

A Study of Vertebral Bone Powder from Patients with Chronic Renal Failure

MICHAEL KAYE, A. J. FRUEH, and M. SILVERMAN with the technical assistance of MRS. J. HENDERSON and MRS. T. THIBAUT

From The Renal Service, Department of Medicine, Queen Mary Veterans' Hospital until 1966, and thereafter from the Division of Nephrology, Department of Medicine, The Montreal General Hospital, Quebec, Canada

ABSTRACT Bone powder from patients dying with chronic renal failure of more than 1 yr duration was shown to release less calcium and more phosphate when equilibrated with a buffer solution, pH 7.4 at 4°C. This change persisted after removal of the organic component and was associated with a reduction in the bone carbonate content. Crystal size and surface area showed no consistent changes from the controls and it was concluded that an alteration in the apatite crystal composition had occurred in long-standing uremia with carbonate-phosphate interchange. Support for this was provided by synthesis of apatites which were carbonate deficient and behaved in a similar manner to the uremic bones.

INTRODUCTION

With the development of renal excretory failure and the syndrome of uremia, multiple alterations in the chemical composition of the extracellular fluid occur. While these are many and varied in type, an almost uniform finding is the development of metabolic acidosis with a fall in the level of plasma bicarbonate due to hydrogen ion retention, together with an increase in the level of serum inorganic phosphorus.

It would be surprising if the changes taking place in the chemical composition of the plasma in uremia were not reflected in bone which is being formed at the time, because the ionic composition at the calcification front must be in part a reflection of the same fluid analyzed by venipuncture. While many bone changes have been described in uremics, these have generally involved the failure of calcification of new bone (osteomalacia) or excessive resorption of deposited bone (osteitis fibrosa)

Dr. Frueh's address is The Department of Geological Sciences, McGill University, Montreal, Quebec, Canada.

Received for publication 20 May 1969 and in revised form 15 September 1969.

and refer to morphological changes related to structure. The bone salt itself has been considered normal in type (1). Recent evidence that bone is an important buffer source for hydrogen ion neutralization in uremia (2) has led to the suggestion that bone consists of a composite salt with one component being calcium carbonate which is available for exchange and buffering. In uremia, depletion of the carbonate fraction was found thus identifying an abnormality in the crystalline salt as distinct from any histopathological condition (3).

In a previous study (4) of "calcium (^{45}Ca) kinetics in chronic renal failure in patients who had not yet reached the terminal stages of their disease, it was noted that the disappearance of tracer into bone tended to be slower than in healthy subjects. Furthermore, the serum calcium was often decreased even though the amount of calcium in the rapidly exchangeable pool was normal; and the calcium in the bone, as determined by subsequent vertebral analysis, might actually have been increased. It was decided, therefore, beginning in 1964, to investigate the exchange rate of bone in uremia in vitro where the numerous other variables present in the living subjects could either be eliminated or controlled. This paper describes our findings from that time until the present.

METHODS

Dried and defatted bone powder from the lumbar vertebra of patients who had died from uremia and bone from control subjects who had died from causes other than renal, neoplastic, or wasting diseases, were used in this study. Many of the samples had been analyzed for a previous paper (5) and had been prepared at that time. The mean age of the control group was 51 yr. The uremics had been known to have renal failure for at least 1 yr (blood urea nitrogen (BUN) > 25 mg/100 ml), except where stated in Table VII. None had been treated by either dialysis or transplantation. A summary of relevant clinical details about each patient is given in Table I.

TABLE I
Clinical Data on Uremics

Name	Diagnosis	Age	Duration* renal disease	Duration* azo- temia	Biochemistry before death				Alk. P' tase	Radio- logical changes	Bone histol- ogy	Para- thyroids hyper- plastic
					BUN	CO ₂	Ca	P				
			yr	yr	mg/ 100 ml	mEq	mg/ 100 ml	mg/ 100 ml	KA units			
I. D.	CGN	53	25	2	304	11	7	11.2	11.7	None	Normal	Yes
N. D.	DGS	30	4	1	140	13	9.5	—	—	None	Normal	No
	CPN											
R. D.	GGN	54	?	1.5	112	—	7.6	6.9	—	OS	OS, OF OP	No
J. G.	CPN	52	17	8	195	15	9.6	6.5	10.0	OS	OS, OF	No
E. J.	CPN	33	6	6	166	15	10.3	5.0	8	OS, OF	OS, OF	Yes
E. L.	CGN	45	14	5	206	12	5.5	8.6	8.5	None	OS	No
A. L.	CGN	42	4	2	134	12	8.3	8.8	—	None	Normal	No
W. L.	CPN	63	2	1	68	18	8.6	4.5	8	OS	OS, OF OM	No
J. L.	CPN	38	5	1	127	7.2	5.0	5.5	10	OS, OM OF	OS, OF	Yes
I. M.	CGN	31	12	1	268	8	4.8	13.2	11	OF	OM, OF	Yes
J. P.	CGN	54	2	2	248	12	6.3	6.4	—	None	OS, OF OM	Yes
Mean		43	9.1	2.7	179	12.3	7.5	7.7	9.6			

DGS = Diabetic glomerulosclerosis, CPN = Chronic pyelonephritis, CGN = chronic glomerulonephritis, OS = Osteosclerosis, OF = Osteitis fibrosa, OM = Osteomalacia, and OP = Osteoporosis.

* Indicates minimal duration.

The bones were cut into fragments, dried at 100°C for 16 hr, and defatted using a Soxhlet apparatus (Kimble Products, Owens-Illinois Inter-America Corp., Toledo, Ohio) with refluxing for 6 hr each with ethyl and petroleum ether. Thereafter the bone was ground using a Wiley mill (Arthur H. Thomas Co., Philadelphia) and passed through sieves of known sizes. 200 mg of the powder was incubated for varying times with 4 ml of either barbital or Tris buffer pH 7.4 at 4°C to inhibit bacterial growth in stoppered tubes which were constantly rotated. Particle sizes between 70 and 100 μ were used except where otherwise stated. At intervals, a tube was removed, centrifuged, and the supernatant analyzed for calcium or radioactivity using methods described previously (4). The initial ⁴⁵calcium (⁴⁵Ca) concentration in the solution was 1 μ g in 4 ml when ⁴⁵Ca was used and each tube contained 0.06–0.08 μ Ci of ⁴⁵Ca.

