THE TOXICITY OF Sn-PYROPHOSPHATE: CLINICAL MANIFESTATIONS PRIOR TO ACUTE LD_{50}

John S. Stevenson, William C. Eckelman, Philip Z. Sobocinski, Richard C. Reba, Edward L. Barron, and Sheldon G. Levin

Armed Forces Radiobiology Research Institute, Bethesda, Maryland, Washington Hospital Center, and George Washington University, Washington, D.C.

Following the administration of large amounts of technetium-Sn-phosphate compounds in rats, tetany appears to be the primary clinical manifestation. To evaluate this phenomenon acute LD_{50} (5 min) was determined and serum calcium and arterial plasma pCO_2 were measured and related to the administered dose. The LD_{50} (5 min) for pyrophosphate was found to be 41.0 ± 1.6 mg/kg (2 s.d.) body weight whereas the LD_{50} (5 min) for polyphosphate was found to be 29.4 ± 2.0 mg/kg (2 s.d.) body weight. There was no significant difference between the slopes of the two compounds. When acute LD_{50}s are compared, polyphosphate was 1.4 times more toxic than pyrophosphate.

Arterial blood pCO_2 remained between 30 and 40 Torr until doses of pyrophosphate exceeded 35 mg/kg body weight, after which it fell sharply. Serum calcium concentration was monitored by atomic absorption spectroscopy and electrocardiography. There was a significant drop in the ionized and bound calcium concentrations beginning at an administered dose of 20 mg/kg body weight. Electrocardiographic changes consistent with hypocalcemia were recognized after injection of 12 mg/kg body weight. However, tetany was not apparent until 22 mg/kg body weight of pyrophosphate was given. Therefore, evaluation of the toxic effects of phosphate agents should not be limited to determination of LD_{50} alone but should include appropriate biochemical measurements in blood.

Technetium-99m phosphate compounds are now considered to be the radiopharmaceuticals of choice for bone scanning. We have recently described the preparation and use of ^{99m}Tc-labeled pyrophosphate for skeletal imaging (*1*). By systematically varying the parameters of ^{99m}Tc-Sn-pyrophosphate preparations, we found that the best method of preparation was obtained by adding 1.75 ml of 1.0 *M* sodium pyrophosphate (pH 10) to 0.25 ml of 1 mg/ml stannous chloride, followed by 1.50 ml $^{99m}TcO_4^-$. The final pH was 6.5. Analysis of in vivo organ distribution in rabbits indicated the importance of using doses on an mg/kg basis for comparative animal studies. We concluded that if a proper dose, on an mg/kg basis, of ^{99m}Tc-Sn-pyrophosphate is used, high-quality bone images in both animals and humans can be obtained.

This report describes the determination of the acute LD_{50} and the evaluation of the abnormal clinical manifestations observed in Sprague-Dawley rats when increasing doses of ^{99m}Tc-Sn-pyrophosphate are administered intravenously by standard injection techniques.

MATERIALS AND METHODS

The chemical nature and method of compounding ^{99m}Tc-Sn-pyrophosphate and ^{99m}Tc-Sn-polyphosphate* radiopharmaceuticals used in this study have previously been summarized (*1,2*). The acute LD_{50} for each compound was determined in Sprague-Dawley rats in the standard manner by plotting the percent mortality at 5 min after the injection as a function of injected dose (mg/kg) body weight. The rats weighed 171–246 gm. Groups of two to ten animals were initially studied at 2 mg/kg dose intervals over a 6–50-mg/kg range.

After statistical analysis, five dose levels covering the critical portion of the LD_{50} (5 min) curves for each compound were chosen and restudied using a

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For reprints contact: John S. Stevenson, Armed Forces Radiobiology Research Institute, Radiological Sciences Div., Bethesda, Md. 20014.

