TECHNETIUM-99m-PYROPHOSPHATE: STUDIES IN VIVO AND IN VITRO

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In rats with induced rickets, the uptake of $^{99m}TcO_4$ and ^{99m}Tc-pyrophosphate per gram of bone was increased as compared with weight-matched controls. However, the uptake of radioactive calcium and ^{32}P-pyrophosphate was similar in both rachitic and control animals, suggesting that the ^{99m}Tc label conferred specificity and favored the rachitic lesions. Employing the rat tibia in an in vitro system, $^{99m}TcO_4$ uptake was predominantly in the organic bone matrix; radioactive calcium, ^{32}P-pyrophosphate, or ^{14}C-diphosphonate uptake was mainly in the bone mineral; and ^{99m}Tc-pyrophosphate, ^{99m}Tc-diphosphonate, and ^{99m}Tc-polyphosphate were found in both mineral and organic phases. By removal of both mineral and polysaccharide and by using agents that altered the degree of collagen fibril cross-linking, evidence was obtained suggesting that $^{99m}TcO_4$ and ^{99m}Tc-pyrophosphate are preferentially bound by immature collagen.

In recent studies (*1*) we have shown that the skeletal uptake of ^{99m}Tc-pyrophosphate was increased in patients with osteomalacia. The present study was initiated to see whether this finding could be confirmed in an animal model and to investigate the biochemical basis for the uptake of technetium-labeled, bone-seeking phosphate compounds.

METHODS AND MATERIALS

In vivo. Weanling Holtzman rats were made rachitic by feeding a high calcium, low phosphorus, vitamin D-deficient diet (No. 160040, General Biochemicals, Chagrin Falls, Ohio) for 3 weeks at which time they had radiologic and histologic evidence of rickets. The rachitic animals together with normal controls, matched for weight, were injected through the jugular vein with ^{99m}Tc-pyrophosphate (^{99m}TcPP) which had been made from a kit (supplied by New England Nuclear) containing sodium pyrophosphate (10 mg) and stannous chloride (1.3 mg) by adding pertechnetate in 5 ml isotonic saline. The degree of binding was checked routinely by silica gel thin-layer chromatography using acetone as solvent (*2*). Less than 1% of the radioactivity migrated with the solvent front. Similar studies were also made substituting stannic for stannous chloride to determine the bone uptake of pertechnetate. The metabolism of the pyrophosphate and tin in the absence of technetium was studied by adding a tracer quantity of the radioactive compound sodium ^{32}P-pyrophosphate to the kit in 5 ml of isotonic saline. In a separate study, ^{45}Ca was injected intraperitoneally 2 hr prior to the intravenous injection of the ^{99m}Tc-pyrophosphate-tin complex. All animals

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were killed by cervical fracture 45 min following the intravenous injection. The left femur and tibia were dissected free of adherent tissue. Each bone was sectioned with a scalpel. The marrow in the shafts of the femur and the tibia was aspirated and discarded. Eight bone samples were obtained: proximal and distal epiphyses of femur and tibia, shaft of femur and tibia, cartilaginous epiphyseal growth plate of the distal end of the femur, and cartilaginous epiphyseal growth of the proximal end of the tibia. The bone fragments were counted in an automatic gamma counter to a total count that gave a statistical accuracy of less than 2% error. The bones were subsequently dried overnight at 70°C and weighed. More prolonged drying caused no further decrease in weight. Samples containing either ^{32}P or ^{45}Ca were ashed overnight at 650°C, the ash dissolved in 10% trichloracetic acid, and 0.1 ml taken for counting in Bray's solution in a liquid scintillation counter. The radioactivity has been expressed as a percent of the injected dose per gram of dry tissue.

