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POLYPHOSPHATES: A CHEMICAL ANALYSIS OF AVERAGE CHAIN LENGTH AND THE RELATIONSHIP TO BONE DEPOSITION IN RATS

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Technetium-99m-tin-polyphosphate for bone visualization has great potential for the diagnosis of skeletal disease. However, there is a controversy in the current literature as to the chemical analysis of the polymer and its distribution in bone.

The average chain lengths ($\bar{n}$) ranging from 8 to 47 of several different polyphosphates were determined by end-point titrations and by phosphorous NMR spectra obtained with a Varian HA-100 D-15 spectrometer. Biological distribution of the respective chain lengths was assessed in Sprague-Dawley rats and the results reported as percent of injected dose per gram tissue.

The data indicated that the shorter chain lengths of polyphosphate yielded the highest bone concentration. As the chain length increased, the percent of uptake of the activity in the bone decreased. The highest uptake among the polyphosphates tested was associated with degraded long-chain polyphosphates.

Technetium-99m-tin-polyphosphate could provide the clinician with a valuable radiopharmaceutical for the diagnosis of skeletal disease (*1*). Of the radionuclides available for bone imaging, the 6 hr half-life of ^{99m}Tc, its 140 keV energy, and its ready availability have made this ^{99m}Tc-Sn-polyphosphate complex a most promising diagnostic tool.

Polyphosphates are dehydrated orthophosphate units connected by phosphorous-oxygen bonds. The number of connected units of a straight chain-polyphosphate is designated as $\bar{n}$, the average chain length. Recent literature reports (*2–4*) indicate that several of the ^{99m}Tc-polyphosphate complexes ($\bar{n}$ = 3–46) are useful for skeletal imaging in diagnostic nuclear medicine.

Since polyphosphates hydrolyze under conditions such as time, moisture, and temperature (*5*), the chain lengths of the polyphosphates used in this study had to be determined first by titration and NMR. After these determinations were made the distribution of seven average chain lengths in rat bone were studied to discover which $\bar{n}$ produced maximum skeletal uptake.

METHODS

The chemical analysis of chain phosphates has been studied extensively by Van Wazer (*6*) who states that end-point titration and nuclear magnetic resonance spectrum are reproducible methods for determination of average chain lengths. Both methods were used in this study to obtain the average chain length, $\bar{n}$, of seven samples of polyphosphates. Six of these samples were labeled by the manufacturers to have average chain lengths of 6, 12–14, 21, 35, 46, and 47 ± 10. The seventh sample was obtained by autoclaving a solution of $\bar{n} = 21$.

End-point titration. According to the literature (*6*), a titratable proton that reacts as a weak acid is associated solely with the terminal end groups of the chain phosphate. Assuming that the polymer is a straight chain, a titration between pH 4.5 and 9.0 is related to $\bar{n}$ by the following (*6*):

$$\bar{n} = 2\,(\text{Total P})/(\text{End Group P}). \qquad (1)$$

By substitution, then

$$\bar{n} = 2\left[\frac{\%\ \text{P} \times \text{mg sample}}{(\text{Ml}_A)\,(\text{M}_A)\,(\text{R})\,[3100]}\right]$$

where A = titrant

M = molarity

R = 1, reacting ratio.

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TABLE 1. AVERAGE CHAIN LENGTH ASSIGNMENTS TO POLYPHOSPHATES

$\bar{n}$ (as indicated by manufacturer)	$\bar{n}$ (titrations)*	$\bar{n}$ (NMR)*
47 ± 10 (kit)‡	10.5 ± 1.6‡	9.1 ± 0.3
46‡	6.7 ±0.4‡	8.3 ± 0.3
35	29.8 ± 1.2	21.0 ± 0.8†
21	17.1 ± 1.1	20.0 ± 1.3
12 — 14	12.1 ± 1.2	9.1 ± 0.1
6	11.0 ± 0.4	12.0 ± 1.1
21 (autoclaved)	3.5 ± 0.2	—

* Statistical variations for three determinations.

