Relation between renal calcium content and renal impairment in 246 human renal biopsies

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Relation between renal calcium content and renal impairment in 246 human renal biopsies. Tissue calcium content from 246 diagnostic human renal biopsies was measured to assess whether elevated tissue calcium concentration could be demonstrated to exist early during the course of human renal disease or was only a manifestation of advanced renal impairment. Renal calcium content correlated significantly with serum creatinine ($r = +0.23$, $P < 0.001$, $N = 246$); serum phosphate ($r = +0.27$, $P < 0.001$, $N = 169$) but not with serum calcium ($r = -0.10$, $P > 0.1$, $N = 193$). Fivefold greater calcium content was measured in biopsied patients with normal renal function than in normal postmortem renal tissue (35.7 ± 5.2 vs. 7.6 ± 0.7 mgCa/100 g wet renal tissue, $P < 0.001$). Those biopsied patients with significant functional impairment (SCr > 1.5 mg/dl) had a higher mean level of serum phosphorus and serum [Ca] × [P] product than patients with normal renal function (5.19 ± 0.22 vs. 3.92 ± 0.11 mg P/dl and 44.8 ± 1.8 vs. 35.7 ± 1.2 mg2/dl2, respectively), and slightly higher renal calcium content (85.3 ± 32.2 vs. 35.7 ± 5.2 Ca/100 g wet renal tissue, $P = 0.06$), which correlated with histologic calcium deposition ($r = +0.52$, $P < 0.02$, $N = 20$). These findings are consistent with the hypothesis that renal calcium deposition begins early in the course of a variety of renal diseases and hence may play a secondary pathogenetic role that accelerates progression to chronic renal failure. Severity of renal calcium deposition is equally closely related to hyperphosphatemia and to the level of renal impairment.

Nephrocalcinosis with ensuing chronic interstitial inflammation may accelerate deterioration in renal function and progression of renal disease in humans [1–3]. Calcium deposits have been well documented in kidneys and other tissues of uremic patients [4, 5], particularly in conditions such as hyperparathyroidism [6, 7], vitamin D intoxication [8] and milk alkali syndrome [9]. In experimental uremia progressive functional renal impairment can be easily induced by increased dietary phosphate [10], administration of PTH [11, 12] or vitamin D [12], parenteral loading of phosphate [13] or calcium [14], and reduction in the amount of functional renal mass [15]. The common histopathologic feature in these experiments is the development of nephrocalcinosis with chronic interstitial inflammation and scarring. There is resulting functional impairment and ultimately, death of the animal follows. This pattern of events and morphologic alterations was first demonstrated in phosphate induced nephrotoxicity by MacKay and Oliver [16].

Data relating to calcium deposition have been unavailable in humans early in the course of renal failure when serum creatinine is still within normal range. In animals with nephrocalcinosis produced by the infusion of phosphate [13], calcium or PTH [17], however, this deposition has been found to be an early event which not only worsens as renal impairment progresses but correlates with the degree of functional renal deterioration.

Ibels et al [18] recently reported that the calcium content of kidneys from patients with ESRD is eightfold greater than that found in normal kidneys, a finding consistent with a pathogenic role for nephrocalcinosis as a contributor to the progression of renal failure. It is still not known whether abnormal calcium deposition occurs early in human renal disease, and if so, whether it is possible to prevent or reduce such early deposition. We have addressed the first of these issues by examining the calcium content of renal tissue obtained at diagnostic biopsy from patients with a wide range of severity of renal impairment. The present study is focused particularly upon three questions. 1.) Can a correlation be demonstrated between renal calcium content and the degree of functional impairment? 2.) Is the process of calcium deposition an early or late event in the natural history of progressive renal disease? 3.) Can hyperphosphatemia be related to increased deposition of calcium and thus possibly incriminated as a determinant of renal calcification in human renal disease?