Red cell and plasma labels in bone were studied in a separate experiment. Rats were injected with 0.4 ml of a mixture of ^{131}I-human serum albumin and ^{59}Fe-labeled rat erythrocytes from a donor animal that had been injected with ^{59}Fe 10 days previously. The animals were sacrificed 15 min later. The separate bone fragments were counted as above using suitable discriminator settings to separate the two nuclides. By injection into the aorta through a 25-gage needle of ^{85}Sr microspheres (3M Company), 15 ± 5 microns in diameter in 0.2 ml isotonic saline, the relative bone-blood flow was estimated in rachitic and weight-matched control rats. The animals were killed by cervical fracture 1 min following the injection. Both femurs and both tibias were removed for analysis as previously described.

In vitro. The in vitro incubation studies were done employing the proximal one-third of the rat tibia that had been cut into equal halves in the coronal plane. Preliminary experiments showed similar bone uptake in front or back segments and also in right or left legs. Each tibial fragment was incubated in saline at a pH of 5.5 in a shaking water bath at room temperature for 30 min. The solution contained either $Na^{99m}TcO_4$, $^{99m}TcPP$, radioactive calcium (either ^{45}Ca or ^{47}Ca), ^{14}C-diphosphonate or sodium ^{32}P-pyrophosphate. Except for the study using free pertechnetate, the radiopharmaceutical being investigated was added in saline to vials supplied in the kit. These vials contained stannous chloride and the phosphate compound under study so that the quantity of tin and phosphate was constant in all incubations. To maintain ion concentrations in the free $^{99m}TcO_4$ studies, stannic chloride was substituted for stannous chloride. A reducing agent, i.e., stannous chloride, is necessary for the formation of ^{99m}Tc-phosphate complexes (*3*). The TcO_4 preparation contained no such complexes as confirmed by thin-layer chromatography.

In separate studies, tris buffer at pH 7.4 was compared with saline at pH 5.5. No difference was found. Incubations at either 37°C or 22°C also showed similar findings. Only the saline data at room temperature will be described. At the end of the incubation period the bone was washed briefly in two changes of nonradioactive saline. If the radionuclide was gamma-emitting, the bones were counted directly. The bones containing beta-emitters were dried and weighed and then dissolved in 1 ml of 10% trichloracetic acid for 5 days. An aliquot of 0.1 ml of the supernate was added to Bray's solution and counted. The counting efficiency for each radionuclide was similar with an accuracy greater than 2%. The uptake in freshly excised tibia was compared with (A) rachitic bone, (B) normal bone that had been demineralized in 0.5 *N* hydrochloric acid for 2 days, and (C) normal bone from which the organic matrix had been removed by ashing at 400°C for 1 hr. This ashing procedure has been shown to remove all nitrogen from the sample and is called anorganic bone (*4*). In preliminary studies, additional samples were treated with hot ethylenediamine. This treatment leaves the crystalline lattice structure unaltered but removes all organic material (*5*). In vitro exchange of these ethylenediamine-treated samples gave the same results as the ashed samples and only the latter were employed here.

In studies shown in Table 1, the anionic dye Orange G was used in a citric acid solution (pH 2.2). The Orange G dye is known to block anionic binding sites (*6*). The bone sample was exposed for 17 hr to a dye concentration of 2%. In other experiments (Table 2), the tibias were demineralized for

TABLE 1. IN VITRO BINDING OF $^{99m}TcO_4$ AND $^{99m}TcPP$ TO FRESH AND TREATED TIBIA

	$^{99m}TcO_4$	$^{99m}TcPP$
Fresh tibia	1	1
Tibia + HCl	3.1 ± 0.2*	11.1 ± 0.6*
Tibia + HCl + Orange G	1.3 ± 0.1*	6.6 ± 0.4*
Fresh tendon	—	1.7 ± 0.2†
Tendon + HCl	6.2 ± 0.7*	9.2 ± 1.6*
Cartilage (rib)	1.3 ± 0.1	1.4 ± 0.1*
Umbilical cord	4.2 ± 0.2*	2.3 ± 0.1*

* $p < 0.001$ between fresh tibia and treated sample.
† $p < 0.01$ between fresh tibia and treated sample.