In the experiments where the bone was labeled with ⁴⁵Ca as a preliminary step, the initial incubation was for 6 days after which the bone powder was centrifuged and the supernatant discarded and washed once with buffer and then incubated as above with nonradioactive buffer.

Acid elution was carried out as described by Pellegrino and Biltz (3) using 1% ammonium chloride added to 100 mg of bone powder on a filter funnel. Successive 5 ml eluates were analyzed for calcium and phosphorus.

Carbonate was measured in a Warburg apparatus with all analyses being in duplicate (6). Synthetic apatite was prepared using a modification of the method described by Neuman and Mulryan (7). 100 ml of 0.1 M KH₂PO₄ and 0.16 M CaCl₂ were infused using a single Harvard pump and a double carriage into 500 ml of barbital buffer, pH 8. During the addition the pH was maintained constant by addition of 6 N KOH using a Metrohm Multidosimat (Metrohm Ltd., Herisau, Switzerland). All experiments were at room temperature and with free exposure to air. The precipi-

tate was washed with 7 liters of distilled water, dried overnight at 100°C, ground in a mortar, washed twice more each time with 1 liter of water, and again dried at 100°C. For the synthesis of carbonate containing apatite, the buffer contained NaHCO₃ varying in concentration from 0.03 to 0.1 M.

Pyrophosphate and other organic phosphates were estimated before and after hydrolysis. 1 ml of sample was heated in a boiling water bath for 10 min with 2 ml of 3 N H₂SO₄. Complete recovery of inorganic pyrophosphate was obtained with this method.

Calculations of fluxes assuming a two compartment system were made at three intervals of time. Their mathematical derivation is given in the Appendix.

X-ray powder diffraction patterns were obtained using both the Debye-Scherrer (Philips, Eindhoven, Holland) and the Guinier focussing cameras (Nonius, Delft, Holland). Quantitative measurements of crystal length parallel to the C axis were made by Stokes' technique employing the 002 reflection and a Phillips diffractometer.

Specific surface areas and nitrogen adsorption isotherms of both the dry defatted powdered samples and the same sample after glycerol ashing were determined with a volumetric gas adsorption apparatus previously described (8).

Fluoride content was measured on the dry fat-free bone of 100–250 μ particle size (9).¹

Experiments using the anorganic bone fraction were carried out in the same fashion after either ashing the powder at 400°C for 1 hr or by heating at 200°C with alkaline glycerol for 1.5 hr. Both procedures resulted in destruction of the organic fraction as determined by subsequent nitrogen analysis.

¹ We are indebted to Mr. P. A. Puxley, head of the Chemical Division, Aluminium Laboratories Limited, Arvida, Canada, for these analyses.

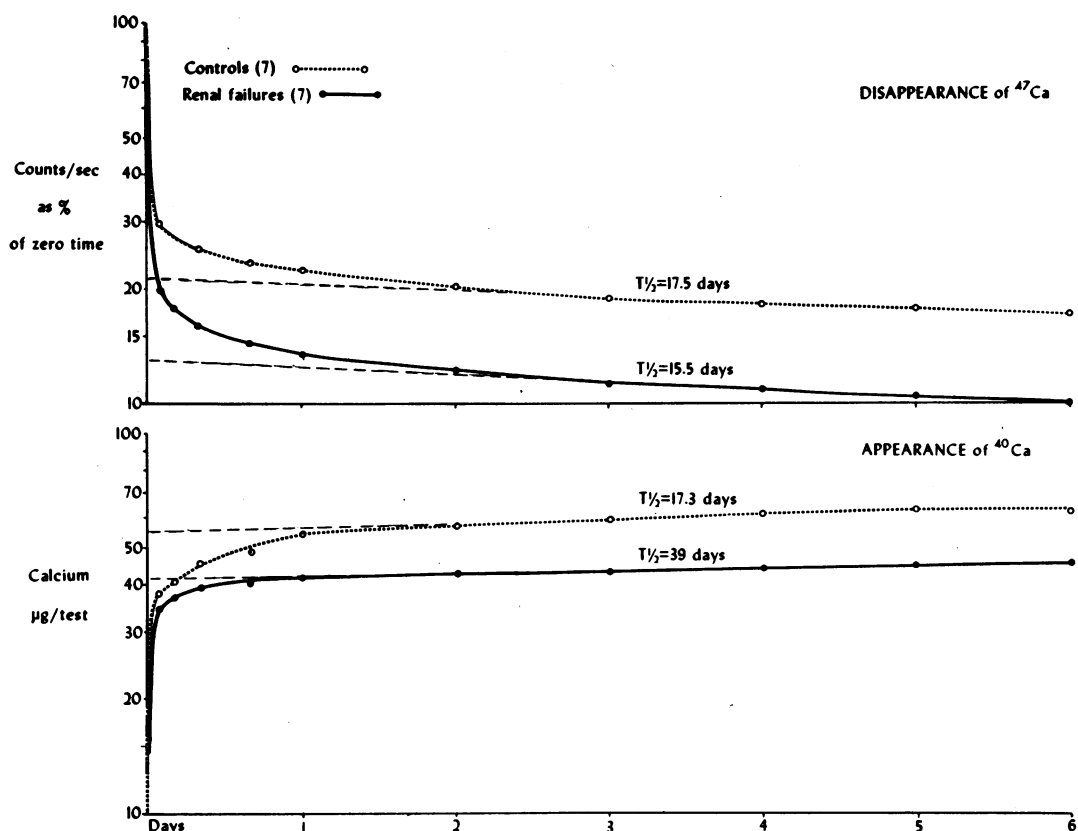


FIGURE 1 Mean values for in vitro calcium exchange between bone and medium. Disappearance of radioactive calcium from buffer solution in the upper half of the figure and appearance of stable calcium in the buffer in the lower half. Uremics, solid line; controls, stippled. SE of means for ^{47}Ca , 0.3-1.5; and for ^{40}Ca , 1.6-2.7. *P* values for differences between means are all less than 0.01 except for the ^{40}Ca appearance where the differences are not significant at 2 and 4 hr, and < 0.05 at 8 and 16 hr.