† NMR performed 10 days after the test solution was prepared. Low NMR value probably indicates degradation of chain due to hydrolysis.

‡ Significantly different at the 0.5% level ($P < 0.005$).

Samples of polyphosphates with varying chain lengths were accurately weighed and dissolved in distilled water. The pH of each solution was adjusted to 4.5 with dilute HCl using a standardized pH meter and titrated with standardized NaOH. Calculations for $\bar{n}$ were performed using the above formula and are summarized in Table 1.

NMR study. The ^{31}P nuclear magnetic resonance spectra were obtained using a Varian HA-100D-15 spectrometer operating at 13.56 MHz. An external reference sample of 85% H_3PO_4, the inner tube of a coaxial tube arrangement, provided the frequency lock signal. The signal-to-noise ratios of the spectra were enhanced by signal averaging using a Varian C-1024 time-averaging computer. Assignments of resonances due to terminal and nonterminal phosphorus atoms in the polyphosphates were made by comparing the observed chemical shifts to those reported by Callis, et al (7) for tri- and tetrapolyphosphates. The chemical shift (relative to 85% H_3PO_4, external) of the terminal phosphorus varied from about +4.9 to +10.3 ppm whereas that of the nonterminal phosphorus varied from about +20.6 to +21.7 ppm apparently depending upon concentration, pH, and presence or absence of $SnCl_2$. The peak areas were broad and showed no measurable spin-spin splitting. Figures 1 and 2 are representative of the spectra obtained.

The $\bar{n}$ for each polyphosphate sample was determined by spectrum analysis using a rearrangement of Eq. 1 as follows:

$$\bar{n} = 2\left[\frac{P_M + P_E}{P_E}\right] = 2\left[\frac{P_M}{P_E}\right] + 2, \qquad (2)$$

where P_M and P_E are the areas under the peaks corresponding to the nonterminal and terminal phosphorus atoms in the polyphosphate chains, respectively.

Labeling procedure. The ^{99m}Tc-Sn-polyphosphates were prepared by dissolving 50 mg of the respective polyphosphate in 1 ml of sterile water, followed by 1 ml (1 mg) of $SnCl_2 \cdot 2H_2O$. The solution was then incubated at 37°C for 30 min. After incubation, 4 ml of sodium pertechnetate (^{99m}Tc) were added and the final solution filtered through a 0.22-micron Millipore filter.

Bio-distribution. The organ distribution of the various chain lengths of polyphosphate labeled with ^{99m}Tc were studied in seven groups of Sprague-Dawley male rats weighing between 150 and 170 gm. The rats were injected i.v. through the tail vein with approximately 10 μCi of ^{99m}Tc-polyphosphate representing 1.7 mg of polyphosphate. Each group received polyphosphates of average chain lengths (as indicated by the manufacturer) of 47 ± 10, 46, 35, 21, 12–14, 6, and the autoclaved $\bar{n} = 21$, respectively. The animals were killed 3 hr postinjection, and the lungs, liver, spleen, kidney, and samples of blood, and muscle were removed and counted for ^{99m}Tc activity in a Nuclear-Chicago Auto Gamma

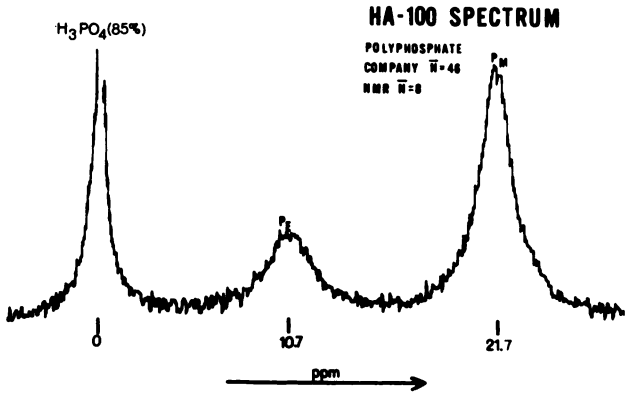

FIG. 1. Ha-100 spectrum—polyphosphate labeled $\bar{n} = 46$ has been proven to be reduced to $\bar{n} = 8$ as shown by NMR spectrum.

