A COMPARISON OF RENAL TISSUE SODIUM AND CALCIUM CONCENTRATION CHANGES INDUCED BY PARATHYROID EXTRACT, CALCIUM EXCESS OR CALCIUM DEPRIVATION IN THE RABBIT

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THESIS

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W. O. Collings

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ABSTRACT

A COMPARISON OF RENAL TISSUE SODIUM AND CALCIUM CONCENTRATION CHANGES INDUCED BY PARATHYROID EXTRACT, CALCIUM EXCESS OR CALCIUM DEPRIVATION IN THE RABBIT

By Charles Henry Wells II

Similar renal lesions have been developed in experimental animals by administering parathyroid extract (PTE) (Cantarow et al., 1938), calcium salts (Mulligan, 1946) or by maintaining the animals on a calcium free diet (Buckner and Nellor, 1960, Dienhart, 1960). The few studies of renal functional changes which accompany the development of these lesions reveal further similarities. Glomerular filtration rate depressions have been reported in animals treated with PTE (Epstein et al., 1959) or calcium gluconate (Chen and Neuman, 1955) while reductions in concentrating ability occur in PTE-treated (Epstein et al., 1959) and calcium gluconate treated animals (Freedman et al., 1958).

Although no common precipitating factor is apparent, the above observations suggest that these factors converge upon a common pathological process. Further evidence for this hypothesis may be derived from the study of vitamin D intoxication, which likewise alters calcium metabolism, and is associated with renal tubular cell calcification and concentrating ability changes like those resulting from PTE, calcium excess or calcium deprivation. The reduction in concentrating ability accompanying the vitamin D lesion has been shown to depend upon renal interstitial ion concentration reductions.

In order to obtain more evidence for use in assessing this theory, lesions were induced in immature rabbits by means of restricted calcium intake, parenteral administration of calcium salts, or by daily injections of parathyroid extract. Renal samples from these and from control animals were histologically examined to verify the presence of the characteristic lesions, and samples of renal cortex, medulla and papilla were analyzed for sodium and calcium concentrations. Daily urine volume and renal calcium excretion measurements were made for each animal before and during induction of the lesion.

Microscopic examination of the renal samples revealed no tenable basis for differentiating the lesions of one treatment from those resulting from either of the others.

The analyses of sodium and calcium concentrations in samples of renal cortex, medulla and papilla from animals subjected to the various treatments revealed similar cortical to papillary concentration gradients for both ions. These gradients existed in all animals studied; however the magnitude of the gradient was significantly ($P=.05$) less in animals subjected to PTE, calcium salt excess or calcium deprivation than in control animals. Cortical sodium and calcium concentrations were significantly ($P=.05$) above control values in one or more groups subjected to each of the above treatments. A high degree of correlation was found to exist between tissue sodium and calcium concentrations in the various renal samples. Urine volume changes induced by the various treatments

were minimal, with a trend toward volume reduction. Urinary calcium excretion and urine calcium concentration were significantly decreased ($P=.05$) in one or more groups subjected to each of the above treatments.

These findings, by demonstrating further similarities between the disease states induced by PTE, calcium salts or a calcium free diet, support the view that these seemingly diverse treatments converge upon a common disease process.

Indirect qualitative measures of interstitial fluid calcium concentration changes induced by these various treatments consistantly show marked reductions in papillary calcium concentrations, despite the formation of calcific lesions in this region. One may thus reason that the observed calcification must be dependent upon increased urine calcium concentration if it is to be explained by the popular metastatic theory, i.e., the precipitation of calcium in otherwise normal tissue because of massive increases in the calcium concentration of the fluids bathing the tissues. Although lesions were seen in the collecting duct epithelium of the papillae, the effluent urine either remained unchanged or was reduced. These observations imply that the tubular epithelial calcification associated with all of these various precipitating causes is not dependent upon a calcium induced metastatic process.

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By

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INTRODUCTION

Experimental animals develop similar renal lesions if given excesses of vitamin D (Goormaghtigh and Handousky, 1938), calcium salts (Mulligan, 1946) or parathyroid extract (PTE) (Cantarow et al., 1938) or if maintained on a calcium restricted diet (Buckner and Nellor, 1960, Dienhart, 1960). The advanced form of the lesion is characterized by scattered calcification and necrosis of tubules, mostly in the renal cortex or corticomedullary zones. Less advanced lesions are characterized by the presence of calcified cytoplasmic granules and of a thickened, calcified tubular cell basement membrane.

Complete functional studies of these various cases have not been published. However, a reduction in urinary concentrating ability is established for animals treated with PTE, vitamin D or calcium salts (Epstein et al., 1958, Carone et al., 1960, Freedman, et al., 1958) while reductions in renal plasma flow and in the glomerular filtration rate have been reported for PTE induced changes (Epstein et al., 1959, Carone et al., 1960). Humans with parathyroid adenomas or vitamin D intoxication also have decreased renal concentrating ability (Gill and Bartter, 1961, Dorhout, 1957). Renal plasma flow and glomerular filtration rate reductions have been reported in persons with parathyroid adenomas (Cohen et al., 1957, Edvall, 1958). The microscopic lesions in man were also much the same as those observed in experimental animals (Thorn et al., 1954, Boyd, 1953).

Although it is apparent that calcium metabolism alterations

are common to all of these situations, a more specific common factor is yet to be found. Examples of elevations and depressions of either serum calcium or parathyroid hormone levels can be found associated with one or more of the precipitating factors. Nevertheless the remarkable similarity of the renal lesions and the changes in renal function which occur with any of these apparently unrelated causes suggests the probability of similar, if not identical, terminal steps in the pathological process.

Further verification of the similarity of these processes and a more complete comprehension of the mechanisms responsible for these changes is of importance not only to the understanding of the disease state but to its prevention and treatment as well.

REVIEW OF LITERATURE

A number of conditions which alter calcium metabolism give rise to similar if not identical renal lesions and renal functional changes. These renal lesions are characterized by tubular basement membrane thickening and calcification, calcification and necrosis of tubular cells and the presence of interstitial calcification and occasional calcified tubular casts. Grossly, the lesions appear in all portions of the kidney, but are most heavily concentrated in the corticomedullary zone. They are remarkably scattered, with normal tubules lying beside others that are completely destroyed (Chown et al., 1939, Hueper, 1927, Hanes, 1939, Grimes, 1957).

Lesions fitting the above description are commonly found in patients suffering from parathyroid adenomas (Thorn et al., 1954) or hypervitaminosis D (Boyd, 1953) and have been reported in patients with peptic ulcers who have been on long-term high alkali, high calcium diets (Becker et al., 1952).

Other examples of lesions of the same apparent nature have been produced in experimental animals by administration of parathyroid extract (PTE) (Cantarow et al., 1938, Epstein et al., 1959, Hueper, 1927), excess vitamin D (Epstein et al., 1958, Goormaghtigh and Handousky, 1938, Wallach and Carter, 1959), calcium salts (Mulligan, 1946, Schneider et al., 1960) and by maintenance of the animals on low calcium diets (Buckner and Nellor, 1960, Dienhart, 1960).

Most of the studies in which these lesions have been experi-

mentally produced have been acute, and it has been pointed out that differences between the acute experimental lesion and the chronic human variety do exist (Anderson, 1939). Chronic experimental lesions, however, are histologically similar to chronic lesions in man. It is probable that the two merely represent different phases of development rather than actual differenced in the disease process (Epstein, 1960).

The striking similarity of the lesions and associated renal functional changes, and the fact that all of the precipitating factors involve calcium metabolism alterations, would lead one to suspect a common etiology.

Despite their apparent involvement, neither the elevation of serum parathormone nor the elevation of serum calcium levels could be a possible common etiological factor. Serum parathyroid hormone levels are elevated in cases of PTE administration, and of a calcium restricted diet; renal lesions are seen in both situations (Nicolaysen et al., 1953, Buckner and Nellor, 1960). However there is no reason to suspect any parathyroid hormone elevation in the case of vitamin D or calcium gluconate administration. On the contrary, one might expect a depression of serum parathyroid hormone levels in these cases (Crawford et al., 1957, Patt and Luckhardt, 1942). Serum calcium levels are elevated after vitamin D (Goodman and Gillman, 1955), calcium gluconate (McJunkin et al., 1932), or parathormone administration (Albright et al., 1929, Canary and Kyle, 1959, Todd et al., 1962), but low to normal with the calcium free diet (Baumann and Sprinson, 1939).

The effects of vitamin D and parathyroid hormone on calcium metabolism have been extensively studied, and there is agreement on the general effects of each. An inverse causal relationship exists between serum calcium levels and the rate of parathyroid hormone release (Patt and Luckhardt, 1942). Parathyroid hormone elevates serum calcium by its action on bone and kidney. It enhances osteoclastic activity, and depolymerization of bone matrix, with a concurrent dissolution of bone salts and thus a release of calcium to the blood stream (Engle, 1952, Johnston et al., 1961).

Phosphaturia and calciuria are the usual result of parathyroid hormone excess. Phosphaturia does not result from an active tubular secretion of phosphate but from a parathyroid hormone induced reduction in phosphate reabsorption by the tubules (Pitts et al., 1958, Samiy et al., 1960). Decreased serum phosphate increases serum calcium by encouraging bone salt dissolution. The complex physico-chemical properties of plasma preclude the possibility of a simple solubility product constant relationship between calcium and phosphate. Nevertheless an inverse relationship between serum calcium and phosphate exists.

The calciuria which often accompanies parathyroid hormone excess is not due to a reduction in the calcium reabsorptive ability of the renal tubular epithelium. On the contrary, recent studies indicate that parathyroid hormone enhances tubular calcium reabsorption (Widrow and Levinsky, 1962, Kleeman et al., 1958). The absence of a demonstrable tubular maximum for calcium (Chen and Neuman, 1955, Poulos, 1957, Epstein, 1960) places the dependence of the cal-

cium reabsorption rate upon the rate of calcium filtration. This has been experimentally verified by Canary and Kyle (1959). Parathyroid extract administration results in calciuria because of an increased filtered calcium load. In this case the urinary calcium loss increases although the calcium reabsorption rate also increases (Canary and Kyle, 1959). The tubular site of maximum calcium reabsorption has been shown to lie between the terminal portion of the proximal tubule and the proximal segment of the distal convoluted tubule (Widrow and Levinsky, 1962, Grollman et al., 1962, Lassiter et al., 1962, Wesson and Lauler, 1959). Parathyroid hormone is thought to have little effect on gastrointestinal calcium absorption. This calcium absorbing process is vitamin D dependent and accounts for serum calcium elevation noted with vitamin D administration (Kimberg et al., 1961, Epstein et al., 1958).