* Polyphosphate, prepared fresh daily, was obtained from Calgon with an average molecular weight of 1,400.

total of 44 Sprague-Dawley rats for Sn-pyrophosphate and 57 Sprague-Dawley rats for Sn-polyphosphate. Each rat was lightly anesthetized by the intraperitoneal administration of 25 mg sodium phenobarbital. The compounds to be tested were administered as a single intravenous bolus in an increasing mg/kg dose. Injections were made directly into the femoral vein through a surgical cut-down. All blood samples were drawn 15 min after the anesthesia was administered and 5 min after the phosphate agent was given intravenously.

Serum protein concentration was determined by the biuret reaction adapted to the Technicon Auto-Analyzer by Sobocinski, et al (*3*). Serum calcium concentrations were measured by atomic absorption spectroscopy (*4*) using the Jarrell-Ash Model 280 Atomsorb. Ionized serum calcium concentrations were evaluated by two methods. The first method involved serial electrocardiographic monitoring before and after administration of the pyrophosphate compound. Each electrocardiogram was obtained from standard limb leads from cutaneously implanted electrodes. Paper tracings were recorded directly on a dual-channel Hewlett Packard Model 7782H with a preamplifier Model 8811A. The second method involved calculating the Ca^{2+} concentration from the relationship between calcium and protein using the following formula based on the original McLean and Hasting's equation (*5*):

$$\text{mg } Ca^{2+}/100 \text{ ml} = \frac{6\,Ca - (P/3)}{P + 6}$$

where Ca equals total calcium in mg/100 ml, and P equals total protein in gm/100 ml.

Blood samples for determination of pCO_2, pO_2, and pH were obtained by direct aortic puncture into a heparinized tuberculin syringe and immediately transferred to the capillary cuvette of the BMS 3 MK acid/base analyzer (Radiometer-Copenhagen). Measurements of pCO_2, pO_2, and pH were obtained within 1 min after the sample was obtained. Final pH was not corrected for body temperature.

Control animals received an equal volume of normal saline and anesthetic. Blood samples for analysis were drawn 5 min after injection of the normal saline and 15 min after intraperitoneal injection of 25 mg sodium phenobarbital. The effects of anesthesia on the blood constituents were evaluated separately.

The experimental results were evaluated by the Student's t-test with the Beherens-Fisher modification (*6*). If the chance occurrence was 5% or less, the data were considered significant.

All blood samples were obtained 15 min after the injection of the anesthesia in the experimental and control animals; pyrophosphate and polyphosphate were injected 10 min after the anesthetic in the experimental animals. Since chemical and tension measurements were obtained only in those animals that survived, presumably the data are biased toward those less affected.

TABLE 1. DOSE-MORTALITY DATA

Dose (mg/kg body wt)	Number tested	Number dead	Percent mortality
Polyphosphate			
26	14	2	14
28	12	6	50
30	11	7	64
32	11	6	55
34	9	8	89
Total	57		
LD_{50}/5 min = 29.4 mg/kg body weight			
Pyrophosphate			
36	8	0	0
38	7	1	14
40	10	4	40
42	9	6	67
44	10	8	80
Total	44		
LD_{50}/5 min = 41.0 mg/kg body weight			

RESULTS

LD_{50} determinations. The critical five dose levels (midpoint of the mortality curve) of polyphosphate were selected and the results are shown in Table 1. The LD_{50} (5 min) was found to be 29.4 ± 2.0 mg/kg (2 s.d.) body weight. The dose mortality curve and the 95% interval are plotted in Fig. 1. Pyrophosphate was also administered at five separate dose levels (Table 1), and the LD_{50} (5 min)

