, VPI&SU), of intact rat kidney stones induced by magnesium deficiency showed that the stones are calcium phosphate apatite, $Ca_5(PO_4)_3 \cdot OH$. Chemical analysis of the calcium and phosphate content in stones, based on dialyzates from the decalcification procedures, are shown in Table 2. It was determined that measurement of calcium and phosphate concentrations in the dialyzate was a valid means of determining their concentrations in the mineral portion of the stones, since a calcium recovery study revealed that 99% of the calcium in intact stones could be recovered in the dialyzate following decalcification and dialysis. A phosphate recovery study was not done, however. No magnesium could be detected in the dialyzable fractions from the stones. In experiments 1-3 rats were given single distilled water, while in experiments 4-7 double distilled water was given. Otherwise, all 7 experiments were conducted as reproducibly as possible in terms of the treatment and feeding of the animals. The higher molar quantity of calcium than phosphate, with respect to the molar ratio for pure hydroxyapatite, suggests some other

TABLE II

Calcium and Phosphate Content in the Crystalline (Dialyzable) Portion of Rat Kidney Stones

Experiment Number[a]	% by Weight of Stones			Calcium/Phosphate Molar Ratio[c]
	Calcium	Phosphate	Apatite[b]	
1 (EDTA)	23.6	31.0	54.0	1.81
2 (EDTA)	28.5	37.2	64.8	1.82
3 (HCOOH)	27.4	35.5	61.9	1.83
4 (HCOOH)	32.2	37.4	65.2	2.04
5 (HCOOH)	30.4	35.4	61.7	2.04
6 (HCOOH)	30.6	35.9	62.6	2.02
7 (HCOOH)	29.0	35.6	62.1	1.93
Average	28.8	35.4	61.8	1.93

[a] Each experiment represents 100 rats. The procedure used for decalcification is indicated in parentheses.
[b] These data are based on the % of phosphate only.
[c] Pure apatite has a calcium/phosphate molar ratio of 1.67.

anion in addition to phosphate. The anionic component could conceivably be organic material in the matrix of the stones.

Appearance of the nondialyzable kidney stone matrix--Electron microscopy of isolated matrix revealed that is is an amorphous material, lacking any visible structure. No cellular components were observed. However, it is likely that the extraction procedures destroyed cellular membranes and organelles. Visually the matrix appeared to be a white, fluffy substance. A photograph of the electron micrograph is shown in Figure 4.

Elemental analysis of the organic (nondialyzable) matrix of stones--About 5.8% (range 3.9-8.0%) of the stone weight can be accounted for as nondialyzable organic material. The carbon, hydrogen, and nitrogen content of rat kidney stone matrix is shown in Table 3. The similarity of the results from two different isolation procedures was encouraging, although this type of analysis could disguise differences in the molecular compositions of the two preparations. These data did suggest, at least, that oxidized organic compounds such as carbohydrates would be likely components of the matrix material.

Calcium, magnesium, and phosphate content of the matrix--Matrix isolated by the formic acid procedure was subjected to wet ashing and subsequent measurement of the calcium, magnesium, and phosphorous (expressed as phosphate) content. Upon analysis only 0.28% of the weight of the matrix could be accounted for as calcium. No magnesium could be detected. Phosphate, however, represented 7.4% of the weight of the matrix. These data indicate that the decalcification and dialysis procedures were effective in the demineralization of the stones, and

FIGURE 4

Appearance of isolated kidney stone matrix

Isolated kidney stone matrix was observed with the electron microscope. The magnification factor is 4000x.

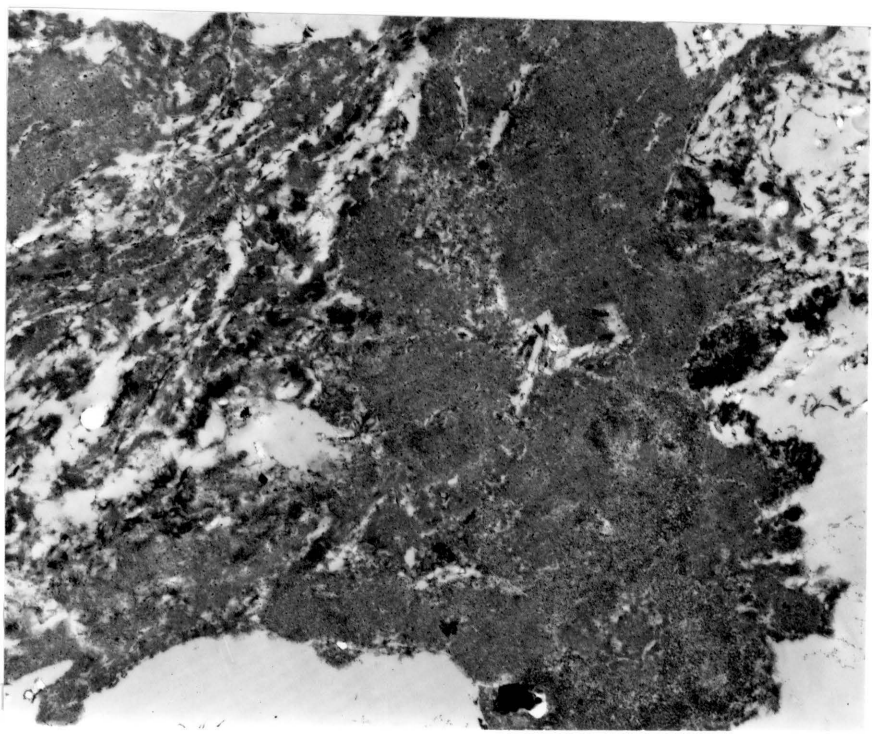


TABLE III

Elemental Analysis of Rat Kidney Stone Matrix

Isolated by Two Different Procedures

	% by Weight of the Matrix[a]		
	Carbon	Hydrogen	Nitrogen
EDTA method	43.188±0.065	6.387±0.255	4.155±0.012
HCOOH method	41.090±0.062	7.000±0.280	4.520±0.014

[a]Matrix from each method was only analyzed once. The deviations for each value represent the expected error of the elemental analysis, based on standards.

that some form of bound phosphate comprises a significant portion of the matrix. Replicate samples were not done in these analyses.