RESULTS

A. Whole bone powder

1. *Equilibration for varying periods with Tris buffer, pH 7.4.* Fig. 1 shows the values for seven patients with renal failure of between 1 and 4 yr duration and seven control bones. There is a more rapid disappearance of ^{47}Ca from the medium into the bone in the patients than in the controls, and a slower appearance of stable calcium

into the medium in the patients. Flux into and out of bone was calculated at 10, 50, and 100 hr and is shown in Table II where it can be seen that at each time the rate of movement from bone to medium is slower in the patients. This was not the case for the first 10 hr for the medium to bone movement where the flux is equal in the two groups but with increasing time the separation between them becomes more marked. The slower fluxes ($\text{SF}_{1\rightarrow 2}$ and $\text{SF}_{2\rightarrow 1}$) in the renal patients could be due

TABLE II
Calcium Flux $\mu\text{g/hr}$

	Time					
	10 hr		50 hr		100 hr	
	Bone to medium	Medium to bone	Bone to medium	Medium to bone	Bone to medium	Medium to bone
Controls (7)	1.301	0.697	0.260	0.125	0.148	0.114
Uremics (7)	1.049	0.743	0.132	0.091	0.098	0.082
<i>P</i>	<0.001	<0.6	<0.001	<0.02	<0.005	<0.005

TABLE III

⁴⁷Ca Added after 15 hr Incubation and Samples Analyzed after a Further 64 hr

Name	⁴⁰ Ca μg	⁴⁷ Ca %
Controls		
I. H.	60.4	22.9
B. A.	64.9	21.4
L. B.	70.4	25.7
N. H.	61.4	24.9
Mean	64.2	23.7
Expected mean from Fig. 1	59.8	20.3
Uremics		
J. P.	42.6	19.0
R. D.	44.2	17.4
N. D.	50.9	19.3
I. D.	51.9	18.2
Mean	47.4	18.5
Expected mean from Fig. 1	43.3	12.2

to either a smaller surface area available for exchange (see Appendix) or to a true decrease in the rate of movement of labeled and unlabeled calcium between the two compartments. As the surface areas in the patients tend to be larger than the controls (Table XI), a true diminution in calcium flux is present.

The difference between the two curves for ⁴⁷Ca is due to a more rapid disappearance by the time the first sample is counted at 2 hr, and there is subsequently no

TABLE IV

Total Calcium and Phosphate in Medium after Equilibration of 1. Dry Fat-Free Bone Powder, or 2. Dry, Fat- and Marrow-Free Powder with Barbitol Buffer for 24 hr

Group	Calcium μg/4 ml	Phosphorus μg/4 ml	Ca:P ratio
1. Controls n = 10			
Mean ±SE	66.1 ±2.5*	35.3 ±1.6	1.90 ±0.09*
Range	50-76	31.2-42.4	1.50-2.26
1. Uremics n = 9			
Mean ±SE	50.4 ±3.3*	40.1 ±3.6	1.35 ±0.17*
Range	38-67.4	21.2-54.2	0.84-2.49
2. Controls n = 3	64, 64, 63	24.5, 25.5, 25	2.61, 2.47, 2.56
Mean	64	25	2.54
2. Uremics n = 3	54, 54, 50	48, 31, 37.5	1.12, 1.74, 1.33
Mean	52	38.5	1.41

* $P < 0.01$.

change in the relative positions of each curve. At first sight this result appears discrepant. However, with the medium essentially calcium-free to begin with, there is less mass movement of stable calcium into the medium initially in the renal patients and this would facilitate diffusion of tracer into the bone from the medium within the first minutes of equilibration. To confirm this, four experiments were carried out in each group in which radioactive calcium was not added until 15 hr of incubation which was then continued, and the samples analyzed after a further 64 hr. The results are shown in Table III where it can be seen that there is

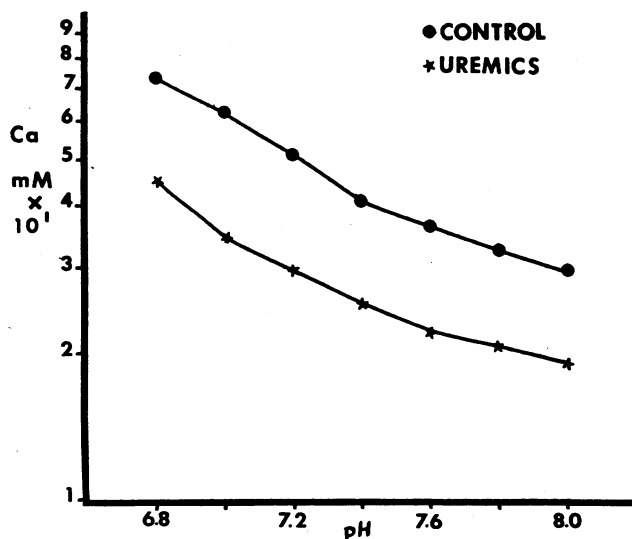


FIGURE 2 Showing less calcium released into medium throughout pH range 6.8-8.0 in the renal patients.

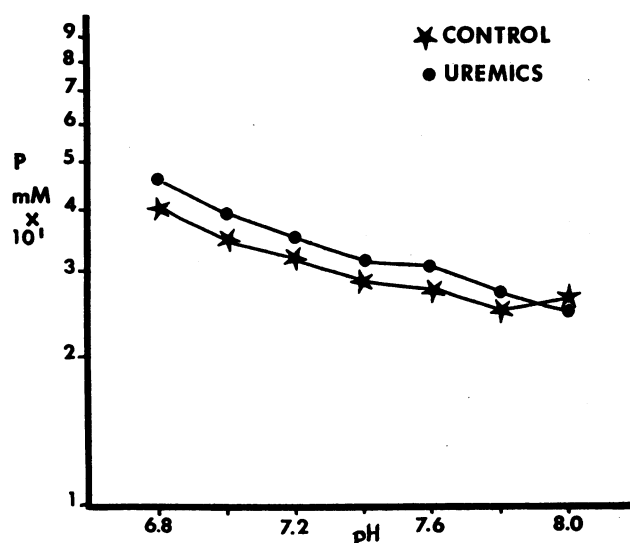


FIGURE 3 Except for pH 8, the controls release less phosphorus into the buffer solution over the pH range indicated.

now less difference between the ^{45}Ca results while the ^{45}Ca difference is unchanged.