Methods

Kidney tissue was obtained from 246 patients who underwent either diagnostic renal biopsy for a variety of renal diseases or nephrectomy at the Johns Hopkins Hospital between 1976 and 1980. The specimens were quickly frozen in liquid nitrogen and kept at −70°C after first being embedded in O.C.T., a commercial mixture prepared by Miles Laboratories (Elkhart, Indiana, USA) which contains distilled water, carbowax, and dimethyl-benzoyl–ammonium chloride which prevented dehydration. Further analysis by atomic absorption spectrophotometry of this fixative in our laboratory did not show evidence of calcium within its components. Tissues for controls were obtained from autopsied patients who died from unrelated causes, had no history of renal disease, hypertension or urinary abnormalities during life. These patients exhibited no clinical or microscopic evidence of diseased kidneys and at time of autopsy their kidneys had a normal appearance; they resembled the biopsied patients otherwise (Table 1).

Table 1. Demographic data for patients grouped by serum creatinine levels

	N	Age yrs	Body wt kg	Sex M/F	Race B/W	SCr mg/dl
Patients SCr < 1.5	122	35.3 + 19.5	61.6 + 26.7	49/73	35/68[a]	0.99 + 0.28
Patients SCr > 1.5	124	40.5 + 19.2	61.0 + 19.3	75/48	21/76[a]	7.66 + 6.06
Controls	19	34.5 + 21.8	65.2 + 18.9	14/5	6/13	1.03 + 0.28

All values are mean + SD.
[a] There were two orientals in each group.
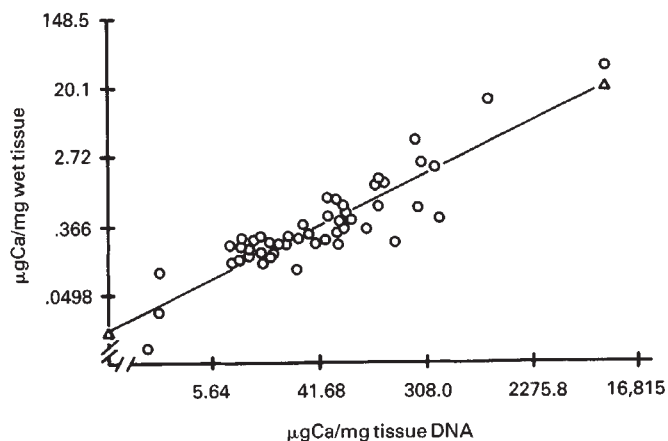
Fig. 1. *Correlation between two methods of expressing renal Ca content.* The scales of both axes in this graph are logarithmic. A spuriously high weight caused by contamination with the preservation media used for freezing should have yielded a negative intercept for the linear regression equation relating the two methods of expressing the tissue calcium content. In fact, the intercept as shown in the equation is +0.36 (±0.185); thus measurement of calcium in frozen needle biopsy specimens and expression/unit wet weight of tissue provides a satisfactory estimate of calcium content.

Table 2. Disease categories for the study group

Histological diagnosis	N
Glomerular diseases	133
Interstitial diseases	31
Vascular diseases	16
End–stage kidneys	27
Miscellaneous	39
'Normals'[a]	19

[a] Postmortem material

Each sample was thawed immediately before assay and weighed individually on a model B5 Mettler high precision analytic balance, (Mettler Instrument Corporation, Highstown, New Jersey, USA) and subsequently placed into a small pyrex assay tube (which had been previously rinsed with diluted HCl) and digested with 0.1 cc of a 12 N solution of HCl under heat to total dissolution. Calcium determinations were made on the acid digests [19], using a model 251 atomic absorption spectrophotometer from Instrumentation Laboratories (Lexington, Massachusetts, USA). Values were expressed as mg calcium/100 g of wet kidney tissue. To check for accuracy and screen for a potential source of variability (particularly for falsely high weight contributed by the O.C.T. fluid), 57 of these

samples were also simultaneously assayed for tissue DNA content [20]. Tissue calcium content was then re-expressed as μg calcium/mg DNA and the results of the two methods compared, $(r = +0.91, P < .001, \mu$gCa/mg tissue = 0.36 + 0.004 × μgCa/mg DNA). Thus the content of O.C.T. in the samples had a negligible influence upon the determination of tissue calcium content expressed on the basis of wet tissue weight (Fig. 1).

