ANALYSIS OF THE RELATIONSHIP BETWEEN ^{99m}Tc-Sn-POLYPHOSPHATE AND ^{99m}Tc-Sn-PYROPHOSPHATE

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Studies of the hydrolysis of ^{99m}Tc-Sn-polyphosphate and ^{99m}Tc-Sn-pyrophosphate have been made. Results show that ^{99m}Tc-Sn-polyphosphates are readily hydrolyzed both in vitro and in vivo. It is suggested that ^{99m}Tc-Sn-pyrophosphate is the stable unit which localizes in the bone.

When physiological quantities of pyrophosphate are added to a solution containing calcium and phosphate, the formation of apatite crystals is inhibited (*1*). When pyrophosphate is added to a solution in which apatite crystals are suspended, the rate at which the crystals dissolve is retarded (*2*). These observations form the basis of the hypothesis that pyrophosphate is in some way involved in the local control of bone mineralization. Experiments in which pyrophosphate has been administered to animals have not supported this hypothesis (*3*). The apparent discrepancy between the effect of pyrophosphate in vitro and the effect in vivo has been explained by the finding of a rapid hydrolysis of pyrophosphate in the extravascular compartment so that pyrophosphate itself never reaches the bone surface at which calcium and phosphate exchanges occur. This same explanation has been offered for the inability of other polyphosphates to inhibit mineralization (*4,5*). In view of these observations, it was surprising to find that pyrophosphate and a variety of polyphosphates labeled with ^{99m}Tc were effective as bone scanning agents. The purpose of this work was to investigate the apparent discrepancy between the observations of Fleisch (*1,2*) and the fact that polyphosphates of various chain lengths can be used successfully as the basis of bone scanning agents.

MATERIALS AND METHODS

Serial blood and urine samples were obtained from patients undergoing routine bone scans using either ^{99m}Tc-Sn-pyrophosphate or ^{99m}Tc-Sn-polyphosphate (chain length 40–50 phosphates). The ^{99m}Tc-Sn-pyrophosphate was prepared by stannous chloride reduction of $^{99m}TcO_4^-$ in the presence of pyrophosphate. Stannous chloride was added as a 2 mg/ml solution in 0.1 *N* HCl. The pyrophosphate solution was prepared in sterile water for injection, the pH being adjusted to 6.5–7.0 using 1 *N* HCl. The final concentration was 25 mg/ml. To 2 ml of pyrophosphate was added ^{99m}Tc-sodium pertechnetate solution followed by 1 ml of the stannous chloride solution. The ^{99m}Tc-Sn-polyphosphate was prepared from New England Nuclear Canada kits.

Separation of phosphate fractions in blood and urine. The blood samples were analyzed for free $^{99m}TcO_4^-$, protein-bound ^{99m}Tc, ^{99m}Tc-Sn-pyrophosphate, and ^{99m}Tc-Sn-polyphosphate of chain length 40–50. Urine samples were also analyzed for the same ^{99m}Tc-Sn-labeled constituents. The analyses were done by gel filtration, paper, and thin-layer chromatography.

Sephadex G50 (100–200 mesh) columns 15 and 45 cm long by 1 cm diam with a void volume of approximately 3 ml were used for gel filtration. Sodium chloride 0.9% was used as the eluting solvent. It was found that ^{99m}Tc-Sn-polyphosphate (chain length 40–50) passed through the column with the same retention volume as $^{99m}TcO_4^-$ whereas ^{99m}Tc-Sn-pyrophosphate could only be removed from the column by elution with 3% hydrogen peroxide solution.

Whatman No. 1 was used for the paper chromatography. Gelman silica gel G (ITLC) was used for the thin-layer chromatography. Chromatograms were examined using a Packard chromatographic analyzer