2. *Equilibration for 24 hr with barbital buffer pH 7.4.* The results of a separate series of experiments using equilibration for 24 hr with barbital buffer and determining both calcium and phosphorus in the solution is shown in Table IV. In the first part of this work an increase in inorganic phosphorus content of vertebral bone was noted after renal failure had been present for at least 3 month (5). Because of the possible inclusion of blood and marrow elements high in phosphate, 0.5-cm slabs from the middle of the fourth lumbar vertebral body were prepared from patients and controls. Each slab was washed with a jet of cold water until all adhering material was removed, then dried, defatted, and ground as previously described. The results are shown in Table IV where it can be seen that the changes are similar to those seen when the bone is not treated specially to remove the marrow contents. It is

apparent that while the decreased release of calcium into the medium is a constant finding in the uremics, equally consistent is the greater amount of phosphate released into the buffer so that the Ca:P ratio is always higher in the normals as compared with the uremics. This presumably is a reflection of the change in the composition of the bone salt as the Ca:P ratio in the vertebral bone was observed to fall from 1.71 in the controls to 1.59–1.64 when renal failure had been present for 3 months or more (5).

3. *Equilibration at varying pH.* These experiments were carried out using barbital buffer varying in pH from 6.8 to 8.0 with a particle size of 100–250 μ , and rotation for 24 hr. As can be seen from Figs. 2 and 3 at every pH tested, the bones from the uremics released more phosphorus and less calcium than the controls. Each curve represents the mean of three controls and three patients. MacGregor and Brown have discussed the theoretical derivation of the stoichiometry of the reactants in such a system (10). Employing their following notation: $\log (\text{Ca}^{++}) (\text{OH}^-)^2 = -1/x \log (\text{H}^+)^3 (\text{PO}_4)^4 + K_{xx}$. The figures in parentheses represent ionic activities and K_{xx} is a constant for each salt. A plot of negative $\log (\text{Ca}^{++}) (\text{OH}^-)^2$ against negative $\log (\text{H}^+)^3 (\text{PO}_4)^4$ will give, as these authors showed, a straight line with a slope of $-1/x$ where x is the Ca:P molar ratio in the reacting salt. The position of the line is determined by the constant K_{xx} . Fig. 4 and Table V show the data from Figs. 2 and 3 plotted in this manner with the method of least squares being used to define the best fit. The second line is within the 95% confidence limits of the first line and the slope of both is -0.5272 giving a Ca:P ratio of 1.89 (Table V). MacGregor,

TABLE V

Derived Ca:P Ratios of Reacting Salt as Compared with Determined Values for the Bone Powder

	Ca:P ratio in original bone powder	Regression coefficient	Ca:P ratio derived from $\frac{1}{x}$
Controls n = 3	1.566	-0.5272	1.8968
Uremics n = 3	1.558	-0.5272	1.8968

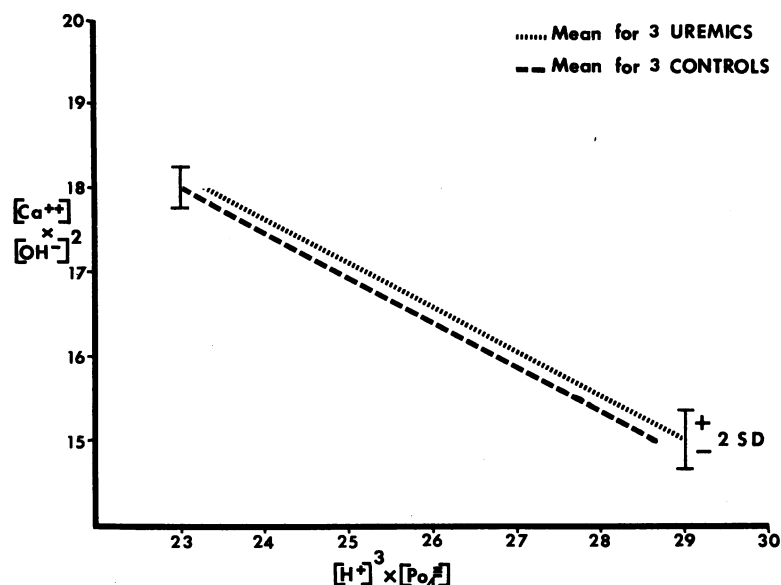


FIGURE 4 Using the least squares method the slopes of $-\log_{10}[\text{Ca}^{++}] \times [\text{OH}]^2$ on the ordinate and $[\text{H}^+]^3 \times [\text{PO}_4^{3-}]$ on the abscissa have been drawn ± 2 SD.

using the data from MacGregor and Nordin (11), with adult femoral bone and a slightly different equilibration system obtained a slope of -0.616 and a Ca:P ratio of 1.62 , results quite similar to the present values. The identical slopes in the patients and controls suggest a similarity of molecular structure.

4. *Preliminary ^{45}Ca labeling.* The results are shown in Table VI where the difference between the two groups persists, and less stable and radioactive calcium appears in the medium.

5. *Exchange with varying duration of renal failure.* The patients were subdivided as in the previous study (5), and the results are shown in Table VII where it can be seen that the difference in exchange becomes more apparent with increasing length of renal failure after 3 months.

6. *Fluoride content.* Fluoride analyses were carried out on the bone powder from 14 controls and nine patients (Table VIII). The mean values in parts per million for the dry defatted samples were 607 for the controls and 963 for the renal patients, $P < 0.025$. However, as can be seen from Table VIII, the difference in calcium exchange was unrelated to the fluoride content being different from the control bone even when the fluoride values were normal.

7. *Bone carbonate.* Bone carbonate was measured in 14 controls in whom the mean value was 1.61 ± 0.02 mEq of carbonate/mg of fat-free dry bone. In the uremics the value was 1.25 ± 0.07 mEq without overlap of individual values between the groups, $P < 0.001$.

Dry ashing at 600°C for 16 hr converts CaCO_3 to CaO . This was verified using pure calcium carbonate when after ashing only 1.2% of the original CO_3^{--} remained. Bone powder was then ashed in the same manner from 17 controls and nine uremics and thereafter equilibrated with buffer. The total calcium released into the medium was 70.1 ± 7.2 μg , range 36–137, for the controls and 26.3 ± 1.8 μg , range 18–35 in the uremics, $P < 0.001$. The same difference, therefore, exists between the groups even in the absence of carbonate.