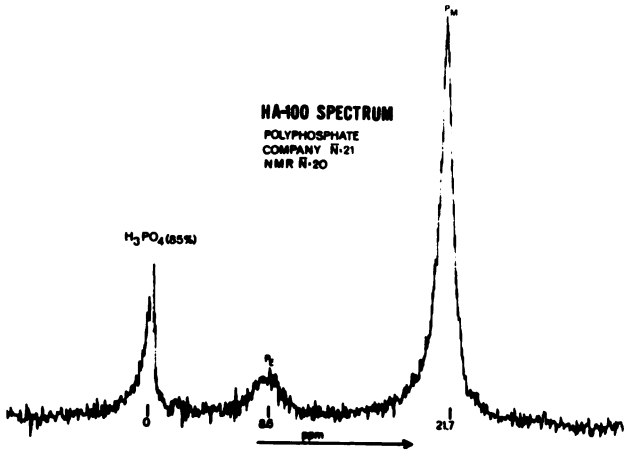

FIG. 2. Ha-100 spectrum—polyphosphate labeled $\bar{n} = 21$ is shown to agree with NMR spectrum.

TABLE 2. ^{99m}Tc-LABELED POLYPHOSPHATE WITH AVERAGE CHAIN LENGTH ($\bar{n}$) AND PERCENT OF ADMINISTERED ACTIVITY PER GRAM ORGAN DISTRIBUTION IN RATS*

Organ	(Autoclaved) $PP_{21(4)}$†	$PP_{6(10-13)}$	$PP_{12-14(9-13)}$	$PP_{21(16-21)}$	$PP_{35(27-30)}$	$PP_{46(6-8)}$	$PP_{47\pm10(9-12)}$
Blood	0.19 ± 0.05	0.89 ± 0.08	0.83 ± 0.08	0.38 ± 0.07	0.82 ± 0.05	0.25 ± 0.19	0.21 ± 0.07
Lung	0.24 ± 0.08	0.28 ± 0.09	0.34 ± 0.04	0.24 ± 0.05	0.36 ± 0.03	0.17 ± 0.07	0.17 ± 0.05
Liver	0.26 ± 0.16	0.75 ± 0.17	0.39 ± 0.08	0.14 ± 0.05	0.43 ± 0.07	0.32 ± 0.10	0.14 ± 0.05
Spleen	0.12 ± 0.09	0.14 ± 0.04	0.25 ± 0.06	0.11 ± 0.05	0.25 ± 0.06	0.13 ± 0.02	0.10 ± 0.06
Kidney	2.3 ± 1.2	6.2 ± 0.7	5.8 ± 1.1	4.0 ± 1.0	10.9 ± 1.8	3.1 ± 2.3	1.9 ± 0.7
Muscle	0.1 ± 0.05	0.12 ± 0.05	0.09 ± 0.01	0.06 ± 0.02	0.09 ± 0.01	0.07 ± 0.02	0.07 ± 0.01
Average bone	2.5 ± 0.4	1.3 ± 0.2	1.3 ± 0.1	1.2 ± 0.3	0.7 ± 0.12	1.8 ± 0.4	2.4 ± 0.3
Limb with marrow	3.0 ± 0.21	—	—	2.2 ± 0.4	—	3.2 ± 0.00	3.6 ± 0.50
Limb without marrow	3.1 ± 0.35	—	—	1.7 ± 0.15	—	3.2 ± 0.25	3.1 ± 0.53
Ratios							
Bone/blood	13.1	1.4	1.6	3.2	0.85	7.2	11.4
Bone/liver	9.6	1.7	3.3	8.5	1.6	5.6	17.1
Bone/muscle	25.0	11.0	14.4	20.0	7.7	25.7	34.1

* Each value represents the average of five rats, 3 hr postinjection except for limb with and without marrow, which is an average of three rats.