Unfortunately the physiological functions of vitamin D and parathyroid hormone give few clues to possible mechanism involved in development of lesions resulting from the administration of excessive amounts of either. However a number of alternative hypotheses have been proposed to explain the precipitation of calcium to form these lesions. Many authors contend that the calcification process resulting from one or more of the treatments discussed above is metastatic, i.e., the calcium is precipitated in otherwise normal tissue. Others favor the concept that calcium is precipitated in damaged tissue. This process is referred to as dystrophic calcification.

The metastatic theory has been presented to explain the

mechanism of calcification of lesions induced by PTE (Shelling et al., 1938, Schneider et al., 1960), vitamin D (Ham, 1932, Goormaghtigh and Handousky, 1938, Mulligan, 1946), and calcium gluconate (Mulligan, 1946, Schneider et al., 1960). Gill and Bartter (1961) studied functional changes induced by excessive calcium intake, vitamin D excess and parathyroid hormone excess. They concluded that calcium excess was the factor primarily responsible for renal function changes which occur in these cases.

McJunkin et al. (1932), however, after a study of calcium gluconate and PTE-induced lesions, were convinced that cell destruction precedes calcification. "In no animal have we seen calcification unassociated with a destructive lesion.... They are toxic lesions which are usually associated with hypercalcemia. The obvious inference is that the calcium salts produce the lesions." The mechanism by which salts inflict damage is still obscure. They noted that injection of calcium salts into kidney tissue failed to produce characteristic lesions and they suggested the possibility of elevated blood calcium resulting in elevated tissue lymph calcium. This was hypothesized to be the damaging agent.

Further support of the theory that these lesions were of a dystrophic nature came from numerous studies of lesion development by various agents. Becker et al. (1952) reported that lesions which developed in renal tissue of their patients who had been on long-term high alkali, high calcium diets were dystrophic. Cantarow et al. (1938) stated that calcification of PTE excess is dystrophic and not dependent upon serum calcium levels per se. Furthermore

Epstein et al. (1959) cited cases where renal tubular cell destruction was seen with calcification following vitamin D treatment.

Other investigators, realizing that the dystrophic and metastatic theories were concerned with only one aspect of tissue calcification, sought to clarify other aspects of the calcification process. Heuper (1927) noted the predominance of lesions in the kidney, stomach and lung and hypothesized that this was due to pH alterations. All of these organs release acid and are consequently left somewhat basic. This reduces calcium solubility. This could be a contributing factor whether the calcification process is dystrophic or metastatic. Another interesting facet was suggested by Engle (1952). Treatment of an animal with PTE results in dissolution of bone matrix as well as calcium salts, and thus results in an increase in serum mucopolysaccharide concentration. It has been observed that PTE depolymerizes renal tubular basement membrane substance, as well as bone matrix (Baker and Francisco, 1959, Grimes, 1957). Intratubular casts associated with PTE treatment have been shown to be composed largely of mucopolysaccharide and are often calcified (Baker and Francisco, 1959). No one has explained how this mucopolysaccharide, which presumably lost its calcium-binding power in bone and thus allowed dissolution of bone salts, regains this property when concentrated in renal tubules. Grimes (1957) suggested that mucopolysaccharide is absorbed into tubular cells where it is further depolymerized to calcium-binding forms by intracellular enzymes. Calcification of casts occurs because of necrosis of tubular epithelium, with rupture and release

of these enzymes to attack intratubular mucopolysaccharides. Grimes studied PTE and calcium gluconate-treated animals and found the tubular epithelial cytoplasmic calcification to be calcification of droplets which can be stained by the Periodic acid Schiff technique (P.A.S.). The P.A.S. positive staining indicates that the substance is a depolymerized polysaccharide (Hotchkiss, 1948), and in this case presumably the residue of bone matrix breakdown. The intratubular casts associated with calcium gluconate or PTE excess are also P.A.S. positive (Engle, 1952, Grimes, 1957). Interestingly, tubular basement membranes also show patchy areas of P.A.S. positive thickening following either treatment. It is felt that this is the basis for calcification of basement membranes seen in the above two cases.

Baker and Francisco (1959), in reference to PTE treated animals, stated "...it would be reasonable to conclude that the parathormone first produced the observed precalcific renal ground substance alterations and thereby specifically prepared these geographical tubule sites for the calcification that subsequently developed."

The study of depolymerization of mucopolysaccharides by PTE produced evidence that both bone matrix (Johnston et al., 1961) and other hexosamine structures are depolymerized (Hausmann, 1960). The concept that depolymerized products may be associated with the renal calcification process is further reinforced by the observation of Boyce et al. (1954) that the total quantity of biocolloids in the urine of patients with calculous disease is 3 to 13 times normal.

Although depolymerization of renal tubular ground substance has

been demonstrated following administration of calcium gluconate or parathyroid extract, there is reason to suspect that differences exist in these depolymerization processes. Animals which had incorporated radioactive sulfate into body tissues were treated with parathyroid extract and with calcium gluconate. The former group released more sulfate than the latter. This was interpreted by the investigator as greater depolymerization of chondroitin sulfate by PTE than by calcium gluconate (Reaven et al., 1960). The relation, if any, of these findings to development of renal lesions following PTE or calcium gluconate administration is yet to be determined.

Another possibility in the development of these lesions was suggested by Schneider et al. (1960). They asserted that PTE had a direct effect on renal tubular epithelium, in addition to its effect on tubular basement membranes and serum calcium levels, which was responsible for development of the lesions. Furthermore, they challenged the single etiology hypothesis, and support their contention with observations made on PTE and calcium gluconate-treated animals. They felt that the calcium gluconate lesion was almost exclusively limited to the tubule basement membrance, with cell necrosis being a secondary effect. In the PTE-induced lesions tubular epithelial necrosis was reported to accompany, but not precede, calcification of cells while the basement membrane thickening was thought to be secondary to epithelial cell necrosis. They further stated that there is more cortical involvement with calcium gluconate-induced lesions than in those ensuing from PTE treatment. However, the corticomedullary zone was the predominate lesion site for

either PTE or calcium gluconate induced lesions.

The observations of Schneider et al. have not gone uncontested. Baker and Francisco (1959) noted basement membrane changes following administration of PTE, even in the absence of calcification of the membrane or tubular cells. Hanes (1939) and Baker et al. (1954) reported that the process involved changes in the basement membrane first, followed by tubular cell necrosis for PTE induced lesions.

Further similarity of the renal changes induced by excesses of PTE, calcium gluconate, vitamin D or by a calcium restricted diet is illustrated by the renal functional changes which accompany the development of these lesions. It is well established that a depression of the glomerular filtration rate occurs concurrently with development of discernable pathological changes from excessive vitamin D (Wallach and Carter, 1959), from PTE (Carone et al., 1960, Epstein et al., 1959) or from calcium gluconate (Chen and Neuman, 1955, Poulos, 1957, Wallach and Carter, 1959). Similar observations have been made with persons suffering from parathyroid adenomas (Cohen et al., 1957, Edvall, 1958).

The tubular maximum (T_m) for PAH has been reported to be depressed in persons with parathyroid adenomas (Cohen et al., 1957), and a similar depression of tubular function was observed by Dorhout (1957) in patients with hypercalcemia. Edvall (1958) felt that this depression of the T_m PAH was a reflection of organic destruction. His studies of patients with parathyroid adenomas before and after surgical removal of the tumors, which failed to show any reversal of T_m PAH values toward normal, support this view.

The concentrating ability of the kidney decreased following administration of excessive PTE (Carone et al., 1960, Epstein et al., 1959), vitamin D (Epstein et al., 1958, Goormaghtigh and Handousky, 1938, Gill and Bartter, 1961, Mulligan, 1946), or calcium gluconate (Freedman et al., 1958). Persons with parathyroid adenomas (Cohen et al., 1957, Edvall, 1958, Gill and Bartter, 1961), or hypercalcemia due to destructive bone lesions (Edvall, 1958) are reported to have similar functional changes.

The best established current theory explaining the concentrating ability of kidney employs an osmotic gradient as the driving force for water reabsorption. The contributions of numerous investigators to the formulation and verification of this theory have been summarized and discussed in a review article by Homer Smith (1959).

The following are essential features of the theory. Osmolar concentration of interstitial fluid increases from the cortex to the papilla. This high papillary osmolarity is developed and maintained by active tubular reabsorption of salts from the tubular urine in conjunction with a high degree of water impermeability exhibited by the ascending limb of the loop of Henle.

The osmotic concentration of tubular urine approximates that of the surrounding interstitial fluid until urine reaches the ascending part of the loop of Henle. This segment, while essentially impermeable to water, nevertheless remains active in salt reabsorption. Urine reaches the distal convoluted tubule in a hypotonic state. The ultimate concentration of urine is thought to occur in the collecting

ducts. This process depends upon the maintenance of a high osmotic concentration in the interstitial space, especially at the tip of the papilla, and upon the permeability of the collecting duct. This latter factor is controlled by the antidiuretic hormone (ADH).

Cohen et al. (1957) observed that exogenous ADH administration in patients with parathyroid adenomas failed to alter the urine concentrating ability of the kidney. This would suggest that the functional change may be an inability to concentrate ions in the renal interstitial fluids and thus develop the concentration gradient necessary for the reabsorption of water.

Manitus et al. (1960) studied sodium and urea concentrations of the papilla and medulla of rats hypercalcemic from vitamin D intoxication. They concluded that the rats' inability to concentrate urine in this case was due, at least in part, to inability to develop and maintain a high concentration of sodium in the interstitial fluid of the medulla and papilla.

Evidence that urine osmolarity often approximates that of the interstitial fluid of the papilla was derived from studies by Ullrich and Jarausch (1956) in which the osmotic concentrations of both papillary fluid and urine were measured under different situations. From their studies they concluded: "Harnstoff ist am Anstieg des osmotischen Druckes im Nierengewebe Von Dursttieren Mengenmassig am starksten beteiligt, dann folgt Natriumchlorid. Die Konzentrationen der untersuchten Stoffe im Gewebe der Papillenspitze zusammengerechnet, ergeben einen osmotischen Druck, der nur wenig von dem des ausgeschiedenen Urins abweicht...."

Early observations of the effect of calcium salts on renal concentrating ability were made by Porges and Pribram (1908). They injected sodium salts and sodium-calcium salt mixtures into the blood streams of dogs and noted that calcium in the salt mixture caused a greater diuresis than injection of similar quantities of pure sodium salts. This might suggest that the circulating calcium concentration is a factor which exercises some control over the urine concentrating mechanism. It is unlikely that the change in the urine concentrating ability is dependent upon the development of destructive renal lesions in view of the report by Dorhout (1957) that the concentrating ability of the kidneys in persons with parathyroid adenomas improves rapidly following removal of the adenoma, while T_m PAH changes do not.