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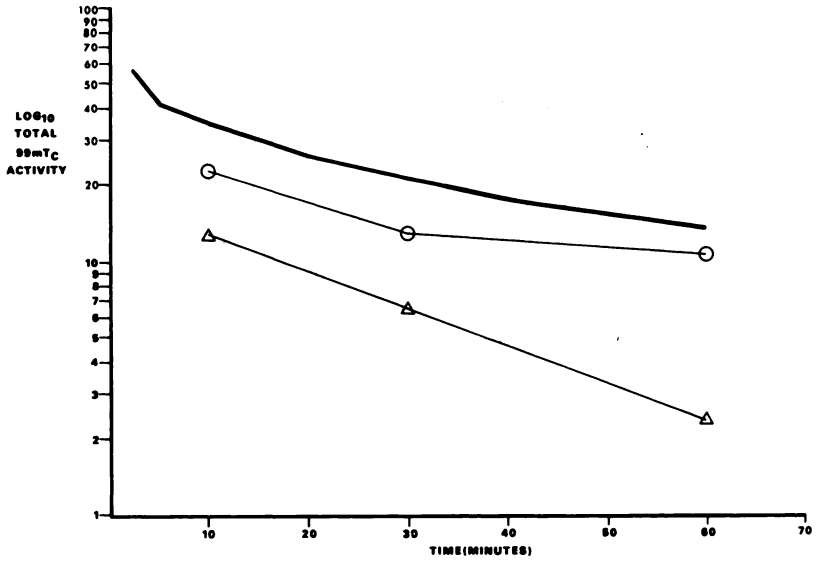

FIG. 1. Changes in concentration of total ^{99m}Tc activity after injection of ^{99m}Tc-Sn-polyphosphate or ^{99m}Tc-Sn-pyrophosphate. (○ - - - ○) indicates contribution to total activity made by unchanged polyphosphate in patients given ^{99m}Tc-Sn-polyphosphate. (△ - - - △) indicates contribution to total activity made by unchanged pyrophosphate in patients given ^{99m}Tc-Sn-pyrophosphate.

(Model 7201). The positions of the phosphates on the chromatograms were determined by their color reaction with acidic ammonium molybdate. The position of the ^{99m}Tc-phosphates obtained from blood or urine was compared with the positions of labeled phosphates of known chain lengths. In this way it was possible to separate $^{99m}TcO_4^-$, ^{99m}Tc-labeled phosphates, and unlabeled orthophosphates.

Hydrolysis of ^{99m}Tc-pyrophosphate and ^{99m}Tc-polyphosphate. The effects of physiological quantities of alkaline phosphatase on ^{99m}Tc-pyrophosphate and polyphosphate were studied in vitro. Technetium-99m-polyphosphate was mixed with borate buffers of pH 7.4, 8.0, and 9.0. Bovine alkaline phosphatase was added to give a final concentration of approximately 19 I.U./ml alkaline phosphatase. Three-milliliter aliquots of these mixtures were placed in viscose extruded bags (Union Carbide Corp. pore size 25 Å) and dialyzed against sterile distilled water at 37°C. The bathing solution was circulated through a continuous loop and its ^{99m}Tc content monitored. The bathing solution was further analyzed by gel filtration and paper chromatography as already described.

The effect of mild acid or enzymatic hydrolysis on ^{32}P-pyrophosphate complexed with ^{99m}Tc was studied by incubating the complex with 0.1 *N* HCl or alkaline phosphatase for 30 min at 37°C.

Hydrazine reduction of pertechnetate. The ^{99m}Tc-pyrophosphate was prepared by substituting hydrazine for tin as the reducing agent. Twenty microliters of 95% hydrazine were mixed with $^{99m}TcO_4^-$ and 12.5 mg pyrophosphate. The yield from the procedure was 70%. Technetium-99m-pyrophosphate made by this method was injected into rabbits and subjected to the hydrolysis experiments described above.

RESULTS

Separation of phosphate fractions in blood and urine. Serial measurements of plasma ^{99m}Tc activity were made in eight patients after injection of ^{99m}Tc-Sn-polyphosphate and in eight patients following ^{99m}Tc-Sn-pyrophosphate injection. The pattern of removal of total ^{99m}Tc activity from the plasma is shown in Fig. 1. No difference in the pattern of removal was found for the two groups of patients. Figure 1 also shows that more than half of the activity in the patients given polyphosphate was due to labeled polyphosphate whereas less than one-third of the total activity in the patients given pyrophosphate was due to labeled pyrophosphate.

The distribution of the ^{99m}Tc activity between the plasma and red blood cells and between the fractions within plasma did not change during the study. Table 1 summarizes the distribution of ^{99m}Tc in the two groups of patients.

