SODIUM TRIMETAPHOSPHATE AS A BONE-IMAGING AGENT. I. ANIMAL STUDIES

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When used in conjunction with stannous ion and ^{99}Tc, the nonsequestering, cyclic, trimeric phosphate anion, $(P_3O_9)^{3-}$, introduced in the form of its sodium salt, exhibits admirable properties as a bone-visualizing agent as demonstrated by animal studies. These studies show that this combination is easily prepared reproducibly and, compared to the agents described in the recent literature (all based on sequestering phosphates), is at least equivalent for bone visualization while being considerably less toxic.

Since 1971 when the use of chain phosphates in bone-visualizing agents was first reported (*1*), there have been a number of published and unpublished studies in which various linear-chain polyphosphates (ranging from the pyrophosphate to polymeric compositions) have been shown to be more or less effective in this application (*2,3*). Because of the ready hydrolysis (both enzymatic and chemical) (*4,5*) of the P—O—P linkages holding these chemical structures together, interest has also focused on a diphosphonate (*6*) in which a carbon atom replaces the bridging oxygen of the pyrophosphate anion to give an extremely stable linkage between the pair of phosphorus atoms.

This paper describes a simple biologic screening test for rating potential bone-scanning agents and its application to the evaluation of several phosphatic materials in comparison with the trimetaphosphate. We have determined the effect of fractional precipitation of several moderately long-chain phosphates on their bone uptake properties. Regarding the use of trimetaphosphate in bone-scanning agents, we have studied several factors including the relative efficacy of various reducing agents, the effect of varying the ratio of phosphate to stannous ion, and the generation of the stannous ion electrolytically. The LD_{50} of the trimetaphosphate-based bone-scanning composition was determined in white mice.

METHODS AND MATERIALS

Chain phosphates. The pyrophosphate, $Na_4P_2O_7 \cdot 10H_2O$, and the tripolyphosphate, $Na_5P_3O_{10}$ (anhydrous Form II), were commercial samples prepared by standard procedures (*4*). The other crystalline phosphate, Kurrol's salt, $(KPO_3)_n$, which was also made by standard procedures (*4*), had been carefully characterized by intrinsic viscosity and ultracentrifuge techniques. They showed that the material [as caused to dissolve in a sodium chloride solution (*4*)] exhibited a number-average number of phosphorus atoms per chain of 5,400.

The samples bearing the PP notation were all vitreous sodium phosphates and except for PP55, which was a well-characterized commercial material, they were prepared here in platinum as melts that were quenched between copper chill plates. The number following the PP symbol is the number-average number of phosphorus atoms per chain. It was checked by end-group titration (*7*) and by ^{31}P nuclear magnetic resonance (NMR) (*8–10*). This number represents the value of n in the average-size linear-chain molecule $Na_{n+2}P_nO_{3n+1}$.

The sample labeled "diphosphonate EHDP" was based on the C-substituted diphosphonate, $Na_4[(O_3P)_2C(CH_3)(OH)]$, which is dubbed sodium "etidronate" and was obtained as a commercial kit, Osteoscan® (Procter & Gamble, Cincinnati, Ohio). Other commercial preparations studied in this work were the pyrophosphate kit TechneScanPYP® (Mallinckrodt, Inc., Hazelwood, Mo.) and the Polyphosphate Kit® (Diagnostic Isotopes, Upper Saddle River, N.J.).

Cyclic phosphates (*11*). Anhydrous sodium trimetaphosphate, $Na_3(P_3O_9)$, and anhydrous sodium tetrametaphosphate, $Na_4(P_4O_{12})$, were made by

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standard procedures (*4*), and a purification procedure was developed for the former. This involved thermal dehydration of its hexahydrate (without any ring scission) to make the anhydride, which does not alter on autoclaving.

Radiopharmaceutical preparation. Unless otherwise noted in the tables of data, the various phosphatic radiopharmaceuticals were prepared by adding 3–6 ml of nitrogen-purged pertechnetate saline solution to 50 mg of the chosen phosphate contained in a nitrogen-purged vial. After the mixture was agitated to ensure homogeneity, 1 mg of stannous chloride (prepared as a 1 mg/ml solution of anhydrous stannous chloride in 0.2 *N* hydrochloric acid) was added with shaking, and the solution was then adjusted to pH 6.0–6.2 with 5% sodium bicarbonate solution.