A careful consideration of the evidence offered in explanation of the renal changes delineated in the above experiments dictates caution in the adoption of a particular theory.

METHODS AND MATERIALS

The previous discussion of literature reveals that similar renal lesions can be developed by conditions which lead to either an elevation of serum calcium or of serum patathormone levels. If the similarity of the pathological manifestations from these dissimilar causes is to be explained by their convergence upon a common final process, the changes in renal function accompanying the development of the lesion should be alike for all precipitating causes. As a test of this hypothesis, renal tissue sodium concentrations were measured in animals subjected to hypercalcemia, PTE excess or both. The importance of decreased renal tissue sodium concentrations in the reduction of renal concentrating ability is established for vitamin D intoxicated animals (Manitus et al., 1960). Furthermore, a decrease in renal concentrating ability has been reported in hyperparathyroidism (Edvall, 1958), although its possible dependence upon reductions in renal tissue sodium concentration has not been established.

Elevated serum parathormone levels, in the absence of hypercalcemia should result from prolonged maintenance on a calcium free diet. Elevated serum calcium levels, associated with a depression of serum parathormone concentrations, should result from injection of calcium salts, while elevation of both serum parathormone and calcium levels should follow PTE injections. Two or more groups of animals were subjected to each of these treatments and these groups were sacrificed after differing spans of treatment so that more information could be accumulated about the lesions in their early stages of development.

PTE was used to induce lesions in two groups, each of which consisted of six immature rabbits. The members of the first group, numbers 43 through 48, were each given 50 units of Lilly Parathyroid Extract[1] per kilogram body weight per day. These animals were sacrificed twenty-four hours after the third injection with a sharp blow to the base of the skull. A 10 ml. blood sample was immediately taken by heart puncture and centrifuged. After centrifugation the plasma was decanted and frozen for future analysis. All syringes and centrifuge tubes used in the above prodedure were rinsed with ammonium heparin prior to use to avoid blood clotting.

The kidneys were removed and samples of cortex, medulla and papilla were collected in tared vessels, weighed and frozen. Other renal tissue samples were preserved in 10% buffered neutral formulin for future histological study.

Members of the second PTE-treated group, numbers 49 through 54, received 50 units of Lilly Parathyroid Extract U.S.P. per kilogram per day for five days, followed by 100 units per kilogram per day for two days and 200 units per kilogram on the final two days of treatment. These animals were sacrificed on the day following the final treatment, and samples of kidney and plasma preserved in the same manner as described for animals of the first group.

Lesions were produced in three groups of six immature rabbits each by the intraperitoneal administration of calcium salts. These salts were administered in the form of Cal-Dextro Solution.[2] The

1. Parathyroid Extract U.S.P., Eli Lilly Co., Indianapolis, Indiana.
2. Cal-dextro, a product of the Fort Dodge Laboratories, Ft. Dodge, Iowa, is a commercial preparation of a d-Saccharate and Gluconate salts in sterile aqueous solution containing 1.68 gm per cent calcium.

dosage for animals 25 through 30 was 1 ml per kilogram per day, for animals 37, 38, 39, 40, 59 and 60 was 2.5 ml per kilogram per day and for animals 41, 42, 55 through 58, and 67 through 72 was 5 ml per kilogram per day.

All animals in the calcium-treated groups were injected with the salt solution on three consecutive days with the exception of animals 67 through 72 which received six consecutive daily injections. These animals were sacrificed twenty-four hours after the final calcium salt injection and samples of blood and tissue were collected in the manner described for sample collection from the Parathyroid Extract treated animals.

All animals discussed so far were allowed access to Wayne Rabbit Ration and tap water at all times. The final two groups, however, were maintained on a restricted calcium intake. They had constant access to a calcium-free diet and distilled water throughout the experimental period. This diet was prepared by Dr. E. R. Miller of the Michigan State University Department of Animal Husbandry to have the following composition:

Protein (casein)	20%			
Fat (lard)	5%			
Carbohydrate (cerelose)	62%			
Fiber (cellulose)	10%			
Mineral	3%			
P	0.6%	Zn	55	ppm
K	0.5%	Mn	40	ppm
Na	0.3%	Cu	15	ppm
Cl	0.45%	Co	15	ppm
Mg	300 ppm*	I	0.5	ppm
Fe	100 ppm			

*parts per million

Vitamins (concentration per kilogram feed)

A	2000 I.U.	Niacin	20 mg
D	500 I.U.	Inositol	200 mg
E	8 mg	Para-aminobenzoic Acid	20 mg
K	2 mg	Folic Acid	400 ug
Thiamine	5 mg	Biotin	80 ug
Riboflavin	5 mg	B_{12}	80 ug
Pantothenic Acid	20 mg	Choline	2 gm
Pyridoxine	5 mg	Ascorbic Acid	128 mg

Animals numbered 61 through 66 were maintained exclusively on this diet and distilled water for twenty-four days, while animals 73 through 78 were similarly maintained for fifteen days. At the end of these periods the animals were sacrificed and samples of plasma and tissue collected in the manner previously described.

Although it has been shown that renal lesions from PTE or calcium gluconate excess, or resulting from a restricted calcium intake are not associated with similar changes in serum calcium concentrations, this should not be interpreted as an indication that consistant changes in the renal tissue calcium levels do not occur. In order to study this possibility, the weighed samples of cortex, medulla and papilla, which were collected and frozen at the time of sacrifice, were homogenized separately with a motor driven Potter-Elvehjem tissue grinder and diluted to concentrations which fell within the measurable range of a Coleman Model 24 flame photometer. All dilutions were made with .02% Sterox in triple distilled water, and the calcium analyses made with the flame photometer calibrated with a standard solution containing 0.2 milliequivalents of calcium and 6.0 milliequivalents of sodium per liter. Mathematical adjustments were made for sample size and dilution so that the calcium concentrations could be reported in milliequivalents per kilogram tissue.

A similar analysis of renal tissue sodium was undertaken so that correlations between tissue sodium and calcium concentrations could be established.

Aliquots of the tissue homogenates prepared for the calcium determinations were diluted to concentrations of approximately one gram tissue per 200 cc solution and quantitatively analyzed by flame photometry. All homogenate dilutions were made with .02% Sterox[1] in triple distilled water, and the direct reading scale for sodium determinations calibrated with a standard solution containing five milliequivalents sodium and 0.2 gm Sterox per liter of triple distilled water. Mathematical corrections for dilution and tissue sample size were made as with the calcium determinations so that the results could be expressed in milligrams calcium per kilogram tissue sample.

It was felt that a knowledge of plasma and urinary calcium concentrations at the time of sacrifice would also be valuable. The plasma samples collected and frozen at the time of sacrifice were later thawed and the calcium concentrations were determined with a Coleman Model 24 flame photometer.

The excretion of urine containing much of its salt in precipitated form, as is so common in rabbits, renders analysis of dissolved urine calcium meaningless. Furthermore, one cannot be sure that the precipitate-to-fluid ratio in bladder urine, taken by needle puncture, is representative of collecting duct precipitate to fluid ratios.

These difficulties were overcome by maintaining the animals in metabolism cages, measuring the urine volume for each twenty-four hour period, acidifying the urine until no precipitates remained and

1. Hartman-Leddon Co., Philadelphia, Penn.

quantitatively assaying the urine-acid mixture for calcium. A theoretical urine calcium concentration, assuming no calcium precipitation, can be calculated thus:

$$Ca_U = \frac{Ca_{U\text{-}HCl} \times V_{U\text{-}HCl}}{V_U}$$

where Ca_U equals the mean theoretical calcium concentration in the twenty-four hour urine sample, $Ca_{U\text{-}HCl}$ is the calcium concentration in the twenty-four hour urine-HCl mixture, $V_{U\text{-}HCl}$ represents the volume of the urine-HCl mixture and V_U represents the twenty-four hour urine volume. The calcium excretion per twenty-four hours (Ca_{24}) can also be derived from these data:

$$Ca_{24} = Ca_{U\text{-}HCl} \times V_{U\text{-}HCl}$$

These determinations were made daily on all animals (except 25 through 30, 61 through 66, and 72 through 78) during the experimental periods, and during a four-day control period which preceded treatment. The twenty-four hour urine volume-calcium excretion analysis schedule varied slightly for animals on the special dietary regimen. Analyses were made daily for the four-day pre-treatment control period, and on every third day thereafter.

In the execution of the above determinations it was found that careful washing of all drainage surfaces of the metabolism cages with HCl and collection of this fluid with the urine-HCl sample was necessary to assure the inclusion of all precipitated urinary salts in the daily sample.

As a final aspect of the study, histological preparations of renal tissue obtained from the various experimental animals were made and studied. These samples were fixed for twenty-four to forty-eight hours in 10% neutral formalin, dehydrated in alcohols, cleaned in xylene and imbedded in paraffin. Eight micron sections were obtained, stained for calcium by the Von Kossa method and counterstained with Kernechtrot. The fixing, dehydrating and staining procedure outlined on page 152 of the Armed Forces Institute of Pathology publication "Manual of Histologic and Special Staining Technics", (1960) was followed without modification.

Several tests for statistical significance were applied to the data obtained in this study. Serum calcium concentration and tissue homogenate concentrations were compared with control values by the Student *T* test.

$$T = \frac{\bar{X}_1 - \bar{X}_2}{\left[\frac{\left(\Sigma X_1 - \frac{(\Sigma X_1)^2}{n_1}\right) + \left(\Sigma X_2 - \frac{(\Sigma X_2)^2}{n_2}\right)}{(n_1 + n_2)(n_1 + n_2 - 2)}\right]^{\frac{1}{2}}}$$

In portions of the study where animals could serve as their own controls, such as measurement of the twenty-four hour urinary calcium excretion, or the twenty-four hour urine output, a variation of the Student *T* test was used, which assesses significance of the differences in the means of control and experimental values for each animal.

$$T = \frac{\bar{d}_{\bar{x}}}{\left[\frac{\Sigma d_{\bar{x}}^2 - \frac{(\Sigma d_{\bar{x}})^2}{n_{\bar{x}}}}{(n_{\bar{x}})(n_{x-1})}\right]^{\frac{1}{2}}}$$

As employed here, $\bar{d}_{\bar{x}}$ represents the mean of the differences between the control and experimental mean values for all animals in the group, and $n_{\bar{x}}$ represents the number of control minus experimental mean value differences included in the analysis.