TABLE 2. IN VITRO UPTAKE OF ^{99m}TcPP BY RAT TIBIA

Treatment	Treated/fresh ratio	p*
None	1	<0.001
HCl	14.7 ± 2.1	<0.001
EDTA	3.4 ± 0.4	—
EDTA + Hy†	6.0 ± 0.1	<0.001
EDTA + Hy + NaH_2PO_4	9.7 ± 0.3	<0.001
EDTA + Hy + $NaBH_4$	2.4 ± 0.2	NS
EDTA + Hy + $NaBH_4$ + NaH_2PO_4	10.7 ± 0.6	<0.001
EDTA + NaOH	2.8 ± 0.2	NS
EDTA + NaOH + NaH_2PO_4	8.4 ± 0.8	<0.001
EDTA + NaOH + $NaBH_4$	4.4 ± 0.3	NS
EDTA + NaH_2PO_4	7.7 ± 0.1	<0.001
EDTA + $NaBH_4$	1.8 ± 0.3	NS

* Between EDTA alone and other treatments.
† Hy, hyaluronidase.

72 hr in 10% EDTA pH 7.0 and exposed to one or more of the following reagents: (A) testicular hyaluronidase, 200 μ/ml in 0.1 *M* acetate buffer at pH 5.3, and containing 0.08 *M* NaCl, for 2 hr at 37°C (to hydrolyze polysaccharides); (B) 0.33 *N* NaOH for 12 hr, to solubilize polysaccharides; (C) sodium borohydride 3.3% in 0.9% NaCl for 2 hr, to stabilize collagen cross-links; (D) 0.5 *M* NaH_2PO_4 for 12 hr, to break collagen cross-links. Except for hyaluronidase, the incubations were all carried out at room temperature. The in vitro data were calculated as counts per minute per milligram of dried bone. In order to compare different studies and amounts of radioactivity, the ratio counts per milligram treated tibia/counts per milligram fresh tibia has been calculated, where the treated tibia represents rachitic, demineralized, or anorganic (ashed) bone. Results are given as mean ± standard error. Differences between means were tested using Student's t-test, p values < 0.01 being taken as significant.

RESULTS

In vivo. The uptake in the different portions of the rat femur and tibia following injection of the various tracers is shown in Table 3. The bone concentration of $^{99m}TcO_4$ was much less than the other three tracers as would be expected (7). Significant differences were seen between rachitic and control animals in the uptake of both $^{99m}TcO_4$ and ^{99m}TcPP, with the rachitic animals consistently accumulating more of each radiopharmaceutical per gram of bone. In comparison, deposition of radiocalcium or ^{32}P-pyrophosphate was the same in the control and rachitic animals. Although the control animals had the same total body weight as the rachitics, their bones were significantly heavier. Expressing the data in Table 3 as the total bone uptake (in either femur or tibia) as a percent of the injected dose, the accumulation of ^{99m}TcPP was still higher in the rachitics, $p < 0.001$. There was no difference between the two groups in the uptake of $^{99m}TcO_4$ or ^{45}Ca and ^{32}P-pyrophosphate uptake was greater in the controls than in the rachitics, $p < 0.001$.

The data in Table 4 show that the number of erythrocytes in the bones were unaltered in the rachitic animals; however, in the epiphyses there were significantly increased amounts of albumin. Blood flow indices, as quantitated by the trapping of microspheres, Table 5, showed the flow in the rachitic animals to be the same as in the controls except for the femoral growth plate, which was less in the rachitic animals.

In vitro. The comparative data shown in Table 6 indicate that the relative uptake by the bone samples was similar for ^{47}Ca, ^{14}C-diphosphonate, and ^{32}P-pyrophosphate, with each one showing increased uptake when incubated with bone from which the organic matrix had been removed, leaving the crystalline structure more available for ionic exchange. After demineralization with acid, ^{47}Ca uptake decreased whereas ^{32}P-pyrophosphate and ^{14}C-diphosphonate were unchanged. The behavior of $^{99m}TcO_4$ was notable for the decreased uptake by the anorganic samples. The ^{99m}TcPP complex reacted differently from all the other compounds showing a strikingly increased uptake onto the demineralized bone and also a considerably increased uptake with the anorganic samples. Uptake of ^{99m}TcPP in fresh rachitic bone was the same as uptake in fresh normal bone.