Sulphate analysis of matrix--No sulphate was detectable in kidney stone matrix under the conditions of the analysis employed. The quantity of matrix and the sensitivity of the assay were such that 2.0% (by weight) sulphate or more would have been detected after the hydrolysis procedure if it were, indeed, present. No replicates were done in this assay.

Neutral sugar content of matrix--The results of the phenol sulfuric acid assay, which is not specific for the carbohydrate chain length, of matrix isolated by the EDTA method revealed a neutral sugar content of 6.9 and 8.0% in two experiments utilizing glucose as the standard. An absorption spectrum of the final reaction mixture was identical to that of the standard, with an absorption maximum at 492 nm. Further characterization by gas chromatography (Table 4) and mass spectroscopy (Figures 5 and 6) only verified the presence of glucose in the matrix. The minute quantity of sugar liberated by the hydrolysis procedure (1 N HCl for 6 hours at 100^{o}) to prepare the sample for gas chromatography indicated that the hydrolysis conditions were not strong enough to enable the analysis to be considered quantitative, since only about 0.3% of the matrix could be accounted for as neutral carbohydrate by this technique. It is likely that other carbohydrates were successfully derivatized but were not present at detectable concentrations. In addition, the matrix visually did not appear to go in solution after the 6 hour hydrolysis. Thus, neutral carbohydrates present in the matrix may not have been exposed to the acid, and, therefore, hydrolysis

TABLE IV

Gas Chromatographic Analysis of the Alditol Acetate Derivative of Glucose from Kidney Stone Matrix

Sugar	Peak Area[a]	% of Matrix by Weight	Sample Retention Time[b]	Standard Retention Time
Arabinose[c]	2.43	--	6.7	6.9
Glucose	0.38	0.30	12.5	12.7

[a]Peak areas reported in square centimeters.

[b]Retention times expressed in minutes.

[c]Arabinose was added to the sample as an internal standard.

FIGURE 5

Gas chromatographic-mass spectral analysis of glucose from kidney stone matrix

A portion of the plot of intensity of total ion current vs. the scan number is presented. The small peak between scans 195-200 corresponded to the retention time of the authentic alditol acetate derivative of glucose.

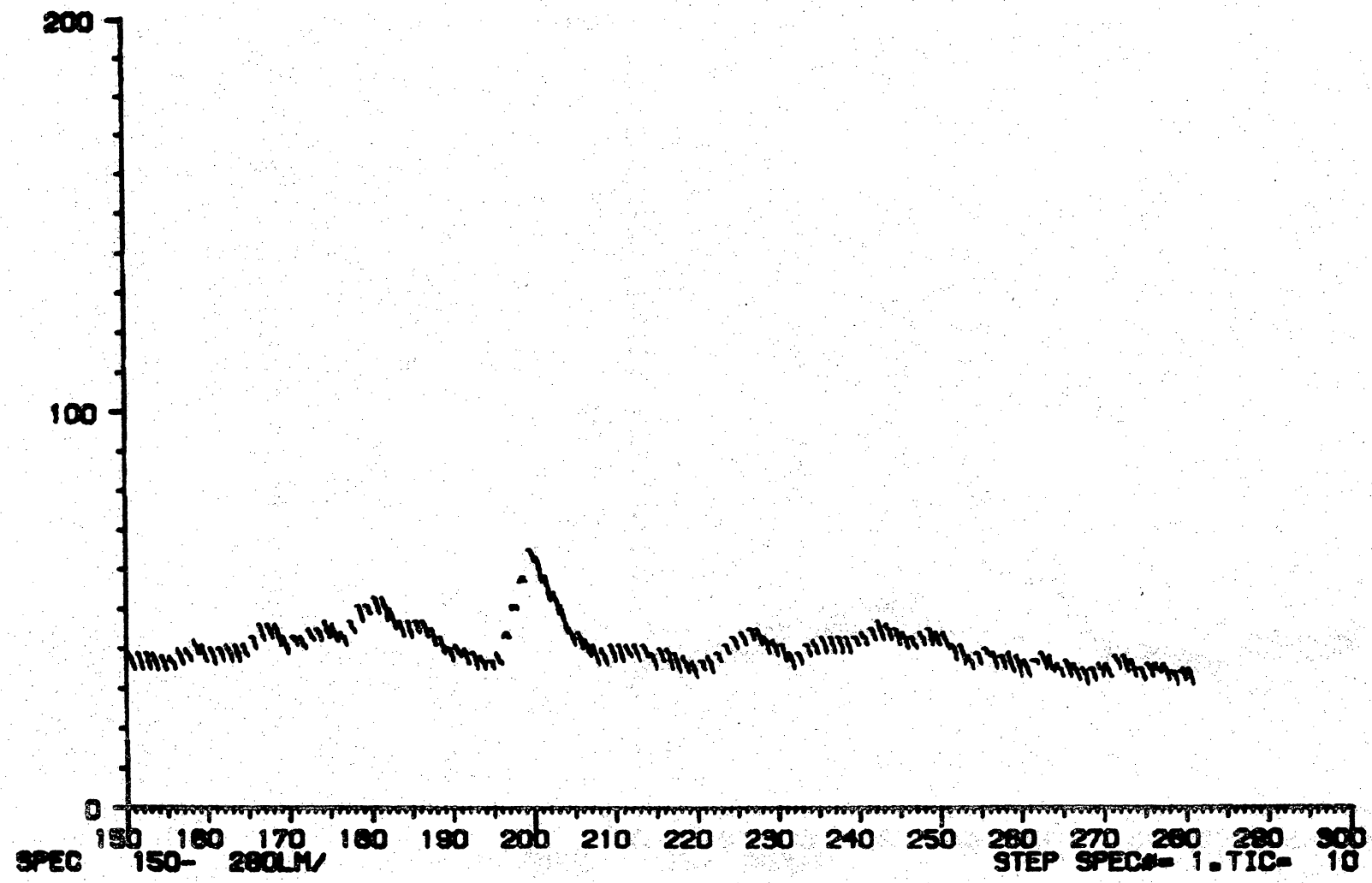
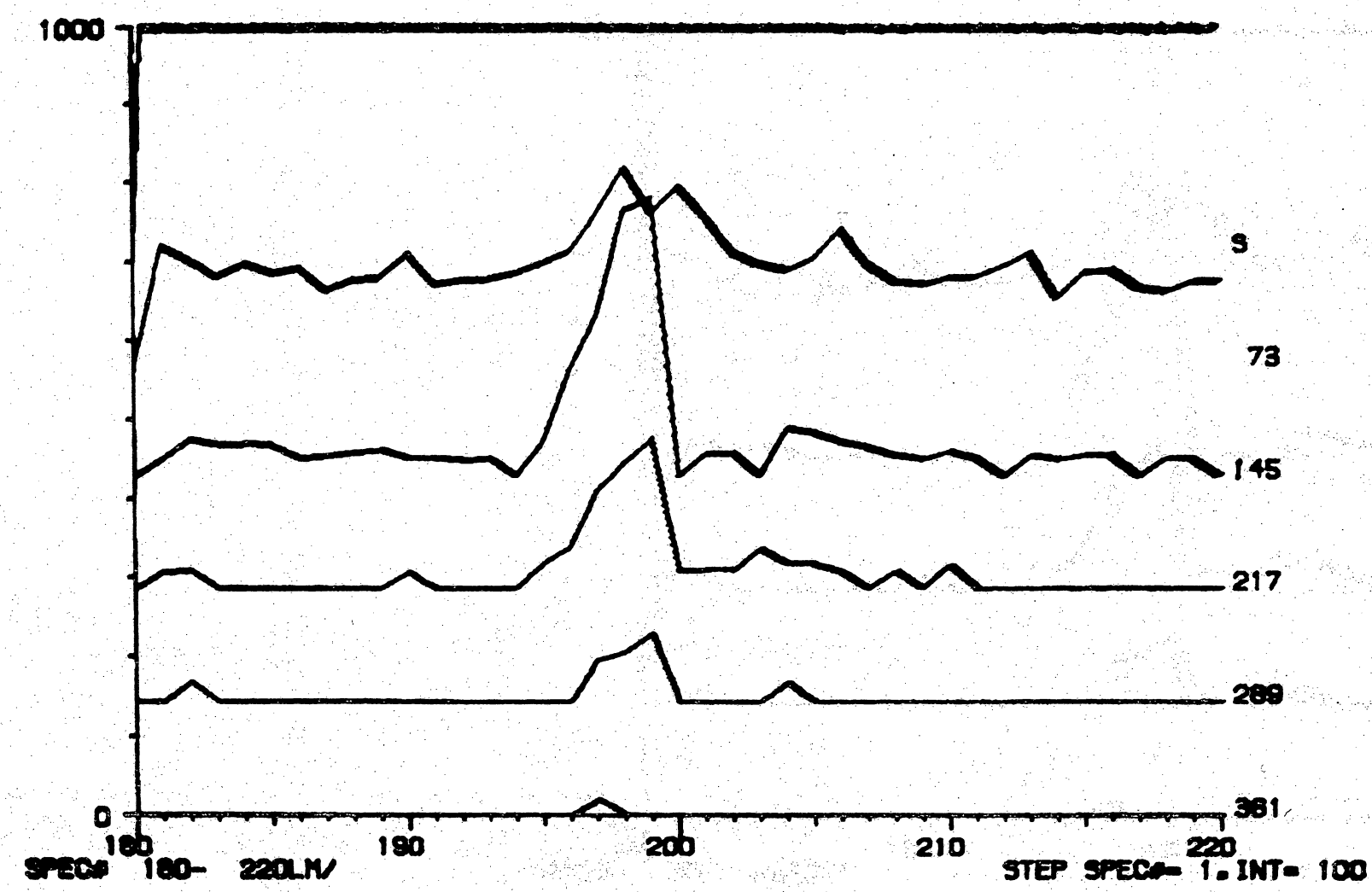