The formula

$$r_{xy} = \frac{\Sigma_{xy} - \frac{(\Sigma_x)(\Sigma_y)}{n}}{\left(\Sigma_{x^2} - \frac{(\Sigma_x)^2}{n}\right)\left(\Sigma_{y^2} - \frac{(\Sigma_y)^2}{n}\right)}$$

was used in calculation of all correlation coefficients except for the correlation of sodium and calcium concentration changes. This calculation employed the follwwing formula:

$$r_{d_{\bar{x}} d_{\bar{y}}} = \frac{\Sigma(d_{\bar{x}})(d_y) - \frac{(\Sigma d_{\bar{x}})(\Sigma d_{\bar{y}})}{n}}{\left[\left(\Sigma d_{\bar{x}} - \frac{(\Sigma d_{\bar{x}})^2}{n}\right)\left(\Sigma d_{\bar{y}} - \frac{(\Sigma d_{\bar{y}})^2}{n}\right)\right]^{\frac{1}{2}}}$$

As applied to this study, $d_{\bar{x}}$ and $d_{\bar{y}}$ represent, respectively, the differences between corresponding control and experimental group tissue homogenate calcium and sodium concentrations.

RESULTS

I. Microscopic Examination

Plates 1 (PTE induced), 2 (calcium salt induced) and 3 (special diet induced) are low magnification photomicrographs showing advanced stages of cortical destruction, as produced in this study. Plates 4 and 5 are similar photomicrographs showing advanced tubular destruction in the medulla and papilla respectively.

Plates 6, 7 and 8 show Von Kossa positive inclusions in tubular cell cytoplasm. Darkened, Von Kossa positive, tubular cell margins may be seen in plates 9, 10 and 11. Plate 12 is an enlarged view of three tubular cell nuclei, showing black, and thus presumably calcified, intranuclear inclusions.

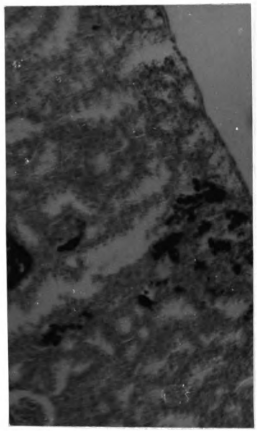


Plate 1

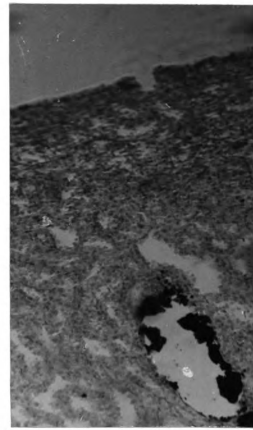


Plate 2

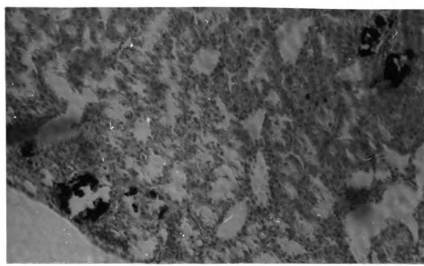


Plate 3

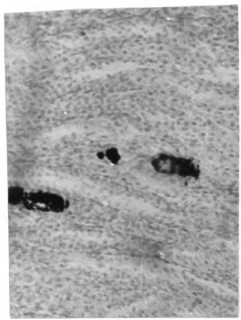


Plate 4

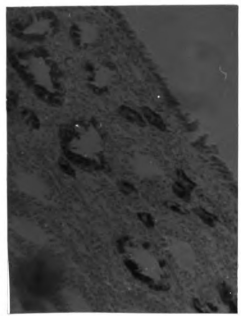


Plate 5

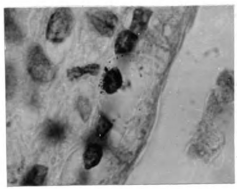


Plate 6

Plate 7

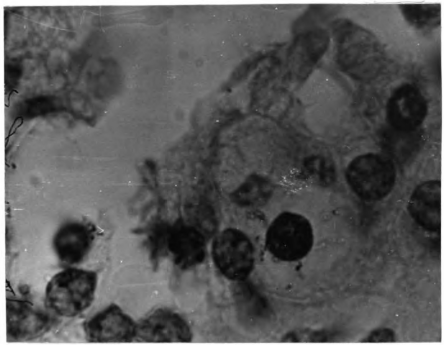


Plate 8

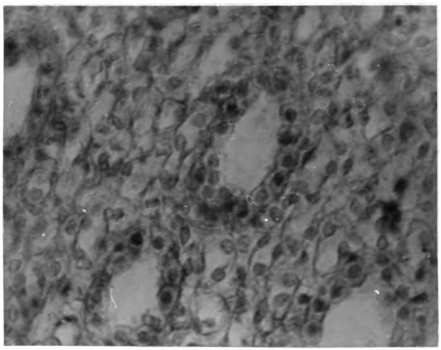


Plate 9

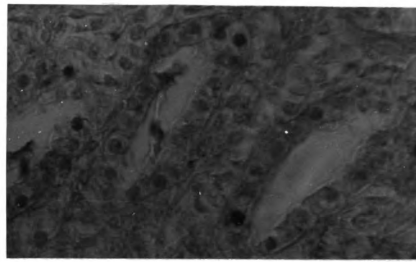


Plate 10

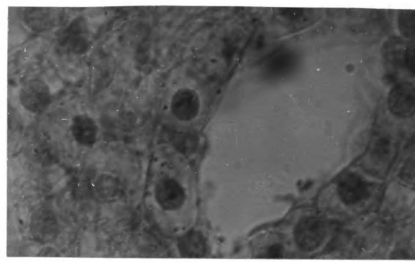


Plate 11

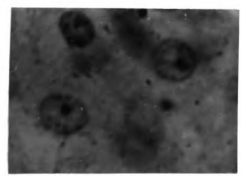


Plate 12

II. Sodium and Calcium Concentrations in Renal Tissue

Table 1 presents the results of calcium analyses of tissue homogenates, and the comparison of the mean cortical, medullary and papillary calcium concentrations of the various experimental groups with those of the control groups. Figure 1 graphically presents the same data. It will be noted that the calcium concentration of renal tissue in the control animals increased progressively from the cortex to the papilla. All of the experimental groups showed similar calcium concentration gradients from cortex to papilla although in three cases (Groups 4, 5 and 7) the cortical calcium concentrations exceeded those of the medulla.

The papillary calcium concentrations of the experimental groups were universally below the corresponding control group values, with depressions significant at the .05 level occurring in groups 3, 4, 5, 6 and 7. All three experimental treatments, i.e., PTE, calcium excess, and calcium-free diet, are represented in these groups.

The mean cortical calcium concentration of each of the experimental groups was elevated above the corresponding control value, although these changes attained .05 significance only with one PTE (Group 8), one special diet (Group 5) and two calcium treated groups (Groups 1, 4).

Figure 2 and table 2 present a summary of determinations of the mean sodium concentrations of the papillary, medullary and cortical tissue homogenates. Table 2 also contains comparisons of the mean tissue sodium concentrations from controls and various

experimental groups. Mean tissue sodium concentrations, of experimental groups and their comparisons with corresponding control values, followed much the same pattern as the calcium concentrations in the same tissues.

The mean cortical sodium concentrations of the experimental groups were elevated above control values in most cases, and elevations significant at the .05 level occurred in groups 4 (calcium treat), 6 (special diet) and 8 (PTE treated).

Depressions of papillary sodium concentration means below corresponding control values were consistantly seen with depressions significant at the .05 level occurring in all cases except groups 1 and 2.

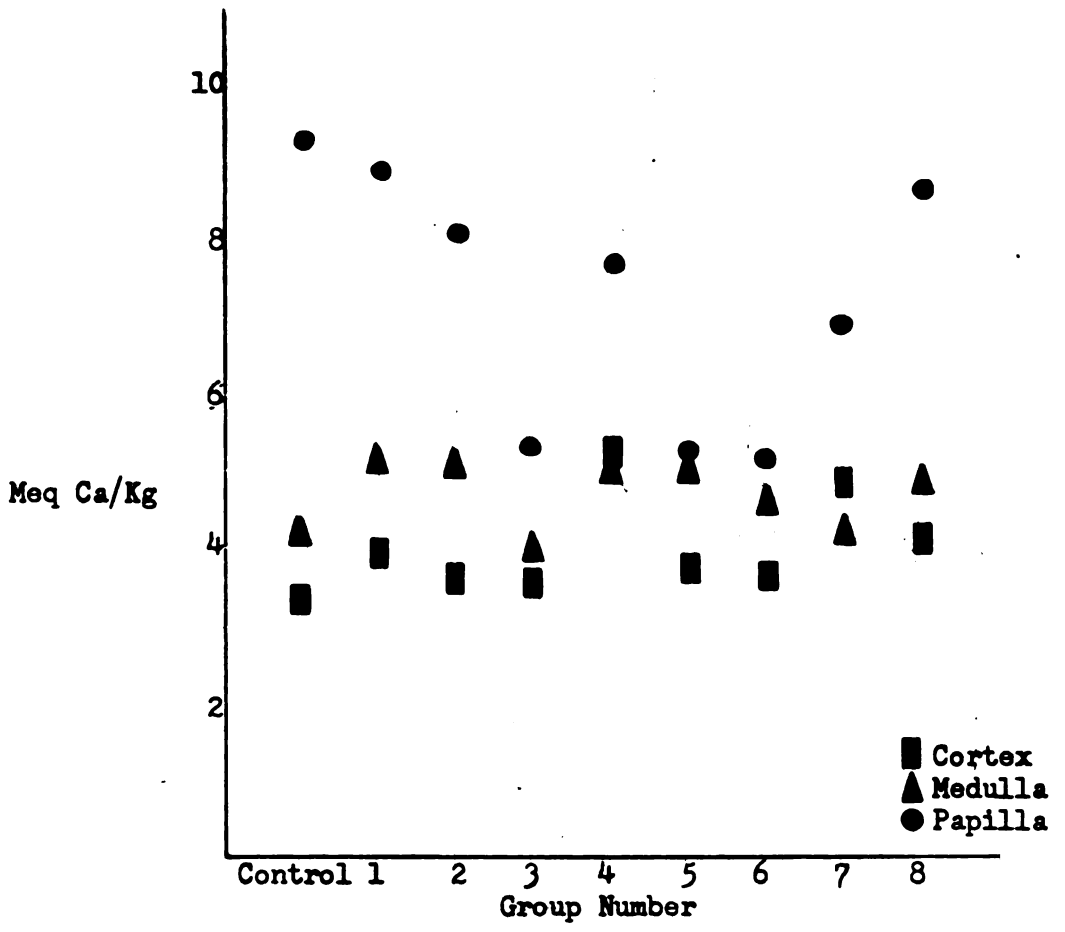
TABLE 1.