Two other compounds were compared with ^{99m}TcPP, one a polyphosphate with a mean chain length of five, the other a diphosphonate, ethane-1-hydroxy-1,1 diphosphonate. Both were complexed to technetium using the same conditions as in the pyrophosphate studies. As can be seen from Table 6, the binding by the bone sample was qualitatively similar for all three technetium phosphate complexes, with high uptakes in anorganic and acid-treated bone, particularly the latter. The ^{99m}Tc-diphosphonate results were the highest of all.

In vitro uptake of $^{99m}TcO_4$ and ^{99m}TcPP, using rat tail tendon or costal cartilage and human umbilical cord, was also examined (Table 1). Fresh tendon was similar to fresh bone but after treatment with acid, uptake for both pertechnetate and TcPP was markedly increased. Cartilage took up only slightly more than bone and umbilical cord bound considerably more. However, direct comparison with bone should probably not be made because of the

TABLE 3. BONE UPTAKE (PERCENT DOSE PER GRAM DRY BONE; MEAN ± S.E.M.)

Bone site*		$^{99m}TcO_4$	^{99m}TcPP	^{45}Ca	^{32}P-pyrophosphate
Femur	Control	0.9 ± 0.1	11.3 ± 0.3	14.8 ± 1.4	19.2 ± 0.3
Proximal epiphysis	Rachitic	1.3 ± 0.1†	13.1 ± 0.4†	14.9 ± 0.8	19.5 ± 1.3
Shaft	Control	0.5 ± 0.1	7.2 ± 0.2	12.8 ± 1.2	14.5 ± 0.5
	Rachitic	0.7 ± 0.1	11.3 ± 0.5‡	13.3 ± 0.5	18.4 ± 1.7
Growth plate	Control	1.1 ± 0.1	22.9 ± 1.0	32.6 ± 4.3	42.0 ± 2.1
	Rachitic	1.5 ± 0.1†	32.3 ± 1.7‡	30.7 ± 1.2	37.7 ± 3.1
Distal epiphysis	Control	1.2 ± 0.1	12.9 ± 0.4	17.1 ± 1.8	25.4 ± 1.5
	Rachitic	1.9 ± 0.1†	18.8 ± 0.5‡	16.9 ± 0.7	24.7 ± 1.1
Tibia	Control	1.3 ± 0.1	15.0 ± 0.4	16.6 ± 1.7	25.3 ± 2.5
Proximal epiphysis	Rachitic	1.9 ± 0.2†	22.3 ± 1.0‡	16.2 ± 0.5	26.0 ± 1.3
Shaft	Control	0.5 ± 0.1	5.4 ± 0.2	10.0 ± 0.9	13.4 ± 0.4
	Rachitic	0.5 ± 0.1	7.7 ± 0.2‡	10.2 ± 0.4	13.3 ± 1.0
Growth plate	Control	1.2 ± 0.1	37.1 ± 2.7	40.3 ± 5.1	48.5 ± 2.6
	Rachitic	1.6 ± 0.1†	38.9 ± 2.3	36.3 ± 3.1	30.1 ± 4.5
Distal epiphysis	Control	0.7 ± 0.1	11.6 ± 0.4	9.2 ± 1.0	16.2 ± 0.4
	Rachitic	0.8 ± 0.1	11.8 ± 0.6	8.8 ± 0.5	14.8 ± 1.0

There were ten animals per group injected with ^{99m}Tc-PP. All other studies had five animals per group.
* Listed from proximal end of limb to distal.
† $p < 0.01$ between rachitic and control.
‡ $p < 0.001$ between rachitic and control.