200
100
0
150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300
SPEC 150- 280LM/
STEP SPEC#= 1.TIC= 10

FIGURE 6

Further evidence for the presence of glucose in matrix by gas chromatographic-mass spectral analysis

A portion of the plot of intensity of selected ion fragments vs. the scan number is presented. The five most common fragments for alditol acetate derivatives of sugars are shown at the right of the plot. The five peaks between scans 195-200 indicate that a sugar derivative is indeed present at that retention time.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145
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SPEC# 180- 220LM/
STEP SPEC#= 1. INT= 100

could not take place. The neutral sugar derivatives that were easily separated from a standard mixture of sugars by this technique included rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose. The insolubility of matrix hampered the quantitative analysis of its composition, as exemplified in this assay, and necessitated the use of caution in the interpretation of results and in the selection of future assay procedures. In one sample of matrix isolated by the formic acid procedure, 22.07% of the matrix could be accounted for as neutral carbohydrate when assayed by a modification of the orcinol reaction, which also is not specific as to the carbohydrate chain length. In this assay glucose was used as the standard. An absorption spectrum of the final reaction mixture revealed identical spectra of the sample and standard with a peak absorbance at 422 nm. It is not clear from these results whether there is a difference in the neutral carbohydrate composition of the matrix isolated by the EDTA and formic acid procedures, or whether the orcinol reaction simply promotes better hydrolysis, than in the phenol sulfuric acid technique, of the neutral carbohydrates from the matrix. When a second version of the orcinol reaction was employed, which is not specific but is commonly used to measure pentoses, only 1.87% of the matrix could be accounted for as pentose, using ribose as standard. An absorption spectrum of the final reaction mixture revealed a difference in the sample and standard; the matrix and standard had a peak absorbance at 665 nm, but the matrix sample exhibited a second peak of strong absorbance at 495 nm. The explanation for this second peak is not clear. It should be pointed out that both the orcinol procedure and the phenol sulfuric acid methods involve the

use of strong acids, but that the orcinol technique includes a period of heating in a boiling water bath and a longer exposure of the sample with the reagents. The phenol sulfuric acid method, on the other hand, depends on the heat generated by mixing the acid with the other reagents and sample. Therefore, the opportunity for a reaction to take place in the phenol sulfuric acid method depends on good mixing and even heat distribution. The matrix has been shown to be insoluble in strong acids, unless it was heated. Thus, the orcinol procedure provides a better opportunity for a reaction to take place when one is dealing with an insoluble sample, and was probably a more reliable assay in this case. It would appear from the latter orcinol assay that pentose is only a minor constituent of the matrix, if at all.