In two of the studies reported here, the ^{99m}Tc-Sn-TMP composition was formed employing stannous ion produced electrolytically from tin electrodes. Sodium trimetaphosphate (50 mg) was added to a 10-ml serum vial that was capped and nitrogen-purged before insertion of the two electrodes. These consisted of tin wire, 0.5 mm diam by 2.5 cm long (Ventron Inc., Beverly, Mass.) and were inserted through the septum so that 1 cm projected into the vial. Placement was facilitated by using a 1-in. No. 18 hypodermic needle to contain the wire during insertion.

In use, 5–8 ml of pertechnetate saline elutrate was added to the vial followed by 0.1 ml of 0.1 *N* hydrochloric acid. After inversion of the vial, a transistorized power supply was connected and various combinations of current and time were used to produce the desired concentration of stannous chloride. The solution was usually swirled during the electrolysis to ensure good mixing.

Screening procedure. Wistar white rats (weight range, 150–250 gm) were injected with 0.25 ml of this solution via the tail vein. After 4 hr (unless otherwise noted) the rats were sacrificed and the bone uptake was determined in comparison to the dose administered. Since it was found that the uptake in the bones (including the marrow) of one hind leg (i.e., the femur, tibia, and fibula) correlated well with the total-bone uptake (about a 20:1 ratio), the radioactive assay was done on the femur, tibia, and fibula of each hind leg and the results for both legs were averaged to give the reported value for one hind leg. Care was taken to avoid contamination of the bones with blood, and the muscles were carefully stripped from the bones without causing damage to the latter. In a separate study, radioactive assays were done on voided urine and specific tissues and organs in order to obtain the detailed distribution of the technetium-labeled trimetaphosphate as a function of time. The radioassays were carried out with either an ionization chamber or a scintillation spectrometer.

TABLE 1. COMPARATIVE BONE UPTAKE MEASUREMENTS

Condensed phosphate	Percent hind-leg bone uptake at 4 hr
Pyro (decahydrate)	1.9 ± 0.5
Tripoly (Form II, anhyd)	2.1 ± 0.7
PP6	1.2 ± 0.4
PP9	0.7 ± 0.3
PP29	0.7 ± 0.3
PP42	0.6 ± 0.2
PP46	0.5 ± 0.2
PP50	1.0 ± 0.4
PP55*	1.8 ± 0.4
PP56	0.6 ± 0.2
PP57	0.8 ± 0.2
PP60	0.8 ± 0.3
PP71	1.0 ± 0.3
PP138†	0.6 ± 0.2
PP143†	0.4 ± 0.1
Kurrol's salt	1.5 ± 0.3
Trimeta (cyclic)	2.1 ± 0.4
Tetrameta (cyclic)	1.4 ± 0.4
Diphosphonate-EHDP®‡	2.2 ± 0.5

* This PP55 is a simple of the original material furnished SUNY investigators by the Monsanto Co.

† These higher polymers gave high liver uptake values as compared to most of the compounds tested (12–25% as opposed to 0.1–0.4%).

‡ Procter & Gamble kit used as instructed.

Animal imaging study. A 3-kg New Zealand white rabbit was given a 1.0 mg/kg body weight dose (containing 5 mCi of ^{99m}Tc) of trimetaphosphate prepared with electrolytically generated tin. The dose was administered via the ear vein and the animal was sacrificed 5 hr after dosing. The rabbit was imaged at a preset count of 500,000, using a Searle Radiographics scintillation camera fitted with a low-energy collimator.

Biodistribution studies. Biodistribution of the trimetaphosphate radiopharmaceutical was determined at chosen times in sets of five rats using a 1.0 mg/kg body weight of the radiopharmaceutical which was produced using electrolytically generated tin. The animals were placed in metabolism cages and the urine (if excreted) was carefully collected. At the chosen time, the animals were sacrificed and the specific organs were radioassayed. The bladder contents were carefully withdrawn with a hypodermic and added to the urine samples. In those instances in which urine contamination of the fur occurred, this fur was excised and combined with the urine/bladder samples.