TABLE 4. ^{59}Fe RED CELL AND ^{131}I-ALBUMIN COUNTS PER GRAM BONE IN FEMUR AND TIBIA OF CONTROL AND RACHITIC RATS (MEAN ± S.E.M.)

	^{59}Fe			^{131}I Albumin		
Site	Control	Rachitic	p	Control	Rachitic	p
Femur						
Proximal epiphysis	432 ± 72	457 ± 23	NS	5.5 ± 0.5	6.8 ± 0.3	NS
Shaft	227 ± 1	220 ± 23	NS	3.2 ± 0.2	3.7 ± 0.3	NS
Growth plate	545 ± 37	601 ± 79	NS	8.1 ± 0.5	10.4 ± 0.7	<0.01
Distal epiphysis	575 ± 48	800 ± 78	NS	7.2 ± 0.3	10.3 ± 0.7	0.001
Tibia						
Proximal epiphysis	614 ± 53	659 ± 29	NS	6.9 ± 0.2	10.0 ± 0.2	<0.001
Shaft	128 ± 16	116 ± 11	NS	2.3 ± 0.2	2.1 ± 0.2	NS
Growth plate	736 ± 84	500 ± 40	NS	10.5 ± 0.6	11.3 ± 0.7	NS
Distal epiphysis	211 ± 26	198 ± 8	NS	3.6 ± 0.3	3.4 ± 0.1	NS
No. of animals	7	8		7	8	

differences in the water content of the tissues and in the geometry of the samples used to measure uptake. Exposure of the acid-treated bone to orange G decreased the binding significantly both for $^{99m}TcO_4$ and ^{99m}TcPP.

The data in Table 2 show that the HCl-treated bones had an uptake of ^{99m}TcPP higher than that expected simply due to removal of mineral, as the EDTA samples were equally demineralized. In this series of experiments using EDTA demineralized samples, hyaluronidase treatment, which hydrolyzes polysaccharide components (*8*), doubled the binding of ^{99m}TcPP. Sodium borohydride, which induces stable cross-linkages in collagen (*9,10*), decreased the binding. Alkali, which solubilizes and removes polysaccharide (*11*), had no effect on the binding. Acid sodium phosphate increased the binding.

DISCUSSION

Calcium, ^{32}P-pyrophosphate, and ^{14}C-diphosphonate, all of which are known to be adsorbed onto or exchange with the ions of the bone crystal (*12,13*), showed similar behavior. In the rachitic animals, bone uptake per gram was the same as in the control animals or was decreased. In vitro, removal of the organic matrix made more mineral available for reaction and the uptake was increased for these radionuclides. Free technetium as pertechnetate, $^{99m}TcO_4$, behaved quite differently. In the rachitic animals, bone deposition of $^{99m}TcO_4$ was increased as compared with the controls although the quantitative change was small. In vitro, the bone with the organic matrix removed had almost no uptake of $^{99m}TcO_4$. In comparison a marked deposition of $^{99m}TcO_4$ occurred in the

TABLE 5. DISTRIBUTION OF ^{85}Sr MICROSPHERES IN CONTROL AND RACHITIC ANIMALS (COUNTS PER MINUTE PER GRAM DRY TSSUE; MEAN ± S.E.M.)

Site	Control	Rachitic	
No animals	10	6	p
Femur			
Proximal epiphysis	87 ± 17	39 ± 11	NS
Shaft	24 ± 7	18 ± 4	NS
Growth plate	282 ± 37	160 ± 25	<0.01
Distal epiphysis	122 ± 13	76 ± 12	NS
Tibia			
Proximal epiphysis	87 ± 18	39 ± 11	NS
Shaft	14 ± 8	6 ± 2	NS
Growth plate	147 ± 21	100 ± 13	NS
Distal epiphysis	60 ± 10	30 ± 3	NS