Amino sugar analysis of matrix--Amino sugars could be not detected in matrix samples isolated by either the EDTA or formic acid techniques. The three methods used for analysis were a 4 N HCl hydrolysis for 4 hours at 100° and analysis by gas chromatography of the alditol acetate derivatives; hydrolysis with 8 N HCl for 2 hours at 95° or 6 N HCl for 24 hours at 110° and analysis by the amino acid analyzer, using the amino sugar program. Sufficient matrix was used in each case so that if amino sugars constituted 1% or more of the matrix, they would have been detected (assuming no destruction). Only one sample of matrix was run per analysis.

Hexuronic acid determination of matrix--The carbazole reaction for hexuronic acids gave negative results for two matrix samples isolated by the EDTA method. The procedure utilized strong acid conditions and a period of heating in a boiling water bath. Under those conditions the

matrix did solubilize. The quantity of matrix used and sensitivity of the assay were such that if hexuronic acid were 1.9% or more of the matrix, it would have been detected (assuming no destruction).

Sialic acid assay of matrix--The thiobarbituric acid assay also utilizes strong acid and a period of heating in a boiling water bath. The matrix was soluble under those conditions, and a reaction took place between the matrix and the reagents in the assay. With N-Acetylneuraminic acid, the standard in the assay, a pink color is produced with a peak absorbance at 549 nm. The matrix sample also produced a pink color, but the absorption spectrum revealed the maximum absorbance was at 532 nm, which is indicative of unsaturated lipids and/or 2-deoxyribose. Therefore, sialic acids were judged not to be a component of matrix. No replicates were done in this analysis.

Fatty acid analysis of matrix--Saponification and methylation of the fatty acids in a matrix sample isolated by the formic acid technique with subsequent separation and analysis by gas chromatography (Table 5) showed that 2.74% of the matrix could be accounted for as fatty acids.

Cholesterol analysis of matrix--Saponification and extraction of the non-saponifiable fraction from a matrix sample isolated by the formic acid technique showed that cholesterol accounts for 5.33% of the matrix. The result of the gas chromatographic separation is shown in Table 6, and the mass spectral confirmation of the presence of cholesterol in the matrix is shown in Figure 7.

Amino acid composition of matrix--The amino acid and ammonia content of matrix isolated by both the EDTA and formic acid methods is shown in Table 7. These data were calculated on the basis of the individual

TABLE V

Separation and Identification by Gas Chromatography

Of Fatty Acid Methyl Esters from Kidney Stone Matrix

Fatty Acid	Peak Area[a]	% of Matrix by Weight	Sample Retention Time[b]	Standard Retention Time
Palmitate	8.33	0.97	6.1	6.3
Heptadecanoate[c]	10.65	--	8.4	8.7
Stearate	8.45	0.92	11.5	11.8
Oleate	5.53	0.64	13.0	13.4
Linoleate	1.79	0.21	15.4	15.9

[a]Peak areas expressed in square centimeters.

[b]Retention times expressed in minutes.

[c]Heptadecanoate was used as an internal standard.

TABLE VI

Gas Chromatographic Analysis of Cholesterol in Kidney Stone Matrix

Peak	Peak Area[a]	% of Matrix by Weight	Sample Retention Time[b]	Standard Retention Time
Cholesterol	4.72	5.33	4.2	4.2

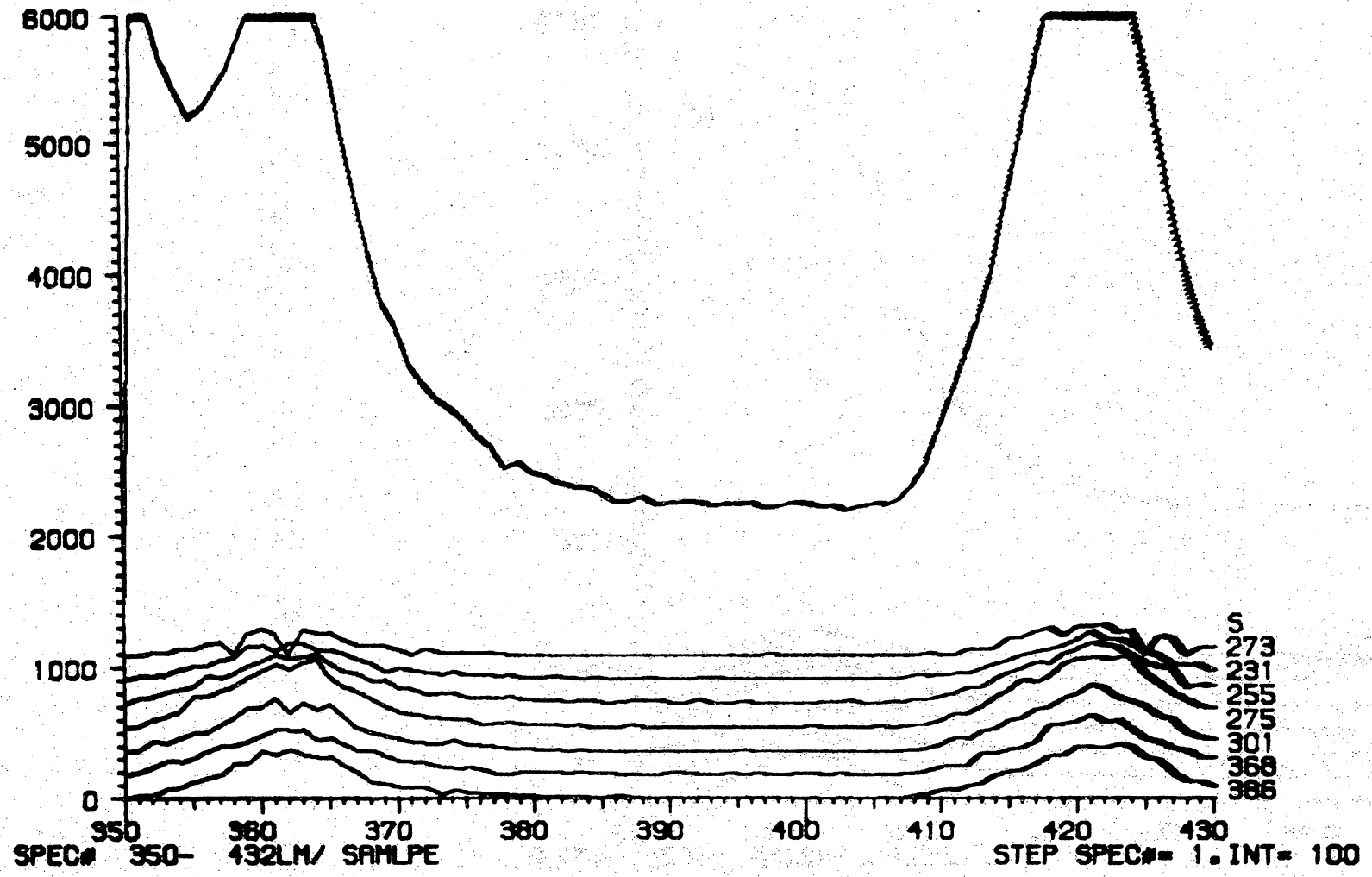
[a]Peak area is expressed in square centimeters.