8. *Acid elution.* Elution patterns were completed for three normal and three uremic bones with similar results in each case. The mean of each group is shown in Fig. 5 where it can be seen that the uremics have con-

TABLE VI
Bone Powder Incubated with Radioactive Calcium for 6 Days Washed and then Nonradioactive Buffer Replaced. Appearance of Radioactivity into the Medium after 2 Days Measured

	Counts per second in medium after 2 days as % of total radioactivity present in bone	Total calcium in medium
	%	μg
Controls (3)	11.8 (10.8–12.8)	48.5 (44–54.3)
Uremics (2)	8.5 (8.0–8.9)	36.6 (36–37)

TABLE VII
Showing Exchange Results after Varying Periods of Renal Failure

Groups	Duration of renal failure	Results after 24 hr equilibration	
		⁴⁰ Ca μg	⁴⁷ Ca %
Controls			
1. n = 7	—		
Mean ±SE		54.3	22.7
Range		45.2–59.9	21.8–25
2. n = 5	<3 months		
Mean ±SE		57.4	20
Range		54.3–66.5	18.2–21.9
3. n = 5	>3 months <1 yr		
Mean		46.1	17.6
Range		37.3–50.0	15.5–20.2
4. n = 7	>1 yr		
Mean		41.8	13.5
Range		33.7–49.8	9.2–15.9

sistently a slightly higher calcium:phosphorus ratio in the eluates which is constant up to the 50th tube. The eluate calcium and phosphorus were both higher in the patients than the controls, the calcium being slightly higher than the phosphorus so that the Ca:P ratio is higher in the uremics. These eluate patterns are entirely different from those found by Pellegrino (3) where the ratio was 3.0 in the normal bone and by the 30th–40th tube had decreased to the level of the uremics of 1.67.

9. Investigation for pyrophosphate and other acid hydrolysable phosphates. This investigation showed similar results in each of three patients and controls. The inorganic phosphorus per milligram of bone powder measured before and after acid hydrolysis was 0.089 ± 0.001 mg and 0.083 ± 0.001 mg in the controls. In the renal patients the values were 0.093 ± 0.003 mg before hy-

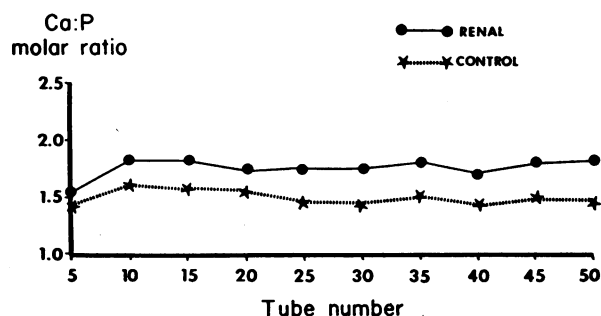


FIGURE 5 Mean Ca:P ratios from three uremics and three controls with 1% NH_4Cl elution.

drolisis and 0.083 ± 0.002 mg afterwards. No increase in measurable phosphorus after acid treatment was found.

B. Anorganic bone

Table IX shows the results after the organic material had been removed. All analyses were after 64 hr of incubation. It can be seen that changes similar to those found with the whole bone are present with decreased stable calcium released into the medium and less ⁴⁷Ca remaining in the solution. Another series using glycerol ashing and powder particles less than 70μ in size was examined at a later date. There were 11 samples in each group which were incubated for 64 hr and the stable calcium in the medium measured. The control mean was $38.5 \mu\text{g} \pm 0.28$ (range 32.5–43.3) and the uremics 27.2 ± 0.37 (range 19.3–33.0), $P < 0.001$.

TABLE VIII
Bone Fluoride Content Compared to Calcium Exchange

Name	Fluoride dry bone powder ppm	⁴⁰ Ca solution at 64 hr μg/4 ml (mean)
Uremics		
A. L.	335	48.7
J. L.	385	36.5
R. D.	835	42.8
I. D.	835	46.8
J. P.	850	39.5
I. M.	960	42.3
N. D.	1050	47.8
J. G.	1625	48.7
E. L.	1805	36.2
Mean ±SE	963 ± 164	43.2 ± 1.7
Controls		
W. F.	280	59
L. B.	350	64.2
E. M.	350	56.1
C. W.	425	51.7
B. A.	450	58
W. A.	460	60
O. B.	475	64.5
L. S.	500	59
M. W.	500	58
N. H.	735	59.6
I. H.	760	63.5
T. R.	1010	48.1
W. H.	1025	61
Y. M.	1185	48
Mean ±SE	607 ± 77	57.9 ± 1.4

$P 963 \text{ vs. } 607 < 0.025$.

Bone fluoride content arranged in order of increasing magnitude with the values for calcium exchange given for comparison. There is no difference in exchange rates within each group whether the fluoride content is low or high.

C. Investigation of crystal morphology, size, and surface area

X-ray diffraction patterns were obtained on the 14 bones that are shown in Fig. 1. They all gave the lines expected with hydroxylapatite, and no uniform difference could be detected either in the spacing or the intensity between the diffraction spectra of the controls and those of the patients. Variation in the sharpness of the diffraction lines was seen in both groups. However, there was no correlation between this and the amount of calcium released into the buffer.

The mean relative crystal length was determined and is shown in Table X where it can be seen that there is no difference between the two groups. When the five renal patients were further divided into two groups on the basis of relative crystal length, the average calcium released into the buffer solution was 45.0 and 44.3 μg , respectively, suggesting that particle size was not affecting calcium release. Surface area was then measured on the intact and anorganic bone, and the results are shown in Table XI. The areas are similar in the two groups with a trend towards larger areas in the uremic bones.