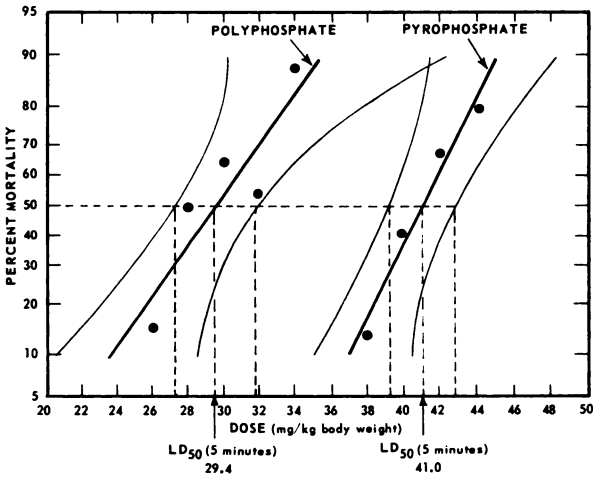

FIG. 1. Plot of acute mortality (5 min) of Sprague-Dawley rats receiving intravenous injections of polyphosphate and of pyrophosphate.

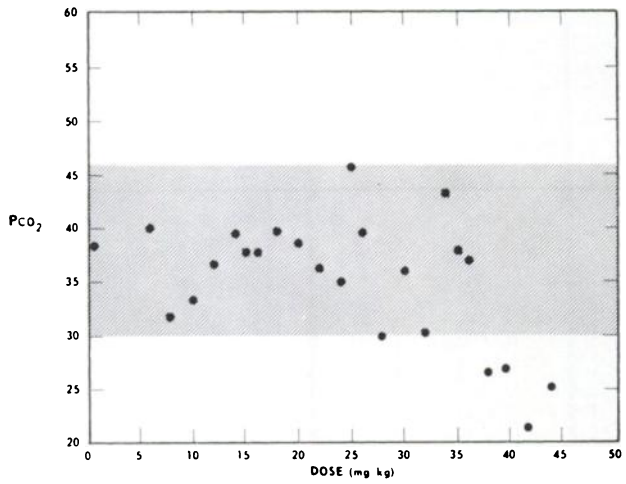

FIG. 2. Arterial blood pCO_2 (Torr) levels in rats 5 min after intravenous injection of pyrophosphate. Each point represents mean of 2–10 animals. Hatched area represents normal range ($\bar{x} \pm 2$ s.d.) of control rats.

was found to be 41.0 ± 6 mg/kg (2 s.d.) body weight. The dose response curves are presented in Fig. 1 for comparison. The LD_{50} (5 min) calculations were performed using the probit analysis methods described by Finney (*7*). The slopes of the two curves were compared and found not to differ significantly. Polyphosphate was 1.4 times more toxic than pyrophosphate over the range of doses examined.

Estimation of arterial acid content. Figure 2 shows that the pCO_2 generally remained between 30 and 40 Torr until doses exceeded 35 mg/kg body weight, after which it fell sharply. The pH (Fig. 3) remained between 7.3 and 7.5 but increased sharply with doses in excess of 35 mg/kg body weight. Acid/base values of eight control rats were done and the pO_2 values did not significantly differ from those of the experimental group.

Changes in calcium concentration. Evaluation of the serum concentrations of bound calcium, ionized calcium, and protein reveals a significant decrease in the serum-bound and ionized calcium concentrations, beginning at a dose of approximately 20 mg/kg (Figs. 4 and 5). Both bound and ionized calcium concentrations responded in a similar way with respect to the dose of pyrophosphate administered. The percent ionized calcium throughout the dose ranges tested remained around 38–42% which would be expected when both the total calcium and ionized calcium concentrations decreased to a similar extent.

Ionized calcium content is difficult to measure by direct chemical procedures so an additional biological index was added to study this phenomenon. The electrocardiogram is one of the more sensitive devices for evaluating the presence of intracellular, i.e., ionizable calcium. The electrocardiographic change consistent with the development of hypocalcemia is prolongation of the Q-T interval (*8*). As seen in Fig. 6, as the dose of pyrophosphate was increased,