The amount of ^{99m}Tc activity excreted in the urine during the first hour was measured in both groups of patients. It ranged from 15 to 35% of the injected dose and the major constituent was always

TABLE 1. ^{99m}Tc DISTRIBUTION IN THE BLOOD OF 16 PATIENTS EXPRESSED AS A PERCENTAGE OF TOTAL ^{99m}Tc ACTIVITY

	^{99m}Tc-Sn-polyphosphate (8 patients)	^{99m}Tc-Sn-pyrophosphate (8 patients)
^{99m}Tc-Sn-pyrophosphate	3–25	12–37
^{99m}Tc-Sn-polyphosphate	53–80	—
$^{99m}TcO_4^-$	10–15	18–58
Erythrocyte-bound ^{99m}Tc	3.7	Less than 1
Protein-bound ^{99m}Tc	10–26	10–69

^{99m}Tc-Sn-pyrophosphate irrespective of the agent injected.

Hydrolysis of ^{99m}Tc-Sn-polyphosphate, ^{99m}Tc-Sn-pyrophosphate, and ^{99m}Tc-pyrophosphate. When ^{99m}Tc-Sn-polyphosphate was hydrolyzed with alkaline phosphatase in vitro, ^{99m}Tc activity appeared at once in the dialysis bath and increased as shown in Fig. 2. The rate of appearance of ^{99m}Tc in the bath was the same as when ^{99m}Tc-Sn-pyrophosphate was put into the viscose bag at pH 7.4 (Fig. 2). When no alkaline phosphatase was added to the ^{99m}Tc-Sn-polyphosphate, the rate of appearance of radioactivity in the bath was markedly decreased (Fig. 2).

Analysis of the bath fluid after 30 min showed that at least 75% of the ^{99m}Tc activity in the bath was due to ^{99m}Tc-Sn-pyrophosphate irrespective of whether ^{99m}Tc-Sn-pyrophosphate or ^{99m}Tc-Sn-polyphosphate was put into the bag. Some $^{99m}TcO_4^-$ was always present in the bath but the amount was constant during the 30 min of dialysis and was due to uncomplexed $^{99m}TcO_4^-$ in the original preparation.

When ^{99m}Tc-Sn-pyrophosphate which had been prepared from ^{32}P-pyrophosphate was incubated with 0.1 *N* HCl or alkaline phosphatase, there was no significant hydrolysis. Further, ^{99m}Tc-pyrophosphate prepared by hydrazine reduction also resisted hydrolysis. The ^{32}P-pyrophosphate to which neither tin nor technetium had been complexed showed some hydrolysis (5–7%) after 30 min in 0.1 *N* HCl.

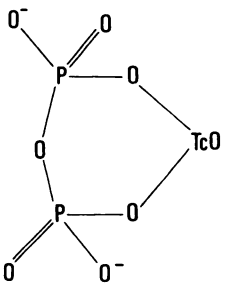

FIG. 3. Possible structure for ^{99m}Tc-pyrophosphate.

Hydrazine reduction of pertechnetate. When Tc(VII) was reduced with hydrazine instead of with stannous ion, a tin-free ^{99m}Tc-pyrophosphate was made. The efficiency of labeling was 70% so that approximately 30% of the original activity remained uncomplexed. Nevertheless, the ^{99m}Tc-pyrophosphate produced using hydrazine reduction was stable in 0.1 *N* HCl and in alkaline phosphatase (18.5 I.U./ml) and produced bone scans in the rabbit.