RESULTS

Comparison studies. The effect of substituting one phosphate for another is shown in Table 1. At least

six rats were used to study each sample and the average percentage uptake values for one set of hind-leg bones are given. In this and the other tables, the standard deviation of each value is reported. Since Samples PP55 and PP60 showed quite different bone uptake values (Table 1), these samples were fractionally precipitated (*12*) from water by ethanol and each fraction was formulated into the standard ^{99m}Tc-Sn-polyphosphate radiochemical preparation. Dosing three rats with each fraction showed no systematic variation with fractionation of the bone uptake property.

Trimetaphosphate. Since sodium trimetaphosphate showed up well in comparison with the other phosphatic bone-visualizing agents (see Table 1), several studies were carried out with the purpose of characterizing and optimizing the radiochemical formulation based on this cyclic phosphate. The effect of the dosage level was studied using groups of three rats at each of five levels. With the usual preparation exhibiting a 50:1 weight ratio of sodium trimetaphosphate (TMP) to stannous chloride it was found that the dosage level could be varied over a range of 0.02–2.0 mg of TMP per kg body weight with the bone uptake percentages remaining essentially constant and the liver uptake staying below 0.5% of the administered radioactivity.

We determined next the optimum weight ratio of sodium trimetaphosphate to stannous chloride for maximum bone and minimum liver uptake. The amounts of sodium trimetaphosphate and of technetium were kept constant at the chosen standard values and the final solution pH was maintained at 6.0–6.2, while the concentration of the stannous chloride was varied. For ratios in the range of 100:1 to 25:1, the liver uptake was constant at 0.4 ± 0.2% of the radioactivity while the hind-leg bone uptake reached its maximum value of 2.1 ± 0.5% at test ratios of 50:1 to 25:1. For 10:1 or 5:1 ratios the preparations were colloidal; and, in accord with other investigators, we have found that colloidal stannous oxychloride absorbs ^{99m}Tc and that this results in liver uptake of the radioactivity. Likewise, coalescence of the colloid leads to lung uptake.

We also determined the effect of standing on the prepared trimetaphosphate radiopharmaceutical. A sample was prepared to contain the usual 50:1 weight ratio of TMP to tin, with a final solution pH of 6.0–6.2. Groups of three rats were given the usual dose at various periods after the radiopharmaceutical was prepared. Three hours after dosing, the animals were sacrificed. The bone uptake of the hind leg was found to remain the same over the period of 0.5 hr to 16 hr after preparation. Liver assays of all of the rats showed no liver uptake exceeding 0.8%, and there was no correlation between amount of liver uptake and the time after preparation.

The final pH of the solution was found to have no effect on the bone and liver absorption in the range of pH 5.5–7.0. However, at pH 7.5, the liver uptake tripled and, at pH 8, it had increased eightfold. This was accompanied by a slight decrease in bone uptake.

A number of attempts were made to prepare the complex between the ^{99m}Tc and the phosphate by using "reducing agents" other than stannous chloride. Since the exact chemical function of these agents has not been thoroughly established, they are merely called adjuvants in Table 2 where these data are reported. Again, groups of three rats were used for each evaluation, which was carried out under the established conditions.

The bone uptake data obtained for the trimetaphosphate radiopharmaceuticals prepared using electrolytically generated stannous ions were measured as a function of the tin concentration, which was adjusted by controlling the electrical current and time of electrolysis. For 50 mg of trimetaphosphate, it was found that, with agitation, 1–3 coulombs gave a radiopharmaceutical exhibiting the optimum hind-leg bone uptake of about 2.2% of the administered dose. Using 0.75 coulombs with agitation or 1.5 coulombs without gave decreased uptake values.