Serum creatinine determinations which provided an index of renal function were performed following the method of Jaffe [21]; serum calcium levels were measured following a method described elsewhere [19] as were serum phosphate levels [22].

Statistical analyses included Wilcoxon's two group rank sum test, Pearson's product moment correlation coefficient and Spearman's rank correlation coefficient where appropriate.

Results

The renal biopsies subjected to this analysis represented a variety of different disease categories (see Table 2). The miscellaneous category includes five with essential hematuria, five with tumors elsewhere in the kidney, eight with polycystic kidney disease, two with cortical necrosis, four with hereditary nephritis, four with hydronephrotic kidneys, six transplant biopsies, one from a patient with sickle cell anemia, one from a patient with a renal stone and three samples which had medullary tissue only.

The complete data set was examined for correlations between renal tissue calcium, serum creatinine, serum calcium, serum phosphate and serum [Ca] × [P] product. Age as a possible confounding variable was evaluated since renal function is known to decline with age [23]. Kidney calcium content correlated with serum creatinine $(r = +0.23, P < .001, N = 246$, Fig. 2), with the level of serum phosphate $(r = +0.27, P < 0.001, N = 169)$ and with serum [Ca] × [P] product $(r = +0.22, P = 0.005; N = 165)$, but not with serum calcium levels $(r = -0.10, P > 0.1, N = 193$, Table 3). Moreover, mean serum levels of phosphate were significantly elevated among those patients who had a serum creatinine of >1.5 mg/dl when compared with a normal (<1.5 mg/dl) serum creatinine, (phosphate levels were 5.2 mg/dl and 3.9 mg/dl, respectively, $P < 0.001$). On the other hand, mean serum calcium levels did not differ between these two groups, $(P = 0.07$, Table 4). Elevation of serum [Ca] × [P] product, found also to be significantly different for these two groups, $(P < 0.001$, Table 4), was due to an elevation in the serum phosphate levels. Furthermore, serum phosphate was strongly correlated with serum creatinine $(r = +0.48, P < 0.001, N = 246)$ (Table 3) indicating that as renal function deteriorates there is a progressive impairment in phosphate
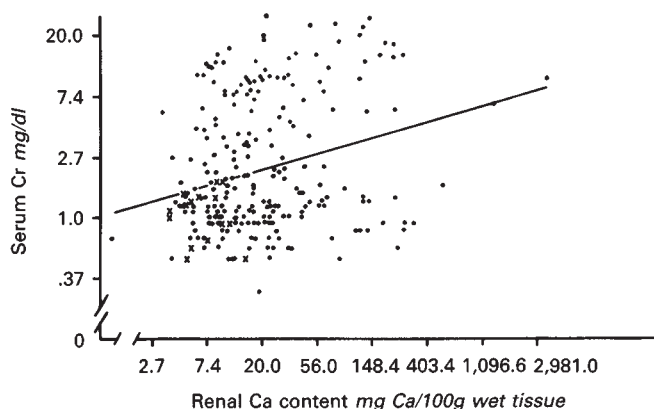
Fig. 2. *Linear regression analysis between renal calcium content and serum creatinine for all tissues analyzed.* Patients are depicted by (●) and controls by (×). The correlation coefficient is +0.27 ($P < 0.001$; N = 265). The linear equation is SCr = 1.029 + 1.288 × tissue calcium.

Table 3. Bivariate correlation coefficients between selected parameters from 246 patients with renal disease

	TCa	SCa	SPO$_4$	Age
SCr	+0.23[a]	−0.09	+0.48[a]	+0.11
TCa		−0.10	+0.27[a]	+0.01
SCa			−0.09	−0.18
SPO^4				−0.13

r values are Pearson's correlation coefficient
[a] $P < 0.001$

excretion with secondary elevation of the serum [Ca] × [P] product.