acid-treated samples. The ^{99m}TcPP reacted with some properties of calcium or ^{32}P-pyrophosphate and some properties of pertechnetate. The fractional uptake in the living animals and the in vitro deposition onto the mineral (anorganic) sample was similar for calcium, ^{32}P-pyrophosphate, and ^{99m}TcPP. However, ^{99m}TcPP resembled pertechnetate in vivo by the accumulation of more radioactivity in the rachitic animals and in vitro by the striking uptake onto acid-treated, demineralized bone. From these considerations it is concluded that pertechnetate has a special affinity for the organic matrix of bone and this property is retained and enhanced after complexing. The ^{99m}TcPP is conceptually "bivalent," being bound to bone matrix because of the technetium and also to the mineral because of the pyrophosphate. Free binding areas for each of these two components must exist on the ^{99m}TcPP molecule. It is also apparent that little or no specificity is shown for the type of phosphate compound with regard to chain length between two and five as far as affinity for the mineral structure is concerned. How specific the technetium requirements are in terms of charge, valence, etc., are presently unknown.

TABLE 6. IN VITRO EXCHANGE WITH FRESH OR TREATED NORMAL RAT TIBIA* (MEAN ± S.E.M.)

		Bone treatment	
Compound tested	Fresh	Demineralized	Anorganic
^{47}Ca-chloride	1.0	0.27 ± 0.01†	2.57 ± 0.15†
Sodium ^{32}P-pyrophosphate	1.0	1.29 ± 0.08	5.40 ± 0.65†
^{99m}Tc-pyrophosphate	1.0	12.63 ± 0.69†	6.34 ± 0.29†
$^{99m}TcO_4$	1.0	7.79 ± 0.16†	0.10 ± 0.01†
^{99m}Tc-polyphosphate	1.0	11.5 ± 0.01†	6.0 ± 0.01†
^{99m}Tc-diphosphonate	1.0	33.2 ± 2.0†	10.6 ± 0.7†
^{14}C-sodium diphosphonate	1.0	1.04 ± 0.11	7.7 ± 0.25†

* For details of calculation see Methods.
† $p < 0.001$ calculated from original data using counts per minute between fresh and treated sample.

The affinity of pertechnetate and the ^{99m}Tc-phosphate complex for the matrix of bone raises the intriguing question as to the tissue component(s) to which binding is taking place. The only known components capable of doing this are the protein polysaccharide complexes or collagen. The polysaccharides, of which the chondroitin sulfates are the most important (*14*), would seem particularly suited for binding positively charged technetium complexes because of their numerous cationic binding sites. Evidence suggesting that these compounds are not the prime binding agents includes the rather low uptake when tissues with high chondroitin sulfate content, such as cartilage, were exposed to ^{99m}TcPP and the fact that pertechnetate is anionic and would not be expected to bind. The reduction in binding using pretreatment with Orange G which blocks anionic sites, would further support this view (*6*). The data in Table 2 show that removal of the polysaccharides either by enzymatic means using hyaluronidase or by alkali actually enhanced ^{99m}TcPP binding, presumably because more of the collagen fibrils were exposed.

By employing agents that changed the degree of intermolecular linking between the fibers, the binding of ^{99m}TcPP was changed. Treatment with strong acid (HCl), which not only demineralized bone but also caused dissolution of the collagen molecule, gave the maximum binding because of the large number of free reactive groups produced. Immature collagen differs from mature or aged collagen by the fewer inter- and intramolecular cross-linkages (*15*) and therefore its greater numbers of potential binding sites. It follows that immature collagen should have greater affinity for $^{99m}TcO_4$, ^{99m}TcPP, or similar analogs. Animals or humans with rickets or osteomalacia, hyperparathyroidism, or Paget's disease are all characterized by the excretion of increased quantities of urinary hydroxyproline when bone disease is active and generalized. The hydroxyprolinuria reflects increased collagen turnover and the presence of increased amounts of immature collagen in the skeleton (*16*). This increase in immature collagen might explain our observations in patients as well as in the rachitic animals in this study.