Group No.	No. of Animals	Treatment	Mean Tissue Homogenate Calcium Conc. (Meq/Kg tissue) Cortex	Medulla	Papilla
Control	10	None	3.34	4.22	9.41
1	6	Cal-dextro 1cc/Kg/day for 3 days	3.85*	5.18*	8.92
2	4	Cal-dextro 2.5cc/Kg/day for 3 days	3.65	5.15*	8.08
3	8	Cal-dextro 5cc/Kg/day for 3 days	3.53	3.96	5.39*
4	6	Cal-dextro 5cc/Kg/day for 6 days	5.17*	5.00*	7.77*
5	6	Calcium restricted diet for 15 days	5.73*	5.05*	5.30*
6	5	Calcium restricted diet for 24 days	3.60	4.60	5.20*
7	6	PTE doses 50 units/day for 3 days	4.82	4.20	6.90*
8	6	PTE doses increasing from 50 to 200 units/day over 7 days	4.08*	4.82*	8.62*

*Significantly different from control values (P=.05). Cortical and papillary samples were tested by a one-tailed test and the medullary samples by a two-tailed test.

FIGURE 1

Experimentally Induced Calcium Concentration
Gradient Changes



Groups 1-4: Calcium Treated
Groups 5-6: PTE-Treated
Groups 7-8: Dietary Calcium Deprivation

For specific group therapies, see table 1.

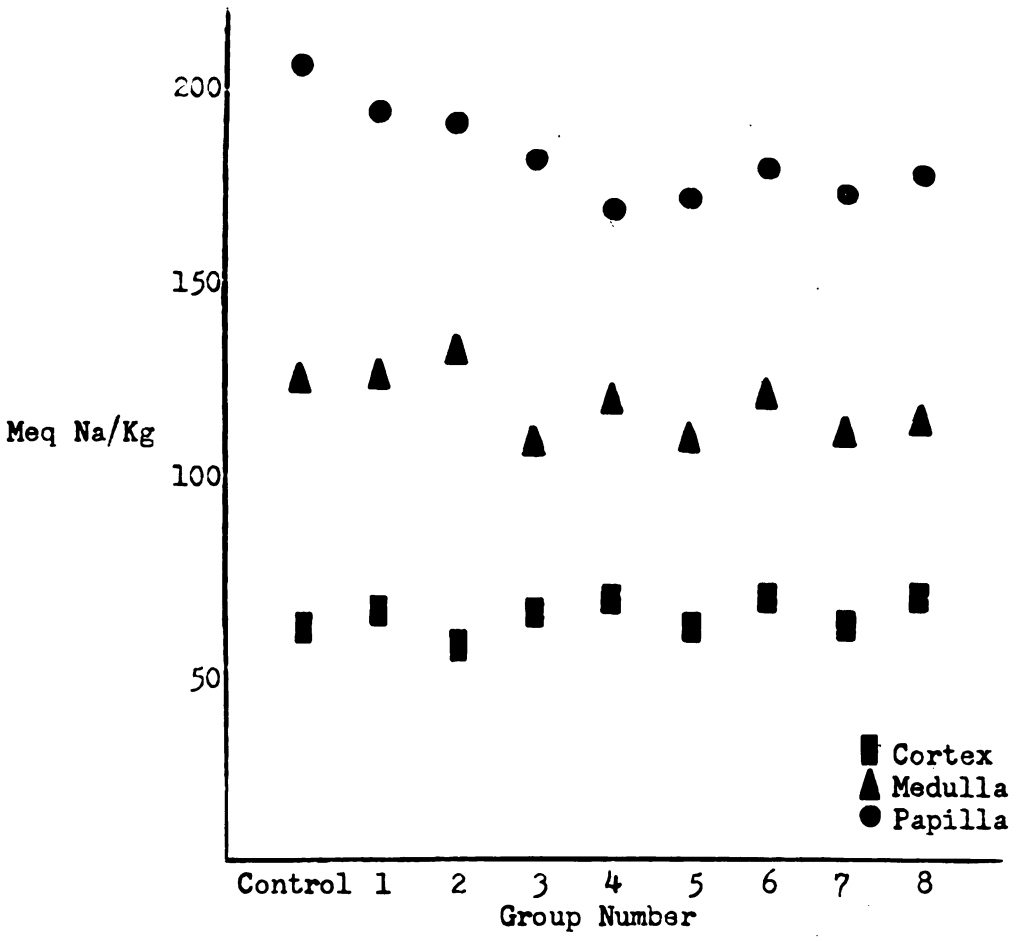
TABLE 2

Group No.	No. of Animals	Treatment	Mean Tissue Homogenate Sodium Conc. (Meq/Kg tissue) Cortex	Medulla	Papilla
Control	10		61.1	123.9	206.9
1	6	Cal-dextro 1cc/Kg/day for 3 days	65.5	125.8	194.7
2	4	Cal-dextro 2.5cc/Kg/day for 3 days	56.6	132.5	191.8
3	8	Cal-dextro 5cc/Kg/day for 3 days	62.0	108.8*	182.0*
4	6	Cal-dextro 5cc/Kg/day for 6 days	68.7*	119.7	168.8*
5	6	Calcium restricted diet for 15 days	60.3	108.0*	172.2*
6	5	Calcium restricted diet for 24 days	68.6*	120.0	179.8*
7	6	PTE doses 50 units for 3 days	61.0	111.2*	173.3*
8	6	PTE doses increasing from 50 to 200 units over 7 days	68.8*	114.5	179.2*

*Significantly different from corresponding control values at the .05 level. Cortical and papillary samples were tested by a one-tailed test and the medullary samples by a two-tailed test.

FIGURE 2

Experimentally Induced Sodium Concentration Gradient Changes



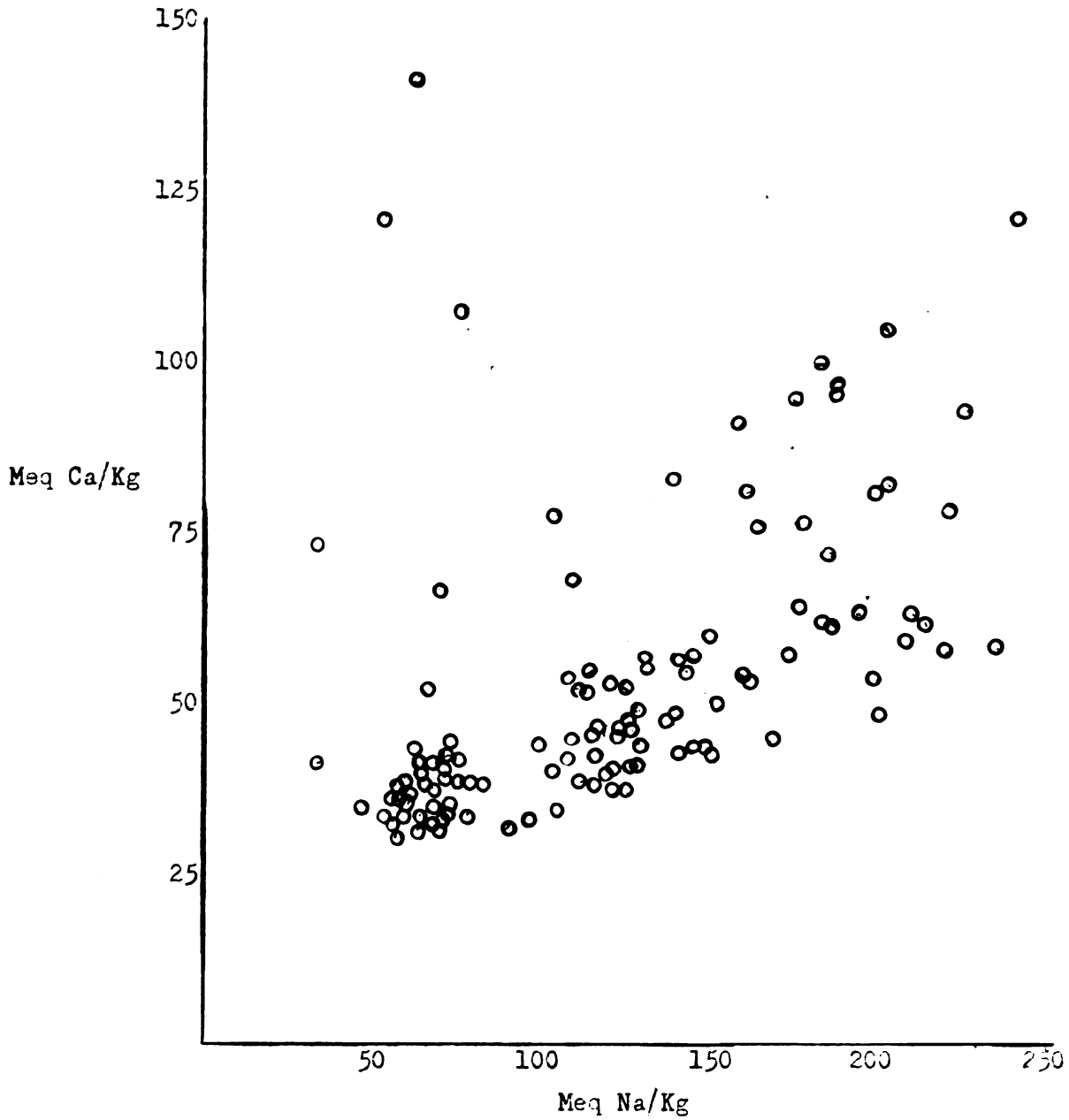
Groups 1-4: Calcium Treated
Groups 5-6: PTE-Treated
Groups 7-8: Dietary Calcium Deprivation

For specific group therapies, see table 2.

Figure 3 is a graphic presentation of the relationship of sodium and calcium concentrations obtained from cortical, medullary and papillary samples of all animals studied.

FIGURE 3

Relation of Renal Tissue Sodium and Calcium Concentration



III. Experimental Alterations of Serum Calcium Concentrations

Changes in plasma calcium concentration, which were brought about by PTE, calcium salt excess or by a calcium restricted diet, are presented in table 3. The decreased plasma calcium in animals maintained on the calcium restricted diet (groups 5 and 6) and increased plasma calcium in calcium salt treated (Group 4) and PTE treated animals (Groups 7 and 8) were significantly different from the control group plasma calcium concentrations ($P=.05$).

The calcium concentrations in plasma samples decline with time if the samples are stored in glass. Figure 4 is a composite of the concentration changes noted in three plasma samples. Calcium concentrations, expressed as percent of the original value, are plotted against time since the collection of the sample.