D. Synthetic apatites

With the addition of bicarbonate to the reactants, the resulting apatite when rotated with buffer solution as in the bone powder experiments freed more calcium and less phosphorus into the solution (Table XII). This change was directly related to the initial bicarbonate concentration when the apatite was synthesized (Fig. 6). After ashing to remove apatite carbonate, a distinct difference remained between the apatite made without bicarbonate and that with 0.1 M NaHCO_3 . All apatites in Table XII showed the characteristic diffraction lines expected on X-ray examination. Pure CaCO_3 examined as a control under the same conditions could not be detected in any of these samples. The apatites without added bicarbonate together with the 0.03 and 0.06 M

TABLE X
Mean Relative Crystal Length in Controls and Uremics

Name	Relative length
Controls	
M. W.	3.12
T. R.	3.70
E. M.	2.85
T. A.	2.87
Mean	3.13
Uremics	
R. D.	4.54
N. D.	4.00
J. P.	3.22
J. G.	2.56
I. D.	2.56
Mean	3.37

bicarbonate addition showed similar diffraction line sharpness. The 0.1 M apatites all displayed line broadening indicative of smaller crystal size or induced crystal defects.

DISCUSSION

These studies have shown that in dried, defatted vertebral bone powder from patients dying with uremia who have been treated by conservative management only, an alteration has occurred in the solubility characteristics of the bone mineral. This change is unrelated to the type of renal disease or bone histopathology and to alterations in the organic matrix, such as coating by osteoid, because it persists after complete removal of all organic material. It is, therefore, a property of the mineral phase only.

TABLE XI
Surface Areas of Whole and Anorganic Bone in Three Controls and Three Uremics

	Surface area	
	Whole bone	Same sample after removal of organic fraction with alkaline glycerol
m^2/g		
Controls		
L. B.	1.24	129.9
N. H.	1.38	139.5
O. B.	1.38	131.5
Uremics		
J. P.	2.24	145.6
J. G.	0.83	144.8
I. D.	2.51	151.4

TABLE IX
Showing Results after 64 hr of Equilibration after Removal of the Organic Fraction

	Ashed at 400°C		Glycerol treated	
	^{40}Ca	^{47}Ca	^{40}Ca	^{47}Ca
	$\mu\text{g}/4\text{ ml}$	%	$\mu\text{g}/4\text{ ml}$	%
Controls (7)				
Mean $\pm$ SE	44.2 $\pm$ 0.89	9.2 $\pm$ 0.14	(4) 46.0 $\pm$ 1.83	7.1 $\pm$ 0.19
Range	36–53.8	8.1–10.9	37.6–55.1	6.0–7.8
Uremics (8)				
Mean $\pm$ SE	36.5 $\pm$ 0.48	6.3 $\pm$ 0.10	(7) 30.3 $\pm$ 0.66	4.8 $\pm$ 0.08
Range	30.6–41.7	5.7–7.0	21.6–36.1	3.9–5.4
P	<0.01	<0.001	<0.005	<0.001

Our data confirm the findings of Pellegrino et al. (3) that a reduction in bone carbonate occurs in chronic renal failure, but we have been unable to verify the findings of the same workers regarding a separate phase, presumably calcium carbonate, with solubility in ammonium chloride and partial loss associated with chronic uremia. Instead the changes found appear to relate to a more intrinsic alteration in the composition of the bone crystal itself. The following evidence is adduced for this.

(a) X-ray diffraction has shown no sign of any crystalline salt other than apatite. This, however, does not exclude the presence of an admixture in quantities not exceeding about 3%.

(b) If the difference between the uremic patients and the controls was due to a reduction in the amount of a separate phase such as calcium carbonate in the uremics, this difference might be expected to disappear after removal of the carbonate by ashing at 600°C. However, the difference between the two groups remained unchanged after this procedure.

(c) The most consistent finding throughout these studies has been the increased amounts of inorganic phosphate appearing in the medium in the uremic samples. Together with the decrease in carbonate an ionic replacement would appear likely, and for this to occur a complex crystalline structure would be required or else molecular disorganization would result.

(d) Synthetic apatite was shown to behave in an identical manner to uremic bone when carbonate-deficient and to resemble normal bone when carbonate-

containing. This carbonate is predominantly within the crystal lattice when prepared by this method as most of the labile carbonate in the hydration layer has been lost on drying (7). Phosphate has substituted for carbonate when the latter is absent from the reactants, and the solution after equilibration contains more phosphate as found in the uremic bone buffer. While this work was in progress, evidence that phosphate can indeed substitute for carbonate in the apatite lattice appeared and our results confirm these findings (12). It is of interest that line broadening on X-ray examination was evident as the bicarbonate concentration increased in the synthesis of the apatites. This suggests either a diminution in grain size with a larger surface area of the crystallites or possibly lattice defects. It should be noted, however, that 0.03 and 0.06 M bicarbonate apatites were indistinguishable by the X-ray techniques employed from those in which bicarbonate had not been added, despite the observed effect of bicarbonate on calcium solubility, as shown in Fig. 6.

Although changes in the amount of other ions and compounds undoubtedly occur in uremic bone, there was no evidence that they were contributing to the process studied. The increased fluoride content is confirmatory of the studies carried out previously that uremics will accumulate skeletal fluoride when renal fluoride excretion is reduced (13). Although fluorapatite is less soluble than hydroxylapatite, there was no relation between the fluoride content and the solubility characteristics in the system under study.

TABLE XII
Release of Calcium and Phosphorus into Buffer Solution from Apatites Synthesized with and without Bicarbonate

Additive	Apatite		Not ashed			Ashed 600°C		
			Buffer solution			Buffer solution		
	Ca:P ratio	CO ₃	Ca	P	Ca:P ratio	Ca	P	Ca:P ratio
	<i>mEq/g</i>							
None	2.13	—	3.4	52.2	0.07	33.8	27.2	1.24
	2.07	1.12	3.8	47.8	0.08	34.1	27.2	1.25
	2.10	0.83	3.9	46.2	0.08	32.4	28.4	1.14
	2.14	0.76	4.3	41.0	0.10	33.8	28.0	1.21
0.03 M NaHCO ₃	2.02	0.61	4.2	41.6	0.1	21.2	27.6	0.77
0.06 M NaHCO ₃	2.09	2.26	17.5	8.4	2.08	32.0	2.4	13.3
0.1 M NaHCO ₃	2.34	3.75	37.5	13.6	2.76	281.0	2.4	117
	2.40	—	32.2	16.8	1.92	327.0	1.2	272
	2.30	—	70.2	6.6	10.6	98.0	2.8	35
	2.19	—	83.7	5.6	14.9	59.0	1.6	37
	2.27	—	60.0	6.4	8.9	218.0	—	—