[b]Retention time is expressed in minutes.

FIGURE 7

Gas chromatographic-mass spectral analysis of cholesterol from kidney stone matrix

A portion of the plot of intensity of selected ion fragments vs. the scan number is presented. The seven most common ion fragments from cholesterol are shown at the right of the plot. By injecting pure cholesterol first and then injecting the sample before the standard emerged from the column, both were plotted on the same graph. Scans 355-370 correspond to the standard. Scans 410-430 correspond to the sample. Due to the back to back injections, the scan numbers do not correspond to retention times in this case. All seven cholesterol fragments were present. The line at the top of the plot represents the gas chromatographic response.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3000
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380
390
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231
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301
368
386
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STEP SPEC#= 1. INT= 100

TABLE VII

The Amino Acids and Ammonia from Acid Hydrolyzed Kidney Stone Matrix

	% of Matrix By Weight			Mole % of Total Amino Acids Detected		
	Exp. 1 (EDTA)	Exp. 3 (HCOOH)	Exp. 4 (HCOOH)	Exp. 1 (EDTA)	Exp. 3 (HCOOH)	Exp. 4 (HCOOH)
Lys	0.71	1.12	1.52	5.54	5.73	6.25
His	----	0.27	0.43	----	1.31	1.66
NH_3	1.12	1.49	2.32	----	----	----
Arg	0.38	1.18	1.43	2.45	5.06	4.95
Asp	1.17	1.60	1.87	10.01	8.98	8.47
Thr	0.52	0.71	0.97	4.95	4.48	4.88
Ser	1.15	0.98	1.71	12.51	6.97	9.80
Glu	1.23	2.34	3.20	9.50	11.89	13.10
Pro	----	0.89	1.04	----	5.83	5.42
Gly	0.95	1.04	1.49	14.24	10.35	11.93
Ala	0.79	1.07	1.34	10.03	8.99	9.09
Cys	----	----	----	----	----	----
Val	0.74	1.06	1.23	7.04	6.76	6.33
Met	----	0.34	----	----	1.72	----
Ile	0.95	0.89	0.81	8.21	5.09	3.71
Leu	1.25	1.84	1.94	10.87	10.50	8.89
Tyr	----	0.40	0.38	----	1.66	1.26
Phe	0.68	1.04	1.17	4.50	4.69	4.28
Total	11.68	18.26	22.85	99.85	100.01	100.02

amino acid molecular weights, and the correction for the peptide bond was not included in the calculations. In addition, the mole percent of each amino acid is presented in order to compare the various preparations. The formic acid procedure affords a better recovery than the EDTA method in terms of amino acids in the nondialyzable fraction from the stones. The source of the ammonia is not known; likely candidates, however, would simply be free ammonia from the urine, or degradation of urea, creatine, asparagine, and glutamine. The preponderence of aspartic acid, glutamate, leucine, and, to a lesser extent, serine is interesting and may be of some importance in the formation of the stone. Also, it should be pointed out that 54 mole % of the amino acids are of the aliphatic and nonpolar classifications. This may contribute to the insolubility of matrix.

SDS-disc gel electrophoresis of matrix--Incubation of a matrix sample isolated by the formic acid technique with SDS, beta mercaptoethanol, and urea with subsequent disc gel electrophoresis did not result in the appearance of any high molecular weight protein bands. The first incubation with just SDS and beta mercaptoethanol with subsequent dialysis of the soluble portion of the incubation mixture, concentration, and electrophoresis did not result in the appearance of any protein bands when the gels were stained with Coomassie Blue. Further incubation of the insoluble material, left after the first incubation, with SDS, beta mercaptoethanol, and urea with subsequent dialysis, concentration, and electrophoresis resulted in the appearance of a band which ran with the tracking dye. In addition, some of the matrix remained insoluble, stayed on top of the gel, and did not stain with Coomassie Blue.

Enough matrix was incubated in this study, based on the amino acid analysis, so that if all of the amino acids were tied up as protein, then 200 micrograms of protein would have been in the sample. As little as 10 micrograms of any one protein species would have been sufficient to produce a very distinct band. These results could indicate that the amino acids in the matrix are either free or are in small peptides such that they migrate with the tracking dye when electrophoresed. An alternative explantion for the absence of protein bands would be that the protein was simply not solubilized under these conditions. The band at the tracking dye was quite distinct, but did not appear to represent 200 micrograms of protein. It is probable that if, indeed, the amino acids are free or in small peptides that many were lost through the membrane during the dialysis step. The fact that these small peptides and/or free amino acids were not lost during the decalcification of the kidney stones and subsequent dialysis step might be explained by the insolubility of the matrix as a whole under those conditions. Thus, the amino acids or peptides could have been inaccessible to the aqueous medium in the dialysis bag and were, therefore, retained in the matrix. Hydrogen bonding or some type of absorption phenomenon are also possible explanations. The disc gel electrophoresis procedure used in this experiment worked well when 200 micrograms of bovine serum albumin was incubated either in SDS and beta mercaptoethanol alone, or if it was incubated with SDS, beta mercaptoethanol, and urea with subsequent dialysis, concentration, and electrophoresis.