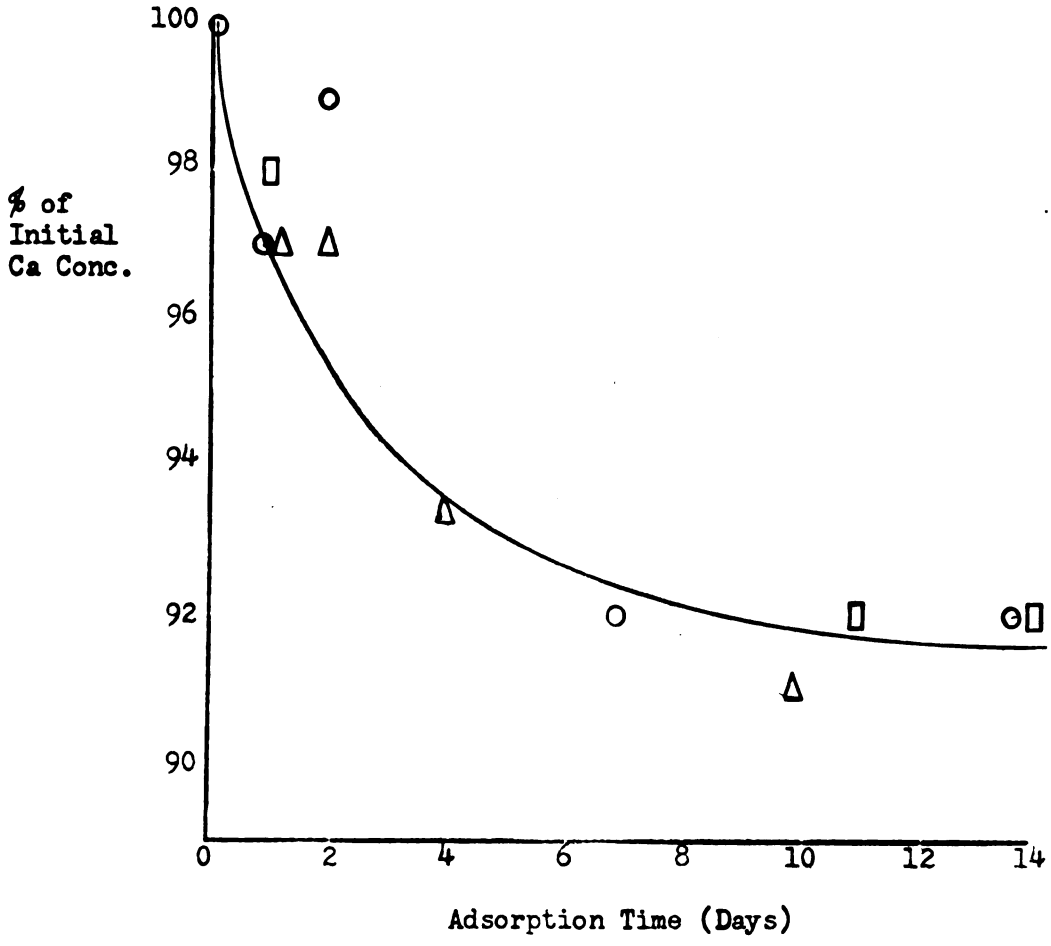
TABLE 3

Group No.	Treatment	No. Animals	Mean Serum Ca Conc (meq/l)
Control	None	6	6.10
3	Cal-dextro 5cc/Kg/day for 3 days	4	6.15
4	Cal-dextro 5cc/Kg/day for 6 days	6	6.43*
5	Calcium restricted diet for 15 days	5	5.66*
6	Calcium restricted diet for 24 days	5	5.80*
7	PTE doses 50 units for 3 days	6	6.72*
8	PTE doses increasing from 50-200 units over 7 days	6	6.43*

*Significantly different from control at .05 level.

FIGURE 4

Calcium Adsorption From Plasma



IV. Urine Volume Changes

A summary of urine volume measurements reported in milliliters per 24 hours per kilogram body weight is presented in table 4.

TABLE 4

Urine Vol/24 hr/Kg body wt.

Group No.	Treatment	Animal No.	Control Mean	Experimental Mean Minus Control Mean
1	Cal-dextro 1cc/Kg/day for 3 days	25	35.85	- 7.43
		26	71.38	-15.83
		27	62.71	-17.82
		28	78.78	-10.03
		29	27.66	- 7.62
		30	28.16	6.23
2	Cal-dextro 2.5cc/Kg/day for 3 days	41	50.20	- 8.87
		42	67.00	1.67
		59	29.75	-14.42
		60	51.25	-40.92
3	Cal-dextro 5cc/Kg/day for 3 days	37	54.20	-12.87
		38	27.80	42.20
		39	77.75	-39.42
		40	37.80	-19.13
		55	63.50	-58.50
		56	37.50	-27.50
		57	15.25	20.08
		58	31.50	-19.17

TABLE 4 (con't)

Urine Vol/24 hr/Kg body wt.

Group No.	Treatment	Animal No.	Control Mean	Experimental Mean Minus Control Mean
4	Cal-dextro 5cc/Kg/day for 6 days	67	59.00	- 7.83
		68	9.00	- 2.33
		69	114.00	-70.83
		70	17.75	-11.92
		71	34.25	-14.75
		72	168.25	-99.58
5	Calcium restricted diet for 15 days	73	70.50	7.70
		74	45.75	4.08
		75	35.75	0.42
		76	113.75	-76.92
		77	31.00	- 3.67
		78	49.50	- 7.83
6	Calcium restricted diet for 24 days	61	69.25	- 0.70
		62	41.00	-21.37
		63	46.47	12.00
		64	21.00	14.56
		65	130.00	-90.14
		66	27.00	4.33

TABLE 4 (con't)

Urine Vol/24 hr/Kg body wt.

Group No.	Treatment	Animal No.	Control Mean	Experimental Mean Minus Control Mean
7	PTE doses 50 units for 3 days	43	104.20	3.80
		44	91.80	-39.47
		45	73.60	25.07
		46	101.20	-12.53
		47	45.80	- 6.80
		48	45.00	- 2.67
8*	PTE doses increasing from 50-200 units over 7 days	49	39.25	-13.75
		50	129.25	-27.75
		51	65.00	-37.37
		52	53.50	-37.87
		53	54.50	-11.00
		54	102.25	-39.50

*Significantly different from control at .05 level.

V. Urinary Calcium Excretion

Table 5 presents a summary of calcium excretion before and after the various treatments. Significant (P=.05) reductions in calcium excretion occurred in 6 of the 7 groups studied.

TABLE 5

Ca Excretion (meq/24hr/Kg body wt)

Group No.	Treatment	Animal No.	Control Mean	Experimental Mean Minus Control Mean
2*	Cal-dextro 2.5cc/Kg/day for 3 days	41	19.55	- 8.30
		42	18.22	- 0.99
		59	18.60	-13.43
		60	13.75	- 8.28
3*	Cal-dextro 5cc/Kg/day for 3 days	37	15.44	- 6.12
		38	14.24	- 9.43
		39	20.38	-15.66
		40	17.02	-13.50
		55	12.98	- 9.08
		56	15.35	-13.62
		57	5.74	1.34
		58	15.30	-10.98
4*	Cal-dextro 5cc/Kg/day for 6 days	67	14.98	- 5.08
		68	11.98	- 8.94
		69	17.15	-12.60
		70	12.43	-11.13
		71	13.75	- 7.38
		72	12.88	- 3.99

*Significantly lower than control values at .05 level.

TABLE 5 (con't)

Group No.	Treatment	Animal No.	Ca Excretion (meq/24hr/Kg body wt) Control Mean	Experimental Mean Minus Control Mean
5*	Calcium restricted diet for 15 days	73	19.23	-18.35
		74	20.25	-19.13
		75	13.60	-12.72
		76	15.15	-12.80
		77	15.65	-13.50
		78	15.75	-14.28
6*	Calcium restricted diet for 24 days	61	18.28	-17.28
		62	9.21	- 6.86
		63	11.63	-10.60
		64	10.95	- 9.14
		65	16.63	-14.27
		66	12.55	-10.63
7	PTE doses 50 units for 3 days	43	12.23	- 4.36
		44	9.42	- 6.02
		45	9.48	0.85
		46	7.86	2.51
		47	9.24	0.06
		48	11.27	- 6.14

*Significantly lower than control values at .05 level.

TABLE 5 (con't)

Ca Excretion (meq/24hr/Kg body wt)

Group No.	Treatment	Animal No.	Control Mean	Experimental Mean Minus Control Mean
8*	PTE doses increasing from 50-200 units over 7 days	49	16.83	- 3.44
		50	11.68	- 1.75
		51	15.98	- 2.08
		52	13.48	- 4.22
		53	14.23	1.17
		54	11.40	- 1.81

*Significantly lower than control values at .05 level.

VI. Correlations between Tissue, Plasma and Urine Calcium Concentrations

An attempt was made to demonstrate interrelations between calcium concentrations in urine and in renal tissue or between those of plasma and renal tissue. A summary of the results of this analysis is presented in table 6.

TABLE 6

Group No.	No. of Animals	Treatment	Correlation Coefficient for: Plasma Calcium-Papillary Calcium Concentration	Papillary Calcium-Urine Calcium Concentration
3	4	Cal-dextro 5cc/Kg/day for 3 days	0.926*	0.597
4	6	Cal-dextro 5cc/Kg/day for 6 days	-0.456	0.505
5	5	Calcium restricted diet for 15 days	0.258	0.715
6	5	Calcium restricted diet for 24 days	0.803	0.469
7	6	PTE doses 50 units for 3 days	0.048	0.636
8	6	PTE doses increasing from 50 to 200 units over 7 days	-0.695	0.444

*Significant at the .05 level.

VII. Urine Calcium Concentration Changes

A summary of urine calcium concentration studies, before and during treatment, is presented in table 7.

TABLE 7

Group No.	Treatment	Animal No.	Urine Ca Conc.(meq/l) Control Mean	Experimental Mean Minus Control Mean
2	Cal-dextro 2.5cc/Kg/day for 3 days	41	.438	- .158
		42	.341	- .075
		59	.633	.068
		60	.299	.936
3	Cal-dextro 5cc/Kg/day for 3 days	37	.289	- .013
		38	.579	- .490
		39	.301	- .148
		40	.462	- .207
		55	.209	1.337
		56	.421	- .238
		57	.343	.081
		58	.507	- .108
4	Cal-dextro 5cc/Kg/day for 6 days	67	.267	.020
		68	1.331	- .871
		69	.150	- .033
		70	.793	- .549
		71	.404	- .001
		72	.077	.192
5*	Calcium restricted diet for 15 days	73	.283	- .271
		74	.447	- .415
		75	.597	- .502

*Significantly lower than control values at .05 level.