TABLE 2. EFFECT OF VARIOUS HEAVY METAL ADJUVANTS

Adjuvant (per 100 mg phosphate)	Phosphate	Percent hind-leg bone uptake at 4 hr
Iron-ascorbic acid (5 mg)	Trimeta	0.3 ± 0.1
Ferrous sulfate (1 mg)	Trimeta	0.6 ± 0.2
Ferrous sulfate (2 mg)	Trimeta	0.6 ± 0.2
Ferrous sulfate (5 mg)	Trimeta	0.3 ± 0.1
Electrolytic zirconium (1.0 mg)*	Trimeta	0.6 ± 0.3
Electrolytic zirconium (0.78 mg)*	Trimeta	0.7 ± 0.3
Electrolytic zirconium (0.50 mg)*	Trimeta	0.6 ± 0.3
Electrolytic zirconium (0.25 mg)*	Trimeta	0.6 ± 0.3
Electrolytic iron (1.0 mg)*	Trimeta	0.8 ± 0.3
Electrolytic iron (0.5 mg)*	Trimeta	1.0 ± 0.4
Ferrous sulfate (3 mg)	PP55	1.0 ± 0.4
Ferrous sulfate (3 mg)	Tripoly	1.1 ± 0.4
Ferrous sulfate (5 mg)	Tripoly	1.0 ± 0.4
Cuprous chloride (3 mg)	Tripoly	0.9 ± 0.4
Cuprous chloride (2 mg)	Tripoly	0.9 ± 0.4
Cuprous chloride (1 mg)	Tripoly	0.8 ± 0.3
Cuprous chloride (2 mg)	Trimeta	1.1 ± 0.4
Chromous chloride (2 mg)	Trimeta	0.9 ± 0.3
Chromous chloride (3 mg)	Trimeta	0.9 ± 0.4

* Electrolytic reductions were undertaken using the appropriate metal electrodes, current, and time so as to produce the required amount of cation as calculated from Faraday's laws.

TABLE 3. BIOLOGIC DISTRIBUTION OF ^{99m}Tc-LABELED TRIMETAPHOSPHATE IN RATS

Tissue	Percentage of administered radioactivity		
	2 hr	4 hr	6 hr
Lung	0.24 ± 0.01	0.11 ± 0.03	0.18 ± 0.06
Heart	0.08 ± 0.02	0.06 ± 0.03	0.05 ± 0.02
Blood	2.72 ± 0.62	0.68 ± 0.17	0.42 ± 0.10
Kidneys	3.34 ± 0.40	3.05 ± 0.80	2.47 ± 0.50
Gastrointestinal tract	1.72 ± 0.45	3.60 ± 0.51	2.66 ± 0.70
Spleen	0.06 ± 0.02	0.07 ± 0.01	0.08 ± 0.02
Liver	0.60 ± 0.14	0.33 ± 0.15	0.42 ± 0.18
Muscle	3.22 ± 0.50	2.18 ± 0.35	1.17 ± 0.32
Tibia and fibula	1.00 ± 0.05	0.84 ± 0.05	0.99 ± 0.05
Femur	1.15 ± 0.10	1.26 ± 0.15	1.21 ± 0.14
Marrow	0.82 ± 0.10	0.51 ± 0.14	0.58 ± 0.20
Total bone (average)	42.3	44.9	45.1
Urine (including bladder contents)	34.2 ± 11.0	38.2 ± 8.0	41.8 ± 6.5

Table 3 illustrates results of biodistribution studies as a function of time. Another time study showed that the bone uptake level remained constant from 2 to 28 hr after dosing with the radiopharmaceutical, with about 2.2% of the administered dose appearing in a hind-leg bone; whereas, for a commercial polyphosphate radiopharmaceutical, the same bone uptake decreased from 1.8% at 3 hr to 1.4% at 24 hr. The blood level was several times higher for the polyphosphate than for the trimetaphosphate at 3 hr or longer. Figure 1 presents scans of a 3-kg rabbit dosed with the trimetaphosphate radiopharmaceutical.

Acute toxicity. Solutions of the tin-trimetaphosphate preparation without addition of the ^{99m}Tc-pertechnetate were administered to groups of 30 white mice (each weighing 25–30 gm) for each dose level. In all cases the trimetaphosphate-to-tin ratio was kept at 50:1, and doses of 50, 100, 250, 400, 500, and 1,000 mg/kg of body weight were slowly administered via the tail vein so that the total time for the injection was 30–60 sec. The mice were maintained for 14 days and, at the end of that period, those surviving were autopsied and the tissues and organs were examined for gross abnormalities.