Preliminary studies of the organic components in the dialyzable fraction--Neutral sugars, assayed by the phenol sulfuric acid technique, and hexuronic acids, measured by the carbazole assay, were not detectable in the dialyzable fraction following the decalcification procedure with EDTA. A sufficient quantity of dialyzate was concentrated by lyophilization and assayed such that if 0.5% of the stones consisted of neutral sugars or hexuronic acids they would have been detected. Similarly, sialic acids, assayed by the thiobarbituric acid method, were not detectable at a 1.8% level of the stone weight, nor were amino sugars, assayed by the amino acid analyzer, at a level of 0.03% of the stone weight, based on the dialyzable fraction from the decalcification procedure using formic acid. A measurable quantity of total nitrogen, assayed by the Micro-Kjeldahl procedure, was detectable in the dialyzate from the formic decalcification procedure, consisting of 0.22-1.33% of the total stone weight. Amino acids were also detectable in these dialyzates. The proportion of each amino acid with respect to total stone weight and mole percent detected is shown in Table 8. Of the total dialyzable amino acids only about 31% were nonpolar and aliphatic. By sulfosalicylic acid precipitation of any protein in a nonhydrolyzed sample of dialyzate from Experiment 4 and subsequent amino acid analysis of the sulfosalicylic acid soluble material, it was determined that about half of the dialyzable amino acids were free (i.e. assayable without prior acid hydrolysis). These free amino acids are likely to have been simply absorbed to the growing calculi while they were in the tubular space.

TABLE VIII

The Total Amino Acids and Ammonia From an Acid Hydrolyzed Sample of the Dialyzable Fraction from Kidney Stones

	% of Stones By Weight		Mole % of Total Amino Acids Detected	
	Exp. 3 (HCOOH)	Exp. 4 (HCOOH)	Exp. 3 (HCOOH)	Exp. 4 (HCOOH)
Lys	0.16	0.05	12.56	5.99
His	0.04	0.02	3.24	2.59
NH_3	1.36	0.26	----	----
Arg	0.01	0.02	0.74	2.04
Asp	0.06	0.06	4.82	7.69
Thr	0.03	0.03	2.90	4.54
Ser	0.06	0.07	6.12	12.84
Glu	0.23	0.14	17.88	17.75
Pro	0.04	0.02	4.31	3.77
Gly	0.13	0.08	20.45	18.28
Ala	0.13	0.05	16.88	9.37
Cys	----	----	----	----
Val	0.03	0.03	3.26	4.31
Met	----	----	----	----
Ile	0.03	0.03	2.83	3.98
Leu	0.03	0.03	2.84	4.59
Tyr	----	0.01	----	0.89
Phe	0.02	0.01	1.17	1.36
Total	2.36	0.91	100.00	99.99

DISCUSSION

The importance of an organic matrix for kidney stone genesis has been an assumption made by some investigators. The alternative hypothesis, that matrix does not play a dominant role in stone genesis, is equally intriguing. Vermeulen and Lyon (43) cite experiments where artificial concretions could be made by rotating a fine wire loop in urine appropriately fortified with added calcium. The wire loop simply acted as a surface upon which the concretion could form. The calcified objects were described as coherent hard masses with a surface texture much like that of genuine urinary calculi. In addition, lamination was observed in the interior of the concretions. The concretions formed in urine revealed a proteinaceous matrix upon decalcification; however, if the concretions were formed in urine ultrafiltrates, there was no residue upon decalcification. The latter results were also observed if an artificial urine (water solution of some important urine components) was used as the developing medium. If an appropriate amount of protein was added to the artificial urine, however, a matrix residue was left from decalcified concretions.

It is well recognized in crystallization processes that small amounts of many substances present in the medium, including protein, will be incorporated by surface adsorption upon the crystalline phase. The ubiquity of matrix in stones could thus be explained by such an adventitious occurrence due to surface adsorption of urinary organic molecules.

Vermeulen and Lyon have proposed that supersaturation of the urine in a stone forming animal becomes so great in the collecting ducts of the kidney that spontaneous nucleation occurs, and fine crystalline particles appear which are either lodged in the tubule or formed upon some foreign surface in the urinary tract to which they are attached. These small crystals then grow in the supersaturated urine to become mature renal calculi. The authors hypothesize that some triggering event must occur to produce the abnormal condition in the urine, but that once started, small cyrstals could grow even in the absence of the pathological urine condition since seed crystals would have been established in the urinary tract.

The nature of this triggering event in humans is not known, but in the magnesium deficient rat an altered calcium to magnesium ratio in the glomerular filtrate could conceivably be the triggering episode. Maintenance of this low magnesium diet for a period of 9 days could thus promote rapid crystal formation in the urinary tract. This concept may explain why calculi were only observed at the cortico-medullary junction since urine supersaturation of calcium phosphate may not reach the critical concentration necessary for precipitation to occur prior to reaching this area of the nephron. It is not clear whether matrix would serve as a template for calcification in this area or whether it is simply nonfunctional adsorbed material.

In the present study, attempts to ascertain the biochemical composition of rat kidney stone matrix were limited by the minute quantities obtainable and by its relative insolubility. About 58% of the matrix has been accounted for by weight as amino acids, neutral

sugars, cholesterol, fatty acids, ammonia, calcium, and phosphate. A summary of these data and the composition of human kidney stone matrix is presented in Table 9.

The origin and function of kidney stone matrix are unknown. Perhaps knowledge of its molecular composition is one step toward an understanding of stone genesis. Certainly, differences are evident between the kidney stone matrices of humans and rats. Two common negative findings in the matrices from magnesium deficient rats and from man are the inability to detect hexuronic and sialic acids. One can conclude that calcification does not depend on a unique matrix composition. The differences in matrix composition may reflect differences in the diet and metabolism between the rat and man and/or a difference in the pathogenesis of the stones. The significance of these differences is not clear, and the interpretation is further complicated by the fact that about 42% by weight, of the rat kidney stone matrix remains unaccounted for in terms of molecular composition.

A balance sheet of certain elements accounted for in kidney stone matrix is presented in Table 10. These data include certain assumptions; some of the fatty acids are assumed to be tied up in lipids, and the neutral carbohydrate is calculated on the basis of glucose only.

If one takes the difference between the percent of each element recovered (Table 10) and the percent of each element actually present in the matrix (Table 3), and divides this difference by 39.56% (the percent of missing weight), an elemental composition can be obtained for the missing material. Based on this calculation and the assumptions

TABLE IX

Comparison of the Compositions of

Rat and Human Kidney Stone Matrix

	Magnesium Deficient Rats	Humans[a]
	g/100 g lyophilized whole calculi	
Total matrix recovered	5.80	3.20
	g/100 g lyophilized matrix	
Total ash	----	13.10
Calcium	0.28	3.76
Phosphate	7.40	5.24
Elemental analysis		
Carbon	41.09	57.24
Hydrogen	7.00	7.03
Nitrogen	4.52	10.20
Protein (Nx6.25)	28.25	63.80
Amino acids (and NH_3)	20.56[b]	----
Nonamino sugars	22.07	9.60
Hexosamine	none detectable	4.90
Fatty acids	2.74	none detectable
Cholesterol	5.33	none detectable
Bound water	----	10.90
Total[c]	58.38	102.30

[a] Data of Boyce et al. (23, 26).
[b] Average from Experiments 3 and 4.
[c] These values do not include data from the elemental analysis. For the deficient rats, the protein estimate (based on N) is not included in the total.