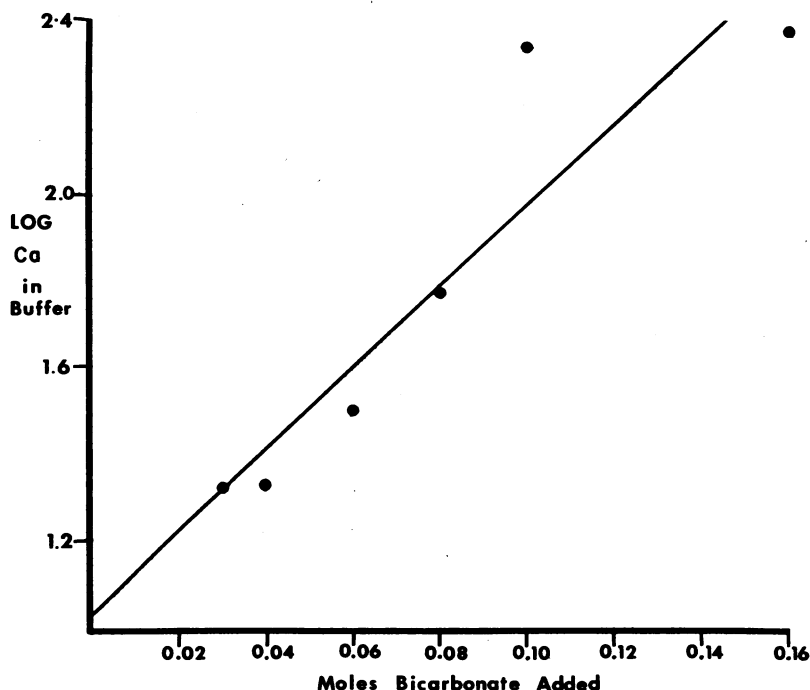


FIGURE 6 Increasing quantities of calcium are released into the buffer from apatite when the bicarbonate content of the reactants is increased. Ordinate scale log to base 10. $r = 0.9269$, $P < 0.01$.

A separate amorphous phase has been demonstrated by Termine and Posner for several different species (14). The possibility of an alteration in the proportion of the amorphous and crystalline phases in the uremics must be considered. This seems unlikely partly because of the phosphate changes previously described and also because with heating, conversion of amorphous to crystalline material would be expected with disappearance of differences between the two groups. Yet, as shown, the differences are if anything more marked. Furthermore, the findings from X-ray diffraction and measurement of relative crystal length indicated no effect on calcium release into the buffer solution even with quite marked variations in "crystallinity," suggesting that if an amorphous phase was present, it was not contributing to these findings.

It should be noted that the method of sample preparation included heating in air to 100°C and that the calcium release data were obtained in aqueous media. Both procedures would markedly reduce the amount of amorphous calcium phosphate present with an increase in crystalline apatite (14). From these considerations an alteration in the proportions of amorphous to crystalline material causing the changes described in this paper appear improbable.

The pH at which these experiments were carried out has been 7.4 for the buffer and 8.0 for the synthetic

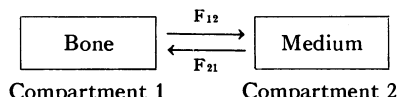
apatites. Recent data would indicate that, in the rat at least, at the calcification front the pH is distinctly alkaline (15, 16). The amount of bicarbonate and inorganic phosphate in the extracellular fluid present at the site of calcification is probably important in determining the final composition of the bone. Sobel, Rockenmacher, and Kramer (17, 18) in an elegant series of experiments have shown that in the rat on dietary regimes with varying Ca:P ratios, "there was an almost direct relationship between the bone phosphorus:carbonate ratio and the serum phosphorus:total carbon dioxide ratio." We suggest that analogous changes are occurring in renal failure as the composition of extracellular fluid is altered by phosphate accumulation and bicarbonate reduction (Table I). This change is then responsible for the formation of carbonate deficient apatite with the defects being filled by PO_4 groups. This exchange is structurally possible with only minimal alterations in crystal symmetry and size (19, 20). Because of slow bone turnover, the extent of the change will be proportional to the duration of renal failure as shown in Table VII, and in less than 3 months of uremia, no alteration can be expected.

The significance of these findings in bone composition can only be speculative at this point. That dissolution of the lattice would proceed more slowly seems likely, but whether this could be detectable in the living subject

is unknown. However, the calcium kinetics described previously (4) are compatible with the in vitro findings and do suggest that this change may be of functional importance. If so, the ready development of hypocalcemia in the uremic and the effect of phosphate (21) in lowering plasma calcium levels could be in part explained.

APPENDIX

The in vitro system consisting of bone powder immersed in an experimentally controlled medium can be represented by a two compartment model.



Let $q_1(t)$ be the amount of radioactivity in compartment 1 at any time "t"; $q_2(t)$, the amount of radioactivity in compartment 2 at any time "t"; $F_{12}(t)$, the flux (labeled and unlabeled) from compartment 1 $\rightarrow$ 2 at any time "t"; $F_{21}(t)$, the flux (labeled and unlabeled) from compartment 2 $\rightarrow$ 1 at any time "t"; $A_1(t)$, the amount of substrate (calcium unlabeled) in compartment 1 at any time "t"; $A_2(t)$, the amount of substrate (calcium unlabeled) in compartment 2 at any time "t"; q_0 , the total amount of radioactivity in the system; S , the surface area across which Ca exchange occurs; and T , the total amount of calcium (unlabeled) as determined chemically, then

$$\frac{1}{S} \frac{dq_1(t)}{dt} = -\frac{F_{12}(t)}{A_1(t)} q_1(t) + \frac{F_{21}(t)}{A_2(t)} q_2(t) \quad (1)$$

$$\frac{1}{S} \frac{dq_2(t)}{dt} = -\frac{F_{21}(t)}{A_2(t)} q_2(t) + \frac{F_{12}(t)}{A_1(t)} q_1(t) \quad (2)$$

where

$$q_1(t) + q_2(t) = q_0 \quad (3)$$

and

$$A_1(t) + A_2(t) = T \quad (4)$$

substituting $q_1(t) = q_0 - q_2(t)$ into equation 2

$$\frac{1}{S} \frac{dq_2(t)}{dt} = -\left[\frac{F_{21}(t)}{A_2(t)} + \frac{F_{12}(t)}{A_1(t)}\right] q_2(t) + \frac{F_{12}(t)}{A_1(t)} q_0 \quad (5)$$

The following two types of experiments are performed. (a) labeled Ca is placed in the medium (compartment 2), and its disappearance is measured as a function of time, i.e., $q_2(t)$ is measured; and (b) bone powder is placed in a controlled medium and the amount of calcium appearing in the medium is measured as a function of time, i.e., $A_2(t)$ is measured.