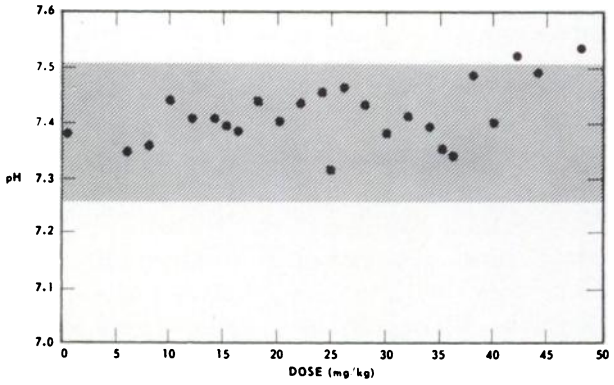

FIG. 3. Arterial blood pH 5 min after intravenous injection of pyrophosphate. Each point represents mean of 2–10 animals. Hatched area represents normal range ($\bar{x} \pm 2$ s.d.) of control rats.

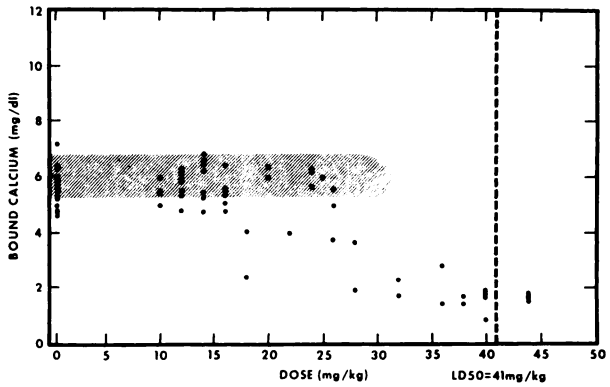

FIG. 4. Serum-bound calcium concentrations, 5 min after intravenous injection of pyrophosphate. Each point represents mean of 2–10 animals. Hatched area represents normal range ($\bar{x} \pm 2$ s.d.) of control rats.

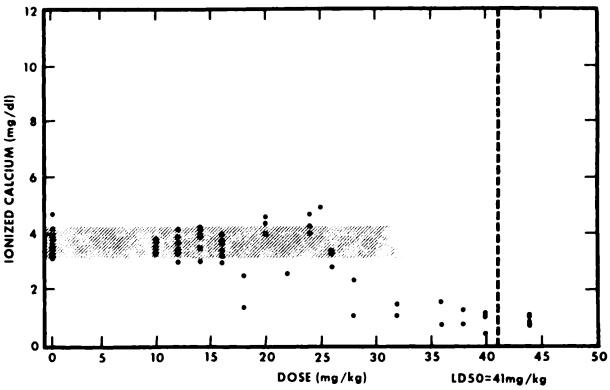

FIG. 5. Serum-ionized calcium levels 5 min after intravenous injection of pyrophosphate. Each point represents mean of 2–10 animals. Hatched area represents normal range ($\bar{x} \pm 2$ s.d.) of control rats.