The results showed the LD_{50} to be slightly greater than 1,000 mg/kg body weight since 14 of the 30 mice dosed at this level died within 2 min of the injection time while none died thereafter. At all other dose levels at least 85% of the mice in each group survived. No gross pathologic changes were found in any of the animals except for some enlargement of the kidneys in three of those surviving the highest dose. In a parallel study, the LD_{50} of the tin-polyphosphate composition derived from commercially available kits was evaluated for acute toxicity in a similar fashion using sets of 25 mice. In this case, the LD_{50} was found to be somewhat greater than 125 mg/kg of body weight since at this level 12 mice died within 5 min of the dosing time, while at a level of 150 mg/kg 19 mice responded similarly.

DISCUSSION

Chain phosphates. Publications over the past few years (*10,13*) have shown considerable disagreement regarding the optimum chain length of the polyphosphates with respect to bone uptake properties. Although this paper is not primarily intended to resolve this question, the data in Table 1 indicate that the maximum bone uptake for the chain phosphates occurs around the tripolyphosphate composition. Indeed, all three of the lower-molecular-weight condensed phosphates—the pyro-, tripoly-, and trimetaphosphate—show approximately the same high level of bone uptake in the rat, with about 45% of the ^{99m}Tc being deposited on the skeleton.

Study of the bone uptake of the chain phosphates has been plagued with apparent irreproducibility

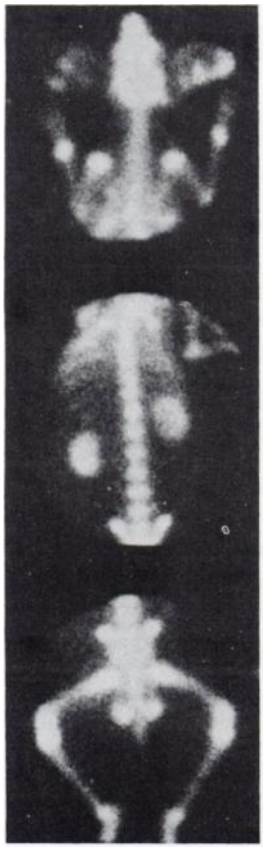
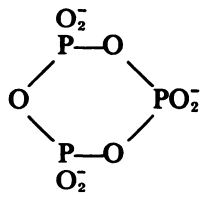


FIG. 1. Whole-body image of a New Zealand white rabbit 5 hr after dosing with 5 mCi of a 1.0 mg/kg body-weight dose of trimetaphosphate radiopharmaceutical prepared with electrolytically generated tin. Imaging was performed on Searle Radiographics camera with a low-energy collimator and a preset count of 500,000.

from batch to batch. The solubility fractionation studies reported here indicate that this is not attributable to the size distributions of phosphate chains in nonhydrolyzed preparations (without appreciable orthophosphate) exhibiting about the same average measured chain length but different bone uptake values. It is possible that the observed differences in bone uptake are attributable to trace impurities such as zirconium or platinum ions picked up from the container in which the melt was held prior to vitrification. However, samples of PP46, PP55, and PP138, as well as the sodium tripoly- and trimetaphosphates, did not show any correlation between detectable trace-metal concentrations and bone uptake. The trace-metals were determined by activation analysis following a 20-min irradiation in the Oak Ridge Research Reactor at a neutron flux of 2.5×10^{13} allowing a long preassay decay for depletion of the ^{24}Na activity. Although no zirconium or platinum was found because of their low analytical sensitivities under the test conditions, trace amounts of antimony, scandium, iron, zinc, and iridium were observed but in similar quantities in both high and low bone-uptake vitreous phosphates.

Trimetaphosphate. When we started the present study in 1971, it seemed to us that the necessary interaction between the chosen condensed phosphate, the ^{99m}Tc, and the reducing agent [which presumably converts the technetium (*14*) from anionic to cationic form so that it may interact] did not demand that the phosphate exhibit calcium complexing. Indeed a phosphatic composition having at least two phosphorus atoms to lend stability to the complex involved in the transport to the bone tissues should be much safer to use if it did not exhibit appreciable complexing of calcium (*15*). Such a combination of properties is found in the family of cyclic metaphosphates, particularly in the smallest stable member, the trimetaphosphate, whose anion structure is shown below (*4*):