TABLE 7 (con't)

Group No.	Treatment	Animal No.	Urine Ca Conc. (meq/l) Control Mean	Experimental Mean Minus Control Mean
5* (con't)		76	.141	.472
		77	.766	- .676
		78	.377	- .304
6*	Calcium restricted diet for 24 days	61	.270	- .247
		62	.239	- .089
		63	.246	- .207
		64	.616	- .546
		65	.130	- .069
		66	.698	- .627
7	PTE doses 50 units for 3 days	43	.116	- .043
		44	.108	.316
		45	.132	- .028
		46	.080	.045
		47	.215	.008
		48	.249	- .120
8*	PTE doses increasing from 50-200 units over 7 days	49	.433	.577
		50	.098	- .001
		51	.255	.683
		52	.267	.495
		53	.283	.095
		54	.110	.048

*Significantly different than control values at .05 level.

VIII. Composite Presentation of Results

Table 8 is included to give the reader a composite view of the results of this study. The values included represent those which were significantly different from corresponding control values at the .05 level. The symbol + indicates an increase of experimental over control values and - a decrease in experimental values; "0" indicates that the observed changes were not of sufficient magnitude to achieve .05 significance. It should not be interpreted as meaning that the control and experimental values were proven to be in the same population.

TABLE 8

	Calcium Deprivation		Calcium Administration		Parathyroid Extract	
	17 days	24 days	3 doses	6 doses	3 doses	7 doses
Serum Calcium	-	-	0	+	+	+
Urinary Calcium Excretion/24 hr	-	-	-	-	0	-
Post Treatment Urine Volume/24 hr	0	0	0	0	0	-
Post Treatment Mean Urine Calcium Concentration	0	0	-	-	0	-
Papillary Calcium Concentration	-	-	-	-	-	-
Medullary Calcium Concentration	+	0	+	+	0	+
Cortical Calcium Concentration	+	0	0	+	0	+
Papillary Sodium Concentration	-	-	-	-	-	-
Medullary Sodium Concentration	-	0	-	0	-	0
Cortical Sodium Concentration	0	+	0	+	0	+

DISCUSSION

I. Microscopic Analysis

A rough microscopic examination of the renal lesions resulting from a calcium free diet (Plate 1), from PTE excess (Plate 2) or from calcium excess (Plate 3) revealed little that would be of value in distinguishing one from another. The lesions were located primarily in cortical tubule segments, although areas of tubular calcification and destruction frequently appeared in the medulla (Plate 4). The occurrence of advanced tubular destruction and calcification in the papilla was less common but did occur (Plate 5).

Advanced destructive lesions, such as those in plates 1-5, have developed to such a degree that little could be determined about the nature of the original lesion.

Various authors have reported calcification of cytoplasmic granules (Grimes, 1957) and of tubular cell basement membranes (Hanes, 1939, Schneider et al., 1960) following PTE or calcium salt treatment. Basement membrane calcification was reported by Hanes (1939) and Schneider et al., (1960) to be the initial change in the development of these lesions.

Animals of this study, whether treated with calcium, PTE or the special diet, developed calcified cytoplasmic granules (Plates 6,7,8) and calcium deposits at the tubular cell margins that could be interpreted as calcification of basement membranes (Plates 9, 10, 11). These changes could be found in all parts of the kidney, and on occasion each appeared without the other. This observation not only

precludes the possibility of differentiating the lesions from one precipitating factor from those of another, but also casts doubt on the concept that basement membrane calcification is universally the initial change in the formation of these lesions.

Tubular cell nuclear inclusions, presumed to be calcium because of positive Von Kossa staining, were also found in animals treated with PTE, calcium salts or a calcium restricted diet. These inclusions were seen in cortical, medullary and papillary cells, and often appeared without other demonstrable changes in the tissue. However, they were more frequently found in cells with either basement membrane calcification or calcified cytoplasmic inclusions, or in cells with both.

The degree of nuclear involvement varied from the presence of one or two small dense intranuclear inclusions (Plate 12) to the development nuclei which stained a dense uniform black by the Von Kossa method (Plates 6, 7, 8). The more completely calcified nuclei were found predominantly in the papillary region, although varying degrees of renal tubular cell nuclear involvements were found throughout the organ.

The occurrence of these nuclear inclusions, in the absence of other demonstrable alterations, and their frequent appearance in cells with pathological changes varying from mild cytoplasmic or basement membrane calcification to total cell destruction would make one suspect that this was the first sign of cell calcification. However, the occurrence of calcified cytoplasmic granules, and basement membrane calcification in the absence of nuclear calcification

casts doubt on this interpretation. The significance of the stainable nuclear inclusions cannot be deduced from results of this study.

Renal lesions, developed in this study by PTE, calcium salt excess or by a calcium free diet, were so similar both in general distribution and in the occurrence of basement membrane, cytoplasmic and nuclear calcific changes that no tenable basis for differentiating one from another could be discovered. Little doubt exists that these lesions were of the same nature as those reported by others (Heuper, 1927, Mulligan, 1946) to have resulted from PTE or calcium salt excess or from a restricted calcium intake despite the appearance of intranuclear inclusions which had not been previously reported.

II. Urine, Plasma and Tissue Sodium and Calcium Concentrations

The mean papillary sodium concentrations of all experimental groups were depressed below control values (Tables 2, 8, Figure 2). The importance of these changes was suggested in a report by Manitus et al. (1960) who studied the reduction of sodium concentration in the papilla of vitamin D intoxicated rats. They reported "that the impaired renal concentrating ability observed in hypercalcemic rats resulted, at least in part, from impaired reabsorption of sodium by the renal tubules and a diminished ability to concentrate and maintain a high concentration of sodium in the interstitial fluid of the medulla and papilla." Further support of the concept that the papillary sodium concentration is directly related to the urine osmolarity can be derived from the studies of Ullrich and Jarausch (1956). They

showed, with remarkable precision, a linear relationship between papillary tissue homogenate sodium concentrations and the osmolarity of the effluent urine. The sodium concentration changes found in figure 2 would lead one to speculate that reductions in papillary sodium concentrations are the cause of decreased renal concentrating ability reported to occur in animals treated with PTE (Epstein et al., 1959) or calcium gluconate (Freedman et al., 1958).

Although the relation of changes in renal concentrating ability to altered papillary sodium concentrations is of interest, the observation that similar changes in papillary sodium concentrations occur following PTE, calcium excess, restricted calcium intake (Table 2) or vitamin D (Manitus et al., 1960) is probably of more significance. No evidence on the mechanism of reduction in papillary sodium is advanced by this study (nor has any been found in the literature) yet it is clear that hypercalcemia is not the cause, nor even a common factor (Table 3). Furthermore, there appears to be a strong logical and experimental basis for the assumption that serum parathyroid hormone elevations occur in animals maintained on a calcium free diet (Buckner and Nellor, 1960) and in animals given PTE excess, whereas the blood hormone level is reduced following vitamin D (Crawford et al., 1957) or calcium salt therapy. Consequently, alterations in blood parathyroid hormone level per se could not be the cause of these similar tissue concentration changes, nor of the formation of the histological lesions.

A plot of tissue sodium versus calcium concentrations (Figure 3) suggests a positive, if somewhat variable, interrelation between the

two ion concentrations. A correlation coefficient of 0.641 was calculated from these data. Its significance, in excess of the .001 level, despite the seemingly low correlation coefficient, is explained by an n of 169. This high over-all positive correlation exists despite the strong negative correlation contributed by the general depression of medullary sodium with concurrent elevation of medullary calcium in most of the experimental groups (Tables 1, 2). Mean medullary calcium concentrations in groups 1, 2, 4, 5 and 8 were significantly elevated above the corresponding control values at the .05 level, whereas the mean sodium concentrations of groups 3, 5 and 7 were significantly less (P=.05) than their corresponding control means.

Plasma calcium concentration increases occurred in PTE and calcium salt treated animals, while decreases were observed in the calcium-free-diet animals (Table 3). The plasma samples from which these analyses were made, were frozen in glass tubes and stored for one to five days prior to analysis. It has since been found that plasma samples stored in this manner undergo a progressive decrease in calcium concentration with time, presumably from calcium adsorption on the glass. If this is correct, the rate and quantity of calcium adsorption will depend on several highly variable factors, such as the glass surface area to fluid volume ratio and the initial calcium concentration of the plasma. Thus an accurate determination of the calcium loss from the plasma is impossible, although the order of magnitude of the change is easily found. It is unlikely that a plasma calcium loss of more than 7% or less than 3% occurred from

any of the samples whose analyses are compiled in table 3. It is probable that the serum calcium losses were approximately equal for all groups. However, this has not been proven, and consequently one should be aware of this limitation while interpreting these plasma calcium data.

Daily urine volume output (Tables 4, 8) consistantly showed small decreases following the initiation of any of the treatments studied. These changes, however, were of significant magnitude at the .05 level only in one of the PTE groups (Group 8). The daily urinary calcium excretion (Tables 5, 8) also decreased in all experimental groups. These decreases proved to be significant at the .05 level for six of the seven groups studied. The biological significance of these changes cannot be established without further study; however, a tentative explanation will be presented later.

Studies of the relationships between calcium concentrations in urine and renal tissue and between those in plasma and renal tissue yielded inconclusive results (Table 6). The correlation coefficients derived from the plasma calcium-papillary calcium concentration relationships varied from -0.695 to 0.926. Despite the indicated significance of this last value, these data give little support to the concept that a strong relationship exists between these two parameters.

None of the correlation coefficients calculated for papillary calcium concentration-urine calcium concentration relationship were significant at the .05 level. However, the consistantly similar values would suggest that a significant correlation might be shown if sufficiently large samples were employed.

III. Relation of Tissue Sodium and Calcium with Renal Lesions

Consideration should be given to the manner in which the data so far presented relate to the formation of the renal lesions. The metastatic theory for the formation of these renal lesions is based on the development of some condition which allows calcium to precipitate in otherwise normal tissue. For this type of lesion the cause is frequently implied to be increased calcium in interstitial fluid, or perhaps in tubular urine. A study of the changes in calcium concentration of these fluids in states which give rise to these lesions would be of value. Since neither samples of tubular urine nor interstitial fluid were available, extrapolations must be made from indirect evidence. Mean daily urine calcium concentrations cannot be directly equated with intratubular urine calcium concentrations because of calcium and water reabsorption which occurs to a variable degree in transformation of the latter into the former. Tubular urine is not, of course, an entity to which any single set of values can be applied. Its concentration and volume varies constantly as it passes down the nephron. Changes in tubular urine calcium concentration may be the cause of the observed lesion through a process of metastatic calcification. If this is true, the concentration change must be reflected in the effluent urine since lesions develop in the collecting ducts of the papilla as well as in cortical tubular cells. Data presented in table 7 raises some doubt about this hypothesis. No significant increases in urinary calcium concentrations were seen, whereas significant reductions in urine

calcium occurred with both dietary groups.