Now

$$\frac{dA_2(t)}{dt} = [F_{12}(t) - F_{21}(t)]S \quad (6)$$

Substituting into equation 5

$$\frac{1}{S} \frac{dq_2(t)}{dt} = -\left[\frac{F_{12}(t)}{A_2(t)} + \frac{F_{12}(t)}{A_1(t)} - \frac{1}{SA_2(t)} \frac{dA_2(t)}{dt}\right] q_2(t) + \frac{F_{12}(t)}{A_1(t)} q_0 \quad (7)$$

Using equation 4 we can, therefore, express $F_{12}(t)$ as a function

of T , $q_2(t)$, $A_2(t)$, and time which are all experimentally measured.

$$SF_{12}(t) = \frac{[T - A_2(t)] \left[A_2(t) \frac{1}{q_0} \frac{dq_2(t)}{dt} - \frac{dA_2(t)}{dt} \frac{q_2(t)}{q_0} \right]}{[A_2(t) - T \frac{q_2(t)}{q_0}]} \quad (8)$$

Once $SF_{12}(t)$ has been determined from equation 8, then $SF_{21}(t)$ can be calculated from $SF_{21}(t) = SF_{12}(t) - [dA_2(t)]/dt$. In this study, SF_{12} and SF_{21} were determined at specific times and compared in control and renal patients.

ACKNOWLEDGMENTS

We wish to thank Dr. T. Salman for the determinations of surface area and use of his BET apparatus, and Mrs. R. Nyeky for secretarial assistance.

This study was supported by the Department of Veterans' Affairs Grant 26-56 until 1966, and Medical Research Grant MA-1981 to one of us (M. K.) from 1966 to 1969.

REFERENCES

1. Stanbury, S. W. 1968. Bone disease in uremia. *Amer. J. Med.* **44**: 714.
2. Lemann, J., Jr., J. R. Litzow, and E. J. Lennon. 1966. The effects of chronic acid loads in normal man: further evidence for the participation of bone mineral in the defence against chronic metabolic acidosis. *J. Clin. Invest.* **45**: 1608.
3. Pellegrino, E. D., and R. M. Biltz. 1965. The composition of human bone in uremia. *Medicine*. **44**: 397.
4. Kaye, M., and M. Silverman. 1965. Calcium metabolism in chronic renal failure. *J. Lab. Clin. Med.* **66**: 535.
5. Kaye, M. 1964. Mineral analysis of the fourth lumbar vertebra in health and renal failure. *J. Clin. Invest.* **43**: 1367.
6. Deakins, M., and R. L. Burt. 1944. The deposition of calcium, phosphorus, and carbon dioxide in calcifying dental enamel. *J. Biol. Chem.* **156**: 77.
7. Neuman, W. F., and B. J. Mulryan. 1967. Synthetic hydroxyapatite crystals. III. The carbonate system. *Calcified Tissue Res.* **1**: 94.
8. Salman, T., and R. F. Robertson. 1965. Gas-phase adsorption and surface area determination of minerals. *Can. Mining Met. Bull.* **58**: 1088.
9. Ashley, R. P. 1960. Zirconyl-alizarin chelate in spectrophotometric determination of trace amounts of fluorine. *Anal. Chem.* **32**: 834.
10. MacGregor, J., and W. E. Brown. 1965. Blood: bone equilibrium in calcium homeostasis. *Nature (London)*. **205**: 359.
11. MacGregor, J., and B. E. C. Nordin. 1960. Equilibration studies with human bone powder. *J. Biol. Chem.* **235**: 1215.
12. LeGeros, R. Z., O. R. Trautz, J. P. LeGeros, E. Klein, and W. P. Shirra. 1967. Apatite crystallites: effects of carbonate on morphology. *Science (Washington)*. **155**: 1409.
13. Call, R. A., D. A. Greenwood, W. H. LeCheminant, J. L. Shupe, H. M. Nielsen, L. E. Olson, R. E. Lamborn, F. L. Mangelson, and R. V. Davis. 1965. Histological and chemical studies in man on effects of fluoride. *Pub. Health Rep.* **80** (No. 6): 529.
14. Termine, J. D., and A. S. Posner. 1967. Amorphous/

- crystalline interrelationships in bone mineral. *Calcified Tissue Res.* 1: 8.
15. Howell, D. S., J. C. Pita, J. F. Marquez, and J. E. Madrugá. 1968. Partition of calcium, phosphate, and protein in the fluid phase aspirated at calcifying sites in epiphyseal cartilage. *J. Clin. Invest.* 47: 1121.
 16. Cuervo, L. A., J. C. Pita, and D. S. Howell. 1969. Measurements of pH, pCO₂ and carbonic anhydrase activity (C. A.) of calcifying sites in normal cartilage. *Clin. Res.* 17: 72. (Abstr.)
 17. Sobel, A. E., M. Rockenmacher, and B. Kramer. 1945. Composition of bone in relation to blood and diet. *J. Biol. Chem.* 159: 159.
 18. Sobel, A. E., M. Rockenmacher, and B. Kramer. 1945. Carbonate content of bone in relation to the composition of blood and diet. *J. Biol. Chem.* 158: 475.
 19. Young, R. A. 1967. Dependence of apatite properties on crystal structural details. *Trans. N. Y. Acad. Sci.* 29: 949.
 20. Legeros, R. Z., O. R. Trautz, E. Klein, and J. P. Legeros. 1969. Two types of carbonate substitution in the apatite structure. *Experientia (Basel)*. 25: 5.
 21. Goldsmith, R. S., and S. H. Ingbar. 1966. Inorganic phosphate treatment of hypercalcemia of diverse etiologies. *N. Engl. J. Med.* 274: 1.