Our results demonstrate that the smallest cyclic phosphate, the sodium trimetaphosphate, is at least as efficacious as any other phosphorus compound with respect to the bone uptake of ^{99m}Tc in rats. As illustrated in Fig. 1, it is also a good bone-visualizing agent. Optimum bone uptake is observed using preparations containing 25–50 mg of this compound per mg of stannous chloride, with minimum localization in the liver. Since all of the trimetaphosphates (whether labeled with ^{99m}Tc or not) are expected to compete for the available bone absorption sites, a desirable material should contain a maximum ratio of labeled to unlabeled trimetaphosphate. This suggests that the phosphate-to-tin ratio should be small so that there would be no unlabeled phosphate left. If, however, the ratio of phosphate to tin were made too low, a stannous oxychloride colloid (*16–18*) could be formed from hydrolysis, particularly in the pH range of 5.5–7.5. On formation, stannous oxychloride appears as a fine colloid, but on standing the particles coalesce and become larger.

In accordance with the known stability (*4*) of the trimetaphosphate ion in the pH region employed, aging of the dosing solution has no deleterious effect on the bone uptake. The use of the trimetaphosphate also seems to be advantageous in that the level of absorption of the ^{99m}Tc (corrected for decay) reaches a constant value in about 2 hr and remains at that level for at least 28 hr. Throughout this time, however, there is constant clearing of the trimetaphosphate from the blood, presumably into the urine. By comparison, the polyphosphate kit exhibited a decrease in bone uptake from 3 to 24 hr, along with a considerably higher blood level at all times. This polyphosphate behavior may be possibly due to an enzymatic breakdown of the deposited complex, leading to release of the technetium.

The pH of the dosing solution should be kept below 7 in order to avoid an increase in liver uptake, and it appears that our pH range of 6.0–6.2 is satisfactory. Without arguing over the exact role of the stannous ion here, we do think that the choice of tin over other heavy metals in their lower oxidation states is an appropriate one, and that a convenient way of injecting the stannous ions is by electrolysis between tin electrodes for a fraction of a minute. The LD_{50} studies conducted on the appropriate 50:1 mixture of sodium trimetaphosphate with stannous chloride have shown a low degree of toxicity, in agreement with a prior study (*19*) on various phosphates including sodium trimetaphosphate. Indeed, the toxicity of the radiopharmaceutical mixture is primarily due to stannous chloride, for which an LD_{50} of 12 mg/kg of body weight has recently been reported for rats (*20*), and an LD_{50} of 20–50 mg/kg weight was found (*21*) earlier for dogs. Our observed LD_{50} of 1,000 mg/kg for rats, using a composition having a 50:1 trimetaphosphate-to-tin ratio, corresponds to 20 mg/kg of body weight with respect to the stannous chloride. Several factors may contribute to the lower tin toxicity in the mixture of stannous chloride with sodium trimetaphosphate. These include the higher pH of the mixture compared to that of stannous chloride, a relatively slow release

into the kidneys (inferred from our ^{99m}Tc data), and the possibility of complex formation between the tin and the trimetaphosphate ions.

Biodistribution studies indicate the radiopharmaceutical is similar in its distribution pattern to that reported for the diphosphonate and pyrophosphate complexes studied in rabbits, swine, and dogs (*3,6,22*). Blood levels are considerably lower at 4 and 6 hr than was observed for the linear polyphosphates in rabbits (*2*), and are at least as low as that cited for the substituted diphosphonate (*3,22*). The excretion rate, as is shown by urine levels, indicates the radiopharmaceutical is similar to pyrophosphate and somewhat slower than that reported for the diphosphonates.

The unusually low toxicity (*19*), plus the wide range of permissible dosage levels without marked liver uptake, indicate that this cyclic phosphate promises to be a better bone-visualizing agent for humans than any of the other phosphorus compounds that are used today for this purpose. Because the cyclic phosphates are poor complexing agents compared to the chain phosphate or the methylene-bridged phosphonates, the former are not usually considered for applications such as the one treated herein. In this particular case, however, we believe that the poor calcium-sequestering ability of the trimetaphosphate ion is highly advantageous from the viewpoint of low toxicity, yet there is sufficient interaction with the technetium to transport it to the bone. Accordingly, we have conducted studies with the ^{99m}Tc-Sn-TMP composition in more than 450 selected human subjects; the results will be presented in another communication.

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