The interstitial fluid calcium concentration could, of course, lead to a metastatic calcification of tubular cells. An increase in tissue homogenate calcium does not necessarily represent a similar increase in interstitial fluid calcium, although such an interpretation may be preferred. Alternative possibilities are increased intracellular calcium concentration and changes in the cell-to-interstitial fluid ratios. The volume contributions of blood and tubular urine to total renal mass are quite low (Weaver et al., 1956) and thus neither could account for the marked ion concentration changes which occur. Similarly, explanation of these marked concentration changes on the basis of changed intracellular ion concentrations is unlikely. The intracellular ion concentration changes necessary to bring about such changes in total tissue calcium and sodium concentrations are undoubtedly beyond the range tolerated by normal cells. Blood pressure dependent changes in renal volume, presumably due to alterations in the interstitial fluid volume, are well established. Although such changes would alter the calcium concentration of tissue homogenates by changing the cellular fluid-to-interstitial fluid ratio, it could not account for the simultaneous increase in cortical calcium concentration and decrease in papillary calcium concentration as shown in tables 1 and 2.

The most logical assumption to explain the data of tables 1 and 2 would be that tissue homogenate ion concentration changes reflect interstitial fluid ion concentration changes. If one is to accept this hypothesis, the relationship of the changes in interstitial fluid

calcium concentration, which can be inferred from table 1, to the development of the observed lesion casts further doubt on the metastatic calcification hypothesis. The increased cortical calcium concentration is quite compatable with the metastatic theory; however, development of lesions in the papilla, where drastic reductions in tissue calcium occur, is not. These findings do not eliminate the possibility of these lesions being metastatic. They are, however, in opposition to the popular concept that tissue calcification in such cases could be due to elevations of calcium concentration in the fluids bathing the tubular cells.

IV. Relation of Sodium and Calcium in Renal Tissue

Aside from the discussion of pathology and associated changes, the correlation of sodium and calcium concentrations in renal tissue (Figure 3), and the striking changes in calcium and sodium concentration from the cortex to the papilla (Tables 1,2) deserve discussion. The observation that tissue sodium concentrations vary in this manner has been substantiated by micropuncture studies of Lassiter *et al.* (1961) and by the tissue homogenate studies of Ullrich and Jarausch (1956). It is currently believed, as discussed previously, that this concentration gradient is maintained by the reabsorption of sodium from tubular urine by the tubular cells. The calcium concentration gradient in renal tissue presented in table 1, can be explained on a similar basis. Contributions to the interstitial fluid ion concentrations made by the reabsorption of sodium from the tubular urine should be closely paralleled by tubular urine

calcium reabsorption. As far as can be determined, the same areas of the nephron are responsible for sodium and calcium reabsorption (Wesson and Lauler, 1959, Globus et al., 1959, Vander, 1961, Grollman et al., 1962). Normal calcium reabsorption rates of approximately 99% of the filtered load have been reported (Chen and Neuman, 1955). These closely parallel the sodium reabsorption fraction (Poulos, 1957). These observations would lead one to anticipate interstitial sodium to calcium ratios approximately equal to the serum sodium to calcium ratio. These factors could, of course, explain the highly significant correlation of tissue sodium and calcium which was reported earlier.

Reductions in calcium and sodium concentration gradients which accompany any of the calcium metabolism alterations induced in this study lack the firm base for their explanation that the correlation of tissue sodium and calcium concentrations enjoyed. Nevertheless a largely speculative explanation of the phenomenon can be given.

The currently favored theory explaining urine formation assumes not only that a cortical to papillary osmotic gradient exists, but that urine is isotonic with the surrounding tissue fluids except for the contents of the ascending limb of the loop of Henle. Throughout this segment ions are removed from the urine by the tubular cells and the urine reaches the cortex in a markedly hypotonic state (Gottschalk and Mylle, 1958). At this point the urine reaches tubule segments which are freely permeable to water, and osmotic equilibrium is re-established. It is necessary to extend the accepted theory and hypothesize the existence of cortical interstitial fluid somewhat hypotonic to plasma. This should result from reab-

sorption of large amounts of water from the hypotonic tubular urine presented to it. If this is the case, then the reduction in renal sodium and calcium concentration gradients by an increase in cortical sodium and calcium concentrations and a depression in papillary sodium and calcium ion concentrations, can be accomplished by any factor which reduces the glomerular filtration rate (GFR). A reduction in filtrate formation will reduce the amount of sodium and calcium reabsorption, and if the rate of capillary reabsorption of renal interstitial fluid is unaltered, the concentration of sodium and calcium in the papilla must fall. At the same time, a reduction in the amount of tubular urine delivered to the cortex will reduce the amount of water reabsorbed, and thus reduce dilution of cortical interstitial fluid. Cortical sodium and calcium ion concentration equilibria will thus be reached at lower levels than normal.

From this theory one would predict a high correlation between tissue sodium and calcium concentration changes. The differences in concentration produced between experimental groups and controls for calcium were correlated with those which occurred for sodium. A correlation coefficient of 0.81 was calculated for the changes in papillary sodium and calcium concentration between the eight experimental groups and their controls. This value is significant at the .05 level. The correlation coefficients for cortical and medullary sodium and calcium concentration changes were not significant however.

The calcified lesion, because of its predominantly cortical

distribution, undoubtedly contributes significant amounts of calcium to the cortical homogenate, while contributing progressively less to the medullary and papillary homogenates. This concept is reinforced by the lack of a demonstrable correlation between sodium and calcium concentrations changes experimentally induced in the cortex and medulla, and also by the occasional elevation of cortical calcium above that in the medulla (Table 1, Groups 4, 5, 7). Although the tissue homogenate calcium concentration increases could be explained by this mechanism alone, such a theory could not account for the concurrent rise in cortical sodium concentration. From the results of this study it is impossible to assess the relative contributions of the calcified cortical lesion, and of the reduction in formation of hypotonic cortical interstitial fluid to the apparent rise in cortical homogenate calcium concentrations. Nevertheless, since neither explanation is adequate alone, both must contribute to the observed cortical calcium elevation.

No indications of the factors leading to the depressed GFR can be derived from the findings of this study, but the occurrence of the phenomenon has been reported in animals given calcium salts (Wallach and Carter, 1959, Poulas, 1957), and PTE (Epstein et al., 1959), as well as in patients suffering from parathyroid adenomas (Edvall, 1958, Dorhout, 1957, Cohen et al., 1957).

The marked reduction in urinary calcium excretion, as shown in table 5 and figures 14-20, can also be best explained by a reduction in the GFR. Such a reduction in the absence of other changes will result in a larger intratubular dwell time for the urine, and

thus a greater extraction of calcium by the tubular cells. The decrease in the filtered calcium load which accompanies a drop in the GFR will contribute still further to the reduction in urinary calcium excretion. Although such an explanation is speculative, it fits the experimental findings more closely than do alternative explanations. A reduction in the filterable calcium for all of the experimental groups could explain the results we see. However, Canary and Kyle (1959) have shown a sharp increase in filterable calcium results from the administration of PTE. An increase in the calcium reabsorptive ability of the tubular cells could also explain the decrease in calcium excretion, but could not simultaneously explain the reduction in interstitial calcium concentrations.

SUMMARY AND CONCLUSIONS

1. No basis could be found for distinguishing lesions of PTE excess from those of calcium excess or from those resulting from a restricted calcium diet. In all cases the lesions were located primarily in the cortex, with heavily calcified tubules lying beside others that appear normal. Distinct calcified cytoplasmic granules and calcification of tubule cell margins, that could be interpreted as basement membrane calcification, were also universally seen. Previously unreported calcified nuclear inclusions were also seen in all groups studied, although the significance of these changes cannot be determined from this study.

2. Analysis of the sodium and calcium concentrations in renal cortical, medullary and papillary homogenates revealed an elevation of both ions in the cortex and a depression of both in the papilla of animals maintained on any of the three experimental treatments, i.e., calcium salt, PTE or a calcium restricted diet. This high papillary sodium concentration is generally thought to be developed and maintained by active tubular reabsorption of sodium from the tubular urine in conjunction with a high degree of water impermeability exhibited by the ascending limb of the loop of Henle. The calcium gradient shown in this study can be explained by a similar mechanism, i.e., active calcium reabsorption from the tubular contents. This theory is reinforced by significant correlation coefficients derived from sodium and calcium relations throughout the organ and from the sodium and calcium changes that occur under

the various treatments.

3. Cortical sodium and calcium increases associated with papillary sodium and calcium decreases were observed in all of the experimental groups. On the basis of the results in this study it is hypothesized that the above phenomenon resulted from a reduction in glomerular filtration rate which occurred in an unspecified manner from PTE, calcium salt or calcium free diet treatment. The maintenance of a concentrated papillary interstitial fluid and a hypothesized hypotonic cortical interstitial fluid depends upon an adequate flow of salts for reabsorption in the papilla and water for reabsorption in the cortex. Furthermore, the papillary sodium concentration decreases can be equated with a reduction in renal concentrating ability, yet no diuresis resulted in this study. A reduction in GFR readily explains this phenomenon.

4. Significant reductions in urinary calcium excretion occurred in six of the seven groups studied. These groups were comprised of PTE treated, dietary treated and calcium salt treated animals. This, too, is explained by the inferred reduction in GFR. Under such conditions the filtered calcium load is not only decreased, but the concommittant increase in intratubular urine dwell time allows for more complete ion reabsorption.

5. The renal calcification resulting from PTE or calcium salt treatment is frequently reported to be metastatic. It is implied that this is due to increases in calcium concentration in the fluids bathing the tubular cells. The calcium increase must,

then, be either in tubular urine or in interstitial fluid. Indirect measures of the interstitial fluid calcium concentration change associated with calcium restricted diet, calcium salt or PTE treatment, indicate that calcium concentration increases do occur in the cortex. However a sharp reduction in papillary calcium concentration is a consistant finding following any of these treatments and lesion formation occurs here also.

If the lesion induced in these cases is of metastatic origin, based on increased tubular urine calcium concentration, that concentration rise must be seen in the effluent urine since lesions occur in the papillary segments of the collecting duct. The calcium concentration of the effluent urine was found to be either decreased or unchanged for animals on any of the three treatments studied. These findings strongly imply that metastatic calcification, based upon elevations of interstitial fluid or tubular urine calcium concentration increases, is not involved in the formation of these lesions.

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