When multiple linear regression analysis (mult r), with renal tissue calcium as the dependent variable, was calculated for serum creatinine, serum phosphate and serum [Ca] × [P] product as independent variables, the relationship remained a significant one, (mult r = 0.34, $P < 0.001$). Partial correlation coefficients were significant for serum creatinine, ($r = +0.25, P = 0.001$) and serum [Ca] × [P] product ($r = +0.22, P = 0.005$) but not for serum calcium ($r = −0.06, P = 0.5$). The highly significant bivariate correlation between serum PO$_4$ and tissue Ca ($r = +0.3, P < 0.001$), is reduced to marginal significance as a partial correlation when creatinine is controlled ($r = +0.15, P = 0.05$), a reflection of the fact that the elevation in serum phosphate is in itself highly dependent on the level of renal function ($r = +0.48, P < 0.001$, Table 3).

Further testing was done by comparing data from patients whose serum creatinine levels were <1.5 mg/dl at time of biopsy, (that is, patients with histologic evidence of renal disease but presumably preserved renal function), and those with serum creatinine levels of >1.5 mg/dl, (patients with functionally as well as anatomically impaired kidneys). Significant differences were demonstrated in serum phosphate ($P < 0.001$) and serum [Ca] × [P] product ($P < 0.001$); tissue calcium content was higher in the group with renal impairment but not significantly so. Both groups, however, did have significantly higher amounts of tissue calcium than did the normals ($P < 0.001$ for both groups, Fig. 3). This observation indicates that the calcium content increases in histologically diseased kidneys even before significant deterioration in renal function occurs.

Neither serum phosphate, tissue calcium, serum calcium or serum [Ca] × [P] product correlated with age, thereby excluding a potentially confounding influence. Moreover, we were unable to show in the population at study a relationship between serum creatinine and age (Table 3) in the biopsy group.

Histologic findings

Histologic sections stained with von Kossa stain for phosphate were examined in 42 randomly selected cases who had a tissue calcium of 30 mg/100 g tissue or higher. These were examined under polarized light (to detect oxalate) and graded 0 to 3+ for calcium phosphate or calcium oxalate deposition by one of us (K.S.). Twenty of the 42 cases had calcium deposits detected histologically. In these 20 cases, there was a significant correlation by the Spearman rank test between histologic grade and tissue calcium content ($r = 0.52, P < 0.02$; Fig. 4). Cases with predominantly calcium oxalate deposition tended to have a lower level of tissue calcium (93 ± 31 mg/100 g, range 35 to 225) compared with cases with predominantly calcium phosphate deposition (471 ± 268 mg/100 g, range 30 to 3721). The calcification observed histologically was not simply a dystrophic phenomenon in heavily scarred tissue but frequently occurred in areas of relatively intact renal parenchyma. Large crystals were seen in tubular lumina and occasional capillaries, as well as free in the interstitium. Finely–granular von Kossa–positive material was also seen in the interstitium in occasional cases with high tissue calcium content (Fig. 5). The mean tissue calcium content tended to be lower in cases with predominantly intratubular calcium deposition (128 ± 46 mg/100 g, range 33 to 542) as opposed to those cases with predominantly–interstitial calcium deposits (254 ± 162 mg/100 g, range 38 to 3721; all values: mean ± SEM). Because of the large scatter, this difference was not statistically significant.

Discussion

These data indicate that at least two conditions are associated with increased calcium deposition in diseased renal tissue in humans: hyperphosphatemia and an elevated serum [Ca] × [P] product. The hyperphosphatemia results from failure in the excretion of the dietary phosphate load by the diseased kidney [24, 25] and almost certainly leads to persistent hyperparathyroidism by decreasing serum ionized calcium [26], resulting in mobilization of calcium from bone. The elevated serum [Ca] × [P] product, which is characteristic of the uremic state, exceeds the solubility product of this ion pair, favoring metastatic calcification [27, 28]. Excess PTH, almost universally present in chronic renal failure [29, 30], seems necessary for the development of nephrocalcinosis. PTH levels were not measured in the present study, but the observation of abnormally low serum calcium (Group I: 8.98 ± 0.90; Group II: 8.76 ± 0.97 mg/dl; both values mean ± SD) and elevated serum phosphate levels in those patients with and without renal functional impairment in the present study, represent findings consistent with elevated serum PTH [30], as well as impaired vitamin D metabolism [31]. This relationship between the serum levels of phosphate and serum levels of PTH has been well–documented in chronic renal failure [30], and the present findings suggest that this probably occurs quite early during the course of