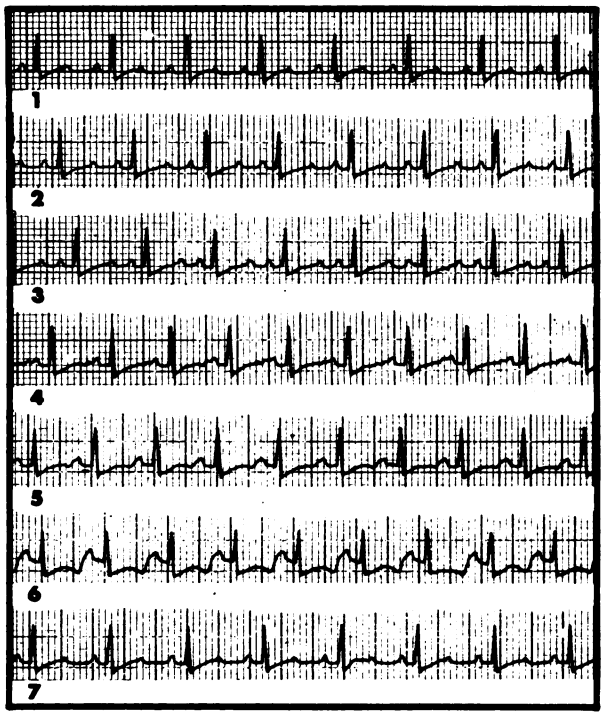

FIG. 6. Representative electrocardiograms from male Sprague-Dawley rats receiving 10 mg/kg (1), 14 mg/kg (2), 16 mg/kg (3), 18 mg/kg (4), 20 mg/kg (5), 41 mg/kg (6), (body weight) pyrophosphate intravenously as single bolus. Following intravenous administration of $CaCl_2$, electrocardiogram (6) returned to normal pattern (7).

the Q-T interval lengthened. Other electrocardiogram abnormalities which were noted included PVCs, tachycardia, ventricular fibrillation, and finally cardiac standstill. The hypocalcemic effects by EKG were not present until the administered dose reached 12 mg/kg or greater. Tetany, however, did not manifest itself until a dose of 22 mg/kg was given.

When $CaCl_2$ was administered intravenously, the hypocalcemic effect was reversed, apparently due to the replacement of the calcium bound by the pyrophosphate. The lengthened Q-T interval returned promptly to its normal duration (Fig. 6). This could only be accomplished if the $CaCl_2$ was administered before the appearance of any severe cardiac arrhythmia.

Control observations. Comparisons of serum calcium and protein concentrations were made between 20 control and 20 anesthetized Sprague-Dawley rats weighing 400–450 gm. Blood was drawn 15 min after the anesthetized animals had received 25 mg of sodium pentobarbital intraperitoneally. The only significant difference observed when anesthetized and unanesthetized animals were compared was that the level of total protein was significantly higher in the unanesthetized animals, an observation that has previously been observed (*9*). Since the formula for calculating the ionized calcium concentrations used the total protein values, the reported absolute values reflect, at least in part, the effect of anesthesia noted in the study.

DISCUSSION

It has long been recognized that after parenteral administration of injected inorganic condensed phosphates or phosphate polymers, in vivo hydrolysis releases unneutralized orthophosphate residues which may lead to metabolic acidosis (*10*). Although the electrocardiographic changes of hypocalcemia were described, Gosselin and coworkers considered severe metabolic acidosis to be the dominant toxic reaction of parenteral phosphate polymer administration (*10*). This conclusion was based on the measurement of arterial CO_2 content and fixed acid excretion in the urine of rats. By direct and indirect measurements, our study confirms the finding of hypocalcemia but we find little or no evidence to support the contention that severe metabolic acidosis is present.

By comparing the values obtained for arterial pH and pCO_2, there was no evidence that significant acidosis was present, i.e., the measured pH that would be expected for a given pCO_2 (*11*) (see Figs. 2 and 3). Indeed, one group of two animals (which received 26 mg/kg) demonstrated a mild respiratory alkalosis with pCO_2 of 39.8 Torr and pH = 7.47 (95% confidence interval for pH, 7.37–7.54).

The decrease in serum calcium concentration appears to be the primary toxic effect when ^{99m}Tc-Sn-pyrophosphate is injected in increasing quantities. The changes in the electrocardiogram and chemical determinations were detected at a per kilogram dose level considerably lower than the acute LD_{50}. Therefore, determination of the LD_{50} for the analysis of toxicity of phosphate compounds is not meaningful without evaluation of the hypocalcemic effects.

Since preservation of the calcium-phosphorus solubility product is continuously operational, it is not unexpected that an abrupt elevation of serum phosphorus concentration be followed by a decrease in calcium concentration. Reiss, et al (*12*) demonstrated in humans that small doses of phosphate resulted in a decrease in serum calcium concentration. This was shown to be accompanied by an increase in parathormone production. Since we did not determine parathormone levels, we do not know whether the effects observed in our study are due to hormone PTH influence. An alternative explanation for the decreased serum calcium levels following phosphate infusion may be that calcium phosphate is precipitated in the blood and rapidly cleared by the reticuloendothelial system, primarily the liver (*13*).