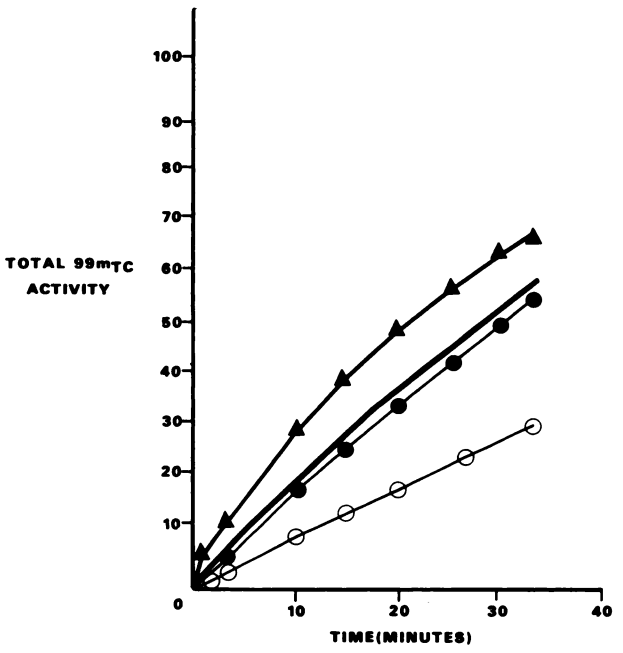

FIG. 2. Appearance of ^{99m}Tc in dialysis bath. Total ^{99m}Tc is shown in arbitrary units. ——— represents activity produced by ^{99m}Tc-Sn-pyrophosphate. (▲ - - - ▲) and (● - - - ●) represent activity produced by ^{99m}Tc-Sn-polyphosphate plus alkaline phosphatase at pH 8.0 and 7.4, respectively. (○ - - - ○) represents activity produced by ^{99m}Tc-Sn-polyphosphate at pH 7.4 in absence of enzyme.

DISCUSSION

The rate at which ^{99m}Tc activity disappeared from the blood in the present studies is very similar to that reported for ^{99m}Tc-Sn-polyphosphate (*6*). Although the rates of disappearance of radioactivity from ^{99m}Tc-Sn-pyrophosphate and ^{99m}Tc-Sn-polyphosphate are similar, the results of the gel filtration experiments (Fig. 1) show that the technetium polyphosphate leaves the vascular compartment more slowly than the ^{99m}Tc-Sn-pyrophosphate. This is not surprising since both phosphates probably leave the blood by diffusion into the interstitial compartment and the molecular weight of the polyphosphate is ~20 times greater than that of pyrophosphate. Furthermore, calculations based on Van Wazer's data suggest that the length of the 40-membered polyphosphate molecule is greater than the diameter of the majority of the pores of the capillary endothelium (*7*).

Two points emerge from the hydrolysis experiments. First, the ^{99m}Tc-Sn-polyphosphate of chain length 40–50 is readily hydrolyzed in vitro by the amounts of alkaline phosphatase normally found in blood. This would suggest that a similar hydrolysis probably accounts for the ^{99m}Tc-Sn-pyrophosphate found in the blood and urine of patients given ^{99m}Tc-Sn-polyphosphate. Unfortunately, the viscose bag experiments do not provide quantitative information

about the rate of hydrolysis of ^{99m}Tc-Sn-polyphosphate. Nevertheless, the fact that ^{99m}Tc-Sn-phosphates produced by hydrolysis left the viscose bag as fast as ^{99m}Tc-Sn-pyrophosphate when it was injected directly into the bag suggests that the polyphosphate was rapidly hydrolyzed. The second point is that ^{99m}Tc-Sn-pyrophosphate prepared by stannous ion reduction is stable in the presence of physiological concentrations of alkaline phosphatase.

Our observations suggest that when ^{99m}Tc-Sn-polyphosphate is injected intravenously, it is hydrolyzed at least in part to ^{99m}Tc-Sn-pyrophosphate. Jung and Russell (*3*) have shown that unlabeled pyrophosphate is rapidly hydrolyzed in the extracellular fluid and the kidney, yet we have found that the bulk of the urinary radioactivity is associated with pyrophosphate when either ^{99m}Tc-Sn-polyphosphates or ^{99m}Tc-Sn-pyrophosphates are injected. It would, therefore, seem that the presence of tin and/or technetium prevents the hydrolysis of ^{99m}Tc-Sn-pyrophosphate to orthophosphate. Since ^{99m}Tc-pyrophosphate prepared by hydrazine reduction is not hydrolyzed in vitro and also produced adequate bone scans, it is apparent that technetium alone can prevent the hydrolysis.

Tin and transition metals such as technetium will complex with a number of diphosphate compounds to produce cyclic structures (*8–10*). Therefore, it seems reasonable to suggest that the structure of ^{99m}Tc-pyrophosphate or ^{99m}Tc-Sn-pyrophosphate is also cyclical. A possible structure is shown in Fig. 3. Finally, it is suggested that this cyclical molecule is the stable unit which localizes in the bone and that ^{99m}Tc-Sn-polyphosphate may be looked upon as a chain of these units.

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