**Table 4.** Comparison of data from biopsy material in patients with normal and impaired renal function

Variable	Controls	P value[a]	Group I SCr < 1.5 mg/dl	P value[a]	Group II SCr > 1.5 mg/dl
Serum cr. *mg/dl*	1.03 ± 0.07 N = 19	NS	0.99 ± 0.02 N = 122	< 0.001	7.66 ± 0.54 N = 124
Tiss. Ca *mg/100 g tiss*	7.6 ± 0.66 N = 19	< 0.001	35.7 ± 5.2 N = 122	0.06	85.3 ± 32.3 N = 124
Serum Ca *mg/dl*	9.02 ± 0.33 N = 13	NS	8.98 ± 0.09 N = 95	0.07	8.76 ± 0.10 N = 98
Serum PO$_4$ *mg/dl*	3.44 ± 0.29 N = 12	NS	3.92 ± 0.11 N = 81	< 0.001	5.19 ± 0.22 N = 88
Serum CaxP *mg^2/dl^2*	30.3 ± 2.5 N = 12	NS	35.7 ± 1.2 N = 80	< 0.001	44.8 ± 1.8 N = 85

All values are mean $\pm$ SEM
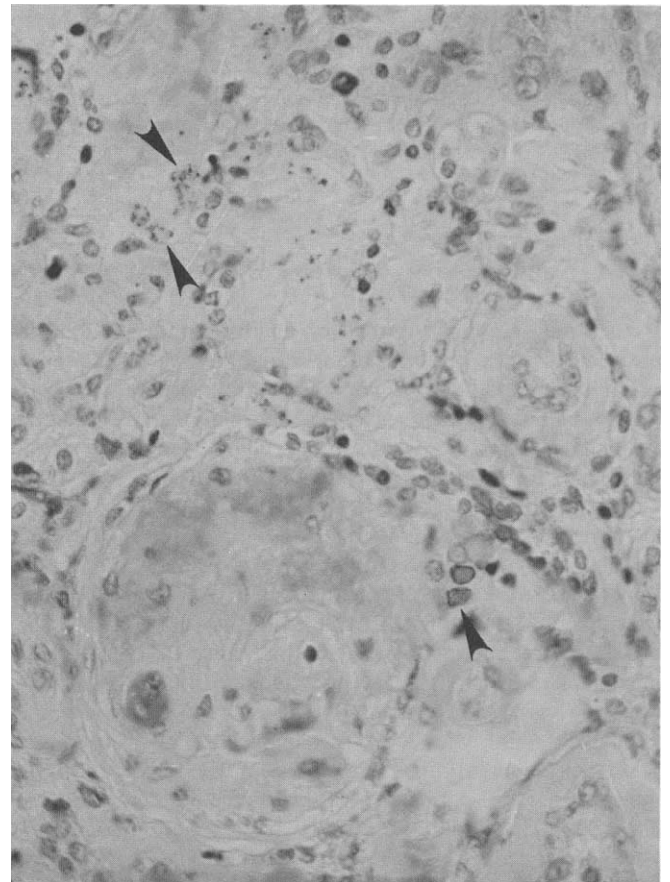[a] Wilcoxon's non-parametric rank sum test for unpaired data
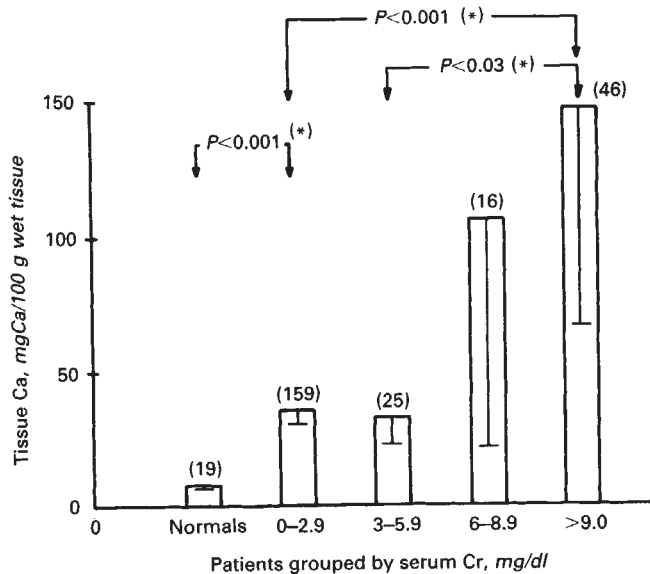
Fig. 3. *Comparison of calcium content measured in biopsy specimens from patients with renal impairment of increasing severity and post-mortem renal tissue obtained by needle biopsy from subjects who had no clinical or laboratory evidence of renal disease and had normal urinalyses.* Wilcoxon's non-parametric rank sum test (*) was used for statistical evaluation of the data because calcium values in the diseased kidneys were not normally distributed. The numbers in parenthesis at the top of each bar represent sample size for each group.
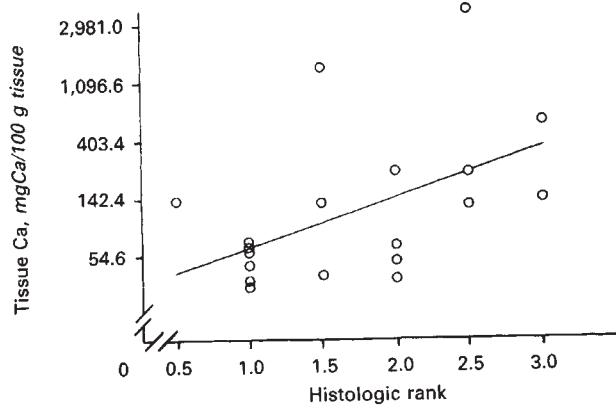
Fig. 4. *The linear regression between histologic rank and quantified tissue calcium for a group of 20 biopsies.* Spearman rank correlation of the same data was also significant ($r = +0.52$; $P < 0.02$).