Recently synthesized bone matrix would be expected to show less mature collagen than the bone formed at an earlier date. Accordingly, the fixation of ^{99m}TcPP to areas of recent bone formation would be expected to be greater than in more aged bone.

Information from a recent autoradiographic study would be consistent with this view (*17*).

The blood flow data in Table 5 show that hyperemia could not have played any part in the selective localization of $^{99m}TcO_4$ or ^{99m}TcPP in the rachitic animals. It was of interest, however, that with an unchanged red cell mass there was more labeled albumin in the rachitic bones in the areas contiguous to the rachitic lesions. As technetium compounds are probably protein-bound, this could be an additional reason for the higher uptake in the rachitics. In this respect the marked difference between the bone deposition of pertechnetate in vivo and in vitro may be related to difficulty in gaining access to the extracellular phase of bone in vivo because of different serum-protein binding characteristics as compared with ^{99m}TcPP (*18*).

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REFERENCES

1. ROSENTHALL L, KAYE M: Technetium-99m-pyrophosphate kinetics and imaging in metabolic bone disease. *J Nucl Med* 16: 33–39, 1975

2. BILLINGHURST MW: Chromatographic quality control of ^{99m}Tc-labeled compounds. *J Nucl Med* 14: 793–797, 1973

3. ECKELMAN W, MEINKEN G, RICHARDS P: Chemical state of ^{99m}Tc in biomedical products. *J Nucl Med* 12: 596–600, 1971

4. KAYE M, FRUEH AJ, SILVERMAN M: A study of vertebral bone powder from patients with chronic renal failure. *J Clin Invest* 49: 442–453, 1970

5. SKINNER HCW, KEMPNER ES, PAK CYC: Preparation of the mineral phase of bone using ethylenediamine extraction. *Calcif Tissue Res* 10: 257–268, 1972

6. VEIS A, COHEN J: The degradation of collagen. A method for the characterization of native collagen. *J Am Chem Soc* 76: 2476–2478, 1954

7. SUBRAMANIAN G, MCAFEE JG: A new complex of ^{99m}Tc for skeletal imaging. *Radiology* 99: 192–196, 1971

8. BAYLINK D, WERGEDAL J, THOMPSON E: Loss of protein polysaccharides at sites where bone mineralisation is initiated. *J Histochem Cytochem* 20: 279–292, 1972

9. BAILEY AJ: Intermediate labile intermolecular cross-links in collagen fibres. *Biochem Biophys Acta* 160: 447–453, 1968

10. DELBRIDGE L, EVERITT AV, STEELE MG: The effect of acid phosphate on the solubility of collagen. *Connective Tissue Res* 1: 311–314, 1972

11. MEYER K, DAVIDSON E, LINKER A, et al: The acid mucopolysaccharides of connective tissue. *Biochem Biophys Acta* 21: 506–518, 1956

12. ROBERTSON WG, MORGAN DB: Effect of pyrophosphate on the exchangeable calcium pool of hydroxyapatite crystals. *Biochem Biophys Acta* 230: 495–503, 1971

13. FRANCIS MD, RUSSELL RGG, FLEISCH H: Diphosphonates inhibit formation of calcium phosphate crystals in vitro and pathological calcification in vivo. *Science* 165: 1264–1266, 1969

14. FARBER SJ, SCHUBERT M: The binding of cations by chondroitin sulphate. *J Clin Invest* 36: 1715–1722, 1957

15. STEVEN FS: Single stage partial depolymerisation of collagen fibrils. *Biochem Biophys Acta* 130: 196–201, 1966

16. PROCKOP DJ, KIVIRIKKO KI: Hydroxyproline and the metabolism of collagen. In *Biology of Collagen*, vol 2, Ramachandran GN, ed, New York, Academic Press, 1968, pp 215–246

17. TILDEN RL, JACKSON J, ENNEKING WF, et al: ^{99m}Tc-polyphosphate. Histological localisation in human femurs by autoradiography. *J Nucl Med* 14: 576–578, 1973

18. HAWKINS D: Personal communication