Toxicity of tin and tin colloids has previously been evaluated by Fisher (*14*). He reported that as much as 350 mg/kg of body weight of tin does not produce any histopathological damage in rabbits. The amount of tin used in the preparation of ^{99m}Tc-Sn-pyrophosphate is so small that no toxic effects from the tin content of this agent should be encountered. Administration of the saline vehicle in quantities used for each mg/kg dose level tested gave no abnormal effects.

We found that in terms of an acute lethal dose (LD_{50}—5 min) polyphosphate was 1.4 times more toxic than pyrophosphate but the evaluation of hypocalcemia and acid-base changes of ^{99m}Tc-Sn-polyphosphate was not made so a detailed comparison was not possible. The method of administration of these compounds was found to play a significant role in their toxic capabilities. If both the polyphosphate and pyrophosphate agents are serially diluted in normal saline and administered slowly or by prolonged intravenous infusion, or conjointly with $CaCl_2$, the reported LD_{50} and clinical manifestations described could be improved (*2*). We have found, however, slow administration or dilution of the pyrophosphate agent in normal saline yields poor bone images and therefore do not feel that the method of administration should be changed. We do feel that evaluation of the toxic effects of phosphate agents should not be limited to determination of LD_{50} alone but should include appropriate biochemical measurements in blood.

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REFERENCES

1. ECKELMAN WC, REBA RC, KUBOTA H, et al: ^{99m}Tc-pyrophosphate for bone imaging. *J Nucl Med* 15: 279–283, 1974

2. DUNSON GL, STEVENSON JS, COLE CM, et al: Preparation and comparison of technetium-99m diphosphonate, polyphosphate and pyrophosphate nuclear imaging radiopharmaceuticals. *Drug Intelligence and Clinical Pharmacy* 7: 470–474, 1973

3. SOBOCINSKI PZ, CANTERBURY WJ, HARTLEY KM: An automated simultaneous method for the determination of serum globulin and total protein. Bethesda, Md, Armed Forces Radiobiology Research Institute, AFRII TN73-11, 1973

4. KOPITOL KE: *Atomic Absorption Methods Manual*, vol III, Waltham, Mass, Fisher Scientific, 1970

5. HENRY RJ: *Clinical Chemistry, Principles and Techniques*, New York, Harper and Row, 1964, pp 375–376

6. CAMPBELL RC: *Statistics for "Biologist"*. Cambridge, Cambridge University Press, 1967, p 121

7. FINNEY DJ: *Probit Analysis*. Cambridge, Cambridge University Press, 1964

8. LIPMAN BS, MASSE E: *Clinical Scales Electrocardiography* (5th ed), Chicago, Year Book Medical Publishers, 1965

9. KOHN HI: Changes in the blood of the rat during ether and barbiturate anesthesia. *Am J Physiol* 160: 277–284, 1950

10. GOSSELIN RE, TIDBALL CS, MEGIRIAU R, et al: Metabolic acidosis and hypocalcemia as toxic manifestations of polymeric phosphate. *J Pharmacol Exp Ther* 108: 117–127, 1953

11. ARBUS GS, HERBERT LA, LEVESQUE PR, et al: Characterization and clinical application of the "significance bond" for acute respiratory alkalosis. *N Engl J Med* 280: 117–123, 1969

12. REISS E, CANTEBURY JM, BERCOWITZ MA, et al: The role of phosphate in the secretion of parathyroid hormone in man. *J Clin Invest* 19: 2146–2149, 1970

13. HERBERT LA, LEMAN J, PETERSON JR, et al: Studies of the mechanism by which phosphate infusion lowers serum calcium concentration. *J Clin Invest* 45: 1886–1894, 1966

14. FISHER HW: Colloidal stannic oxide. Animal studies on a new hepatolienographic agent. *Radiology* 68: 488–498, 1957