Fig. 5. *Von Kossa stain for calcium phosphate showing spherical crystals (right lower arrow) and small punctate calcific deposits (top left arrows), in a nephrectomy specimen from a patient who had end–stage diabetic glomerulosclerosis with a calcium content of 1,377 mg/100 g wet tissue calcium content* ($\times 400$).

disease, before serum creatinine measurements provide a reliable estimate of impairment of glomerular filtration rate.

This relation between tissue calcium and renal function is evident upon examination of the kidney calcium content from patients with histologically impaired kidneys but serum creatinines within the normal range (Group I), and patients with both functional and histopathologically impaired kidneys (Group II Fig. 2). The significant positive correlation for tissue calcium content and serum creatinine for all patients studied represents further evidence that these two variables may be causally related.

This progressive accumulation of calcium in kidneys whose function declines with time is most closely related to and is probably determined by the increased concentration of serum inorganic phosphate which results from progressive decrease in

renal function [32]. The associated increase in serum [Ca] × [P] product may well be the proximate cause of this increased calcium deposition, but the elevated serum phosphate seems to be the most important factor in initiating the sequence necessary for the development of nephrocalcinosis. Serum calcium levels did not differ between patients with and without impaired renal function.

Acute or rapidly developing hyperphosphatemia and the development of severe renal functional impairment in isolated cases has been reported with [33, 34], and without [35] simultaneous development of soft tissue calcification. In two reports [34] the temporal relationship between development of hyperphosphatemia and rise in serum creatinine have been clearly documented; furthermore, in one of the cases [36] when hyperphosphatemia was corrected, renal function improved substantially, although its failure to return to normal indicates that some of the changes produced by severe hyperphosphatemia may indeed be irreversible.

Increased calcium deposition and the accompanying interstitial inflammation accelerate the loss of renal function in animal models of uremia [10], and rigid restriction of dietary phosphate preserves renal function with increased survival [15, 37]. Similarly, our previous studies [38] and others [39] have shown that agents which prevent nephrocalcinosis are also effective in preserving renal function despite increased dietary phosphate intake.

This nephrocalcinosis and associated renal damage may also be reversible in patients with end–stage renal disease as noted by Walser, Mitch and Collier [2, 3].