TABLE X

Balance Sheet of Carbon, Hydrogen, and Nitrogen

From Kidney Stone Matrix

Recovered	Percent of Matrix			
	Carbon	Hydrogen	Nitrogen	Total Weight
Lecithin[a]	1.24	0.20	0.03	1.86
Sphingomyelin[b]	1.83	0.31	0.10	2.71
Palmitate[c]	0.44	0.07	----	0.59
Linoleate	0.16	0.02	----	0.21
Cholesterol	4.47	0.64	----	5.33
Neutral carbohydrate[d]	8.83	1.48	----	22.07
Amino acids[e]	8.44	1.42	2.60	18.68
Ammonia[e]	----	0.38	1.57	1.91
Phosphate[f]	----	----	----	6.80
Calcium	----	----	----	0.28
Total	25.41	4.52	4.30	60.44
Percent recovery[g]	61.84	64.57	95.56	

[a]Assuming fatty acid portion of lecithin is 50% oleate, 50% palmitate.
[b]Assuming fatty acid portion of sphingomyelin is 100% stearate.
[c]Remaining free palmitate after subtracting palmitate in lecithin.
[d]Calculated on the basis of glucose.
[e]Average from Experiments 3 and 4.
[f]Correction has been made for the phosphorous in lecithin and sphingomyelin.
[g]Based on the elemental analysis of matrix (Table 3).

in Table 10, carbon is present at 39.6%, hydrogen at 6.3%, and nitrogen at 0.6%. If it is assumed that the remaining percent of missing weight is oxygen, then this indicates rather oxidized organic compounds such as carbohydrates or organic acids (e.g. citrate and lactate). For example glucose consists of 40.0% carbon and 6.7% hydrogen; citrate consists of 37.5% carbon and 4.2% hydrogen. If the missing weight of the matrix is due to carbohydrate, solubility problems or chemical degradation may account for the fact that it was not detected in the analyses. The actual identification of the missing material will require further investigation.

Several interesting questions persist: (1) Where does matrix come from, (2) Is matrix unique to stone formers, and (3) What is the mechanism of stone genesis in the magnesium deficient rat? Although the data presented in this thesis are not sufficient to enable one to answer these questions conclusively, a presentation of some of the possible answers is in order.

Two possible sources of kidney stone matrix are immediately obvious. Components of the matrix could come from outside the kidney by passage into the glomerular fluid; and/or matrix may be derived from renal cellular constituents. It is doubtful that matrix could come entirely from the urine due to the large amount of carbohydrate in the matrix and due to the presence of fatty acids and cholesterol. Surface adsorption to the growing calculus of a number of molecules from the urine may take place, however, and it is likely that these may account for the 30% of stone weight in the dialyzable fraction that cannot be accounted for as matrix or apatite. The free amino acids in the

dialyzable fraction from stones may be one example of adsorbed molecules from the urine. In addition, it is conceivable that certain proteins from the urine could become incorporated into a growing calculus. This may be the source of the amino acids detected in the matrix. The urine may possibly be the source of the bound phosphate in the matrix. Some urinary monomeric molecules may also become a part of matrix, either by becoming trapped, adsorbed or hydrogen bonded to other matrix components.

The second possibility that matrix could be derived from renal cellular constituents, is strongly implicated. The glycocalyx of the proximal tubular cells and of other tubular cells, or the basement membranes could prove to be the source of carbohydrate found in the matrix. Calcium has been shown to bind to neutral sugars through the interaction of the hydroxyl groups with calcium ions (44). It is known that certain carbohydrate rich membranes contain significant amounts of neutral sugars (45), and calcium binding to glycocalyx has been observed (46). Presumably these neutral sugars are in the form of polysaccharides or glycoproteins. The small quantity of fatty acids and cholesterol in the matrix could come from the microvilli of the brush border, or the membranes from other tubular cells. The small amount of lipid as a whole may suggest a stronger calcium binding to other matrix components, and a degree of selection of those components over those which do not bind calcium as effectively.

Attempts to find a component of matrix that is unique to stone formers, have largely been unsuccessful. In human stones Matrix Substance A is the most potent antigenic component consistently present

in matrix and may account for up to 85% of the matrix weight (26). It is a relatively small urinary macromolecule of 30,000 to 40,000 molecular weight, and is consistently absent from normal urine and kidney tissue. It is present, however, in some other disease states and in tissues other than the kidney. Serum albumin has also consistently been found in human stone matrix. The fact that the matrix from magnesium deficient rats contains considerably less protein than in human stone matrix suggests differences in the mechanisms of stone genesis, if one accepts the supposition that matrix plays a primary role in stone formation.

The kidney stone matrix from magnesium deficient rats may consist of a conglomeration of monomeric and polymeric molecules that are present in the normal urine and kidney. Magnesium deficiency could conceivably cause calcium phosphate to precipitate on the glycocalyx of tubular cells, due to the altered magnesium to calcium ratio in the glomerular filtrate. Small seed crystals could then tear off of the glycocalyx and flow through the tubules until they were lodged in the hair pin bend of Henle's loop at the cortico-medullary junction of the kidney. The small seed crystals could then grow by accretion of urinary organic molecules, membranes of adjacent tubular cells undergoing necrosis, and calcium and phosphate from the supersaturated urine. The carbohydrate rich basement membranes of necrotic cells could thus be incorporated into the matrix. Proteins of the membranes may have a significant function in the binding of calcium, as well.

Calcium reabsorption by tubular cells could also have a role in stone genesis. The theory proposed by Bunce and Bloomer (21) is still

viable. It involves the pinocytosis of calcium phosphate from the tubular fluid in the region of the proximal convoluted tubules, merger of the pinocytotic vacuoles with lysosomes, formation of seed crystals, and regurgitation of crystals back into the tubular space. These seed crystals could then pass through the tubules to the restriction at Henle's loop and grow as previously described.