† Numbers in parenthesis indicate the average chain length ($\bar{n}$) obtained by end-point titration and NMR spectra.

scintillation counter. To determine bone deposition, samples, including the femur, tibia, spine, rib, and pelvic bones, were counted and the percent of administered activity per gram of average bone was calculated. To estimate marrow activity, a femur and tibia were removed, weighed on an analytical balance, and counted. The percent of administered activity per gram of bone was calculated. The same limb was then washed free of marrow and the percent of administered activity per gram recalculated.

RESULTS

Table 1. Table 1 summarizes the NMR and titration data. The two experimental methods of determining $\bar{n}$ agree statistically in the 97% confidence level. The selected samples of $\bar{n} = 12$–14, $\bar{n} = 21$, and $\bar{n} = 35$ agree experimentally with NMR and titration results; however, the polyphosphates labeled 46 and 47 ± 10 were significantly shorter than those indicated by the manufacturer ($p > 0.005$). It is apparent that the polyphosphates labeled $\bar{n} = 46$ are reduced to $\bar{n} = 8$ as shown by the end-point titrations and the NMR spectrum (Figs. 1 and 2). The autoclaved sample $\bar{n} = 21$ reduced to $\bar{n} = 4$. The NMR spectrum of autoclaved $\bar{n} = 21$ occurred at the frequency of the lock signal and was determined to be mostly orthophophosphate units.

Table 2. Table 2 summarizes the organ distribution as percent of administered activity per gram of tissue for the various average chain lengths of polyphosphates. The data indicated that the percent uptake in rats of ^{99m}Tc-Sn-polyphosphate increased with a decrease in average chain length. The increased uptake in the bone was not accompanied by an increase of activity in surrounding tissue as shown by the bone/tissue ratios.

The polyphosphate labeled $\bar{n} = 21$ was autoclaved because it was expected that autoclaving would reduce the average chain length of that sample and consequently increase the bone deposition. As shown in Table 2, the autoclaved PP_{21} ($\bar{n} = 4$) has a higher uptake than the nonautoclaved PP_{21} ($p < 0.001$). There were no significant differences in bone uptake among chain lengths 6($\bar{n} = 10$–13), 12($\bar{n} = 9$–13), and 21($\bar{n} = 16$–21). However, PP_{35}($\bar{n} = 27$–30) had a lower uptake than PP_{21}($p < 0.02$).

It is well documented that condensed phosphates will decompose back to the orthophosphate form (5). Since orthophosphates may localize in the marrow, studies were performed to determine that the higher uptake of the shorter chain lengths was not due to marrow uptake. There was no significant difference in uptake associated with bone containing marrow and bone without marrow for any of the chain lengths studied.

DISCUSSION

The controversy in the literature over which $\bar{n}$ of PP yields the best uptake in bone might be resolved because as this study shows, some longer chain lengths hydrolyze to shorter chain lengths. Therefore, before one reports which $\bar{n}$ has greater uptake in bone, it is imperative that the $\bar{n}$ be accurately determined. Since the NMR and titration methods as described by Van Wazer were accurate and reproducible, it is recommended that one of these methods be used for identifying the average chain

length ($\bar{n}$) before labeling. To determine the $\bar{n}$ after labeling, the methods of labeling used in this study did not significantly change $\bar{n}$ (*8*).

Furthermore, it appears that polyphosphates of shorter average chain lengths give the maximum concentration of activity in the rat skeleton. The implications of these results for clinical work should lead to further research on the bone uptake of various chain lengths.

In a study such as this, the possibility does exist that a minor contaminant with a high specificity for bone may have been introduced through the degradation of the longer chains. This might explain the higher uptakes of the lyophilized kit ($\bar{n} = 10$) and the autoclaved sample $\bar{n} = 4$. The resolution of this issue, however, was not within the scope of this paper.

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