The alternate hypothesis, that calcium deposition may occur as a secondary event in previously scarred tissue and, therefore, not be a direct contributor to further functional deterioration can be excluded by what we have observed in the histologic examination of renal tissue of these patients. Calcium deposits were frequently found in areas of relatively–intact renal parenchyma, in the form of discrete intratubular or interstitial calcification.

Maschio and colleagues [40] reported recently that by restricting dietary phosphorus by 28% of average normal dietary intake and limiting protein intake, in a group of patients with mild renal failure (mean serum creatinine of 2.2 mg/dl), the average rate of decline in renal function was substantially reduced. This reduction was associated with lower levels of serum phosphate.

Our findings extend the initial report of Ibels and colleagues [18], and demonstrate that the calcium content from kidneys with different types of renal disease is significantly higher than that seen in normal kidneys. The findings here indicate that increased calcium deposition begins early in the course of renal disease and can be demonstrated in patients with serum creatinine of <1.5 mg/dl, who have histologic evidence of renal disease. Values for tissue calcium in such patients exceeded that for normal kidneys by more than fourfold. Furthermore, this process seems to be quite dependent on the level of serum phosphate. We interpret these findings to indicate that hyperphosphatemia occurs quite early in renal impairment before significant decline in GFR can be documented by monitoring serum creatinine. It seems most likely that the hyperphosphatemia is transient and recurrent and may occur only after meals,

since fasting phosphates are usually normal in patients with serum creatinines less than 1.5 mg/dl.

Barsotti et al [41] provided evidence that at least part of the benefit of protein restriction could be attributed to reduction in phosphate intake. Although the decrement in nitrogenous dietary intake exerted a beneficial effect, this effect was significantly increased when the dietary phosphorus was also simultaneously restricted by 50%. Our results support their finding of a significant correlation between the level of renal function and serum phosphate concentrations, and we have demonstrated that this also correlated with tissue calcium deposition. Further corroboration of the relation between phosphate intake and renal damage comes from studies in animals with 5/6 reduction of renal mass fed a diet with normal phosphate content [42].

Within 48 to 72 hours of phosphate loading or PTH infusion in rats [17], there are ultrastructural cellular changes in proximal tubular mitochondria, not visible by light microscopy, that appear consistent with apatite deposition, resulting in extensive disruption of normal mitochrondrial structure. These ultrastructural changes, demonstrable within two days after phosphate loading, were still almost undetectable by light microscopy after ten days despite a renal calcium content six times higher than controls [13]. Experimental models of acute renal failure induced by gentamicin, $HgCl_2$ and ischemia [43, 44], have exhibited an early significant increase in the amount of intramitochondrial calcium accumulation. This process correlates with the degree of functional renal impairment [43], suggesting a possible role of calcium in renal cell death. It is known that intramitochondrial calcium accumulation interferes with oxidative phosphorylation [45] and early mitochondrial calcium accumulation has been demonstrated in several experimental models of renal damage.

In clinical renal disease, phosphate may have an etiological role per se in the intracellular accumulation of calcium with calcium phosphate deposition in a sequence analogous to that demonstrated in cultured kidney cells, where phosphate produces an increase in the intracellular calcium pool, probably mostly in the mitochondria [46]. The present study documents an association between progressively–impaired renal function, renal calcium content and serum phosphate in biopsy material from a wide variety of renal diseases. It seems likely that these findings are linked to the early alterations in intracellular calcium and mitochondrial damage described above. This suggests that calcium deposition may be an extremely–important secondary factor in determining the rate of damage seen in many renal diseases. The relation of this progressive increase in abnormal calcium deposition within the kidney to the recently–demonstrated adverse effects of hyperperfusion of the glomeruli in progressive renal impairment [47–49] remains to be determined.

The strong association between this increased tissue calcium and serum phosphate that can be demonstrated quite early in the course of renal disease strongly incriminates dietary phosphate as an important factor in determining rate of renal function decline, and forces attention upon dietary phosphate restriction as an early therapeutic intervention that may be of considerable benefit [50]. At the least, the hypothesis should be examined critically.

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