Water reabsorption and increasing saturation of calcium and phosphate ions in the glomerular fluid as it flows from the cortex to the medulla is likely to be a factor in the precipitation of calcium phosphate at the cortico-medullary junction. Precipitation may be the primary event in the magnesium deficient rat. The crystal could then grow larger, cause necrosis of adjacent cells, and selective incorporation of certain cellular components into the growing calculus.

The time involved for a kidney stone to form in a human is unknown. It is unlikely that severe magnesium depletion could explain human stone genesis, although a somewhat reduced level of dietary magnesium intake may occur. In the magnesium deficient young rat, kidney stones are visible in histological sections within 24-72 hours after consumption of the first low magnesium meal. The severity of the stress placed on the human or the rat may markedly influence which urinary and cellular components become calcified.

If one considers some of the individual components of the matrix from magnesium deficient rats, precedent does exist for their presence in other calcified tissues. Lipid has been shown to be essential for calcification of a calculus matrix prepared from dental calculi (47). Thus, the lipid found in the matrix from magnesium deficient rats could

play a functional role in stone genesis and growth. The soluble organic matrices of both fetal and adult bovine enamel were found to contain relatively large amounts of protein bound organic phosphorous (48, 49). Upon further analysis the phosphate was found to be linked to serine. These phosphate groups, although covalently bound, remain very reactive to calcium ions (48). A newly discovered amino acid, gamma carboxyglutamate, has been shown to be present in proteins solubilized from chicken bone (50). Calcium binding by proteins of matrix has been thought, by some workers, to be an initial step in the mineralization process. Nucleation of calcium phosphate crystallization by components of an organic matrix requires interaction of the mineral ions with sites on the matrix to form ordered ion clusters. Phosphoserine residues and carboxyl groups of glutamic and aspartic acids have been suggested as possible sites for calcification. Gamma carboxyglutamate strongly binds calcium and has been found in prothrombin to be essential for hemostasis. Although it has not been determined that this amino acid is present in kidney stone matrix, it is known that during acidic hydrolysis gamma carboxyglutamate in hydrolyzed to produce glutamate. The relatively large proportion of glutamate observed to be present in the matrix may warrant further investigation of this point. A calcium binding protein has been observed to be present in the urine from humans with hypercalciuria and renal calculi (51). The calcium binding ability of this protein has been investigated *in vitro* but its role *in vivo* is not certain. Carbohydrate has been found in human kidney stone matrix (26) and a PAS positive interior of stones has been observed both in the magnesium deficient rat (20) and in man (26).

With regard to stone genesis, several interesting questions arise: (1) Is the matrix needed at all for calcification to occur, (2) If the matrix is needed, are all or just part of its components functional, and (3) Does crystal formation precede or follow aggregation of urinary and/or cellular organic molecules? These and other questions will have to be answered before the mechanism of kidney stone genesis is understood. Magnesium deficiency provides a convenient method of generating kidney stones for future study and offers an opportunity for progressive biochemical and histological changes to be assessed.

SUMMARY

The factors leading to the formation of kidney stones in humans are unknown. Dietary magnesium deficiency as an experimental method of producing kidney stones in rats in a relatively short period of time, has been employed to gain an understanding of the nature of these kidney stones.

Kidney stones generated by magnesium deficiency have been visualized *in situ* and appear to be primarily at the cortico-medullary junction of the kidney. Histochemically, the stones stain positively for calcium (by the von Kossa stain) and for carbohydrate (by the PAS stain). The stones appear to have a laminar interior.

A technique was devised to isolate the stones from kidney homogenates. The mineral component of isolated stones was analyzed by X-ray emission and found to be hydroxyapatite. The gross morphological character of the stones was examined under the microscope, and was found to vary between stones. Most of the stones were oblong and appeared to be composed of fused, round structures. The sizes of the stones varied as well; the width for most stones was about 24 microns, and the length sometimes reached 94 microns.

Isolated stones were demineralized either with EDTA or formic acid, and fractionated into dialyzable and non-dialyzable fractions. Chemical analysis of the dialyzable fraction revealed that about 60% of the total stone weight could be accounted for as calcium phosphate apatite. The nondialyzable fraction accounted for about 5.8% of the total stone weight. The composition of the reminder of stone weight (in the

dialyzable fraction) is not known.

The nondialyzable material was termed matrix. Visually it was a white fluffy material. Electron microscopy revealed the matrix was essentially amorphous. Various chemical analyses were done on the matrix in order to ascertain its composition on a dry weight basis. These tests included elemental analysis, amino acid analysis, a variety of carbohydrate assays, fatty acid analysis, a cholesterol determination, phosphate analysis, and measurements of calcium and magnesium content. From these studies, about 60% of the matrix composition has been determined, the majority of which are amino acids and neutral carbohydrate.

The origin and function of matrix are unknown. Various possibilities have been discussed with respect to this question, but based upon the limited data, no conclusive answer could be given. Knowledge of matrix composition may be useful in determining its origin.

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The Isolation and Partial Characterization of Rat Kidney Stone Matrix Induced by Magnesium Deficiency

by

Gregory Allen King

(ABSTRACT)

Dietary magnesium deficiency in rats has been employed to generate kidney stones in a relatively short period of time. Stones have been observed in situ and examined histochemically. Techniques have been devised for the isolation of the kidney stones and for the demineralization of the stones. The gross morphological character of isolated concretions has been examined microscopically, and the mineral composition determined by X-ray emission analysis. Hydroxyapatite was found to be the mineral component. Decalcification and subsequent dialysis of stones revealed that about 60% of the stone, on a dry weight basis, consists of apatite, and about 5.8% consists of a nondialyzable organic component termed matrix.

A variety of chemical analyses were done on this matrix material. These tests included elemental analysis, amino acid analysis, a variety of carbohydrate assays, fatty acid analysis, a cholesterol determination, phosphate analysis, and measurements of calcium and magnesium content. From these studies, about 60% of the matrix composition has been determined, the majority of which are amino acids and neutral carbohydrate.

The origin and function of kidney stone matrix are unknown. Possible answers to these questions have been presented, although

insufficient data does not permit any conclusive statement to be made. Knowledge of the composition of matrix may be useful in determining its origin.