# Uptake and Biodistribution of Technetium-99m-MD$^{32}$P During Rat Tibial Bone Repair

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The present study was carried out in order to test the hypothesis that intravenously injected Tc-MDP separates into its technetium and methylene diphosphonate components in the bone, and that the technetium is preferentially taken-up by the newly-formed osteoid, while the methylene diphosphonate is taken up by the forming mineral. Uptake of Tc-MDP was studied in a rat model of primary bone formation following tibial bone marrow ablation. Each of five radiopharmaceuticals ($^{99m}TcO_4$, $^{99m}$Tc-MDP, Tc-MD$^{32}$P, $^{99m}$Tc-MD$^{32}$P or MD$^{32}$P) was injected and their uptake was followed in the whole bone as well as in the organic and inorganic phases of the bone. Irrespective of the radionuclides injected, $^{99m}$Tc was always taken-up preferentially by the organic phase, while the $^{32}$P was preferentially taken-up by the inorganic phase. When $^{99m}TcO_4$ was injected, it was not taken up by the bone at all. These results indicate that the increased incorporation of $^{99m}$Tc, when administered as $^{99m}$Tc-MDP during bone healing, reflects an enhancement in the formation of the organic matrix and not of the calcification process. The study also suggests that the $^{99m}$Tc-MDP dissociates into its technetium and methylene diphosphonate moieties, which are then adsorbed onto the organic and inorganic phases respectively.

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Technetium-99m-labeled phosphates have served as bone scanning agents for the diagnosis of a broad spectrum of pathological conditions affecting the skeleton, with $^{99m}$Tc-methylene diphosphonate ($^{99m}$Tc-MDP) being the most widely used today (*1–8*). A comprehensive review on technetium chemistry and technetium-based radiopharmaceuticals has been published by Deligny et al. (*1*) and Deutsh et al. (*9*).

The theoretical basis for the use of $^{99m}$Tc-labeled phosphates in the diagnosis of bone pathology is based on the high affinity of the phosphates for hydroxyapatite (*10*), enzymes (*11*) and immature collagen (*12*). Various authors have stressed the role of one or more of these factors in the kinetics of $^{99m}$Tc-phosphate uptake. It has been suggested that circulating $^{99m}$Tc-MDP is adsorbed selectively onto the mineral phase of forming bone (via hydroxyapatite crystals). This is based on studies carried out in vivo, in which the use of autoradiographic techniques localized the $^{99m}$Tc to mineralized tissue and not to the unmineralized osteoid or bone cells (*13,14*). In vitro studies using calcifying solutions support this finding in that they indicate that the $^{99m}$Tc is adsorbed onto the hydroxyapatite nuclei that are being formed (*15,16*).

It has also been suggested that the collagen matrix of bone is the target for $^{99m}$Tc (*12,17*). This is based on the observation of its increased uptake by bones in osteomalacia, Paget's disease and hyperparathyroidism when administered as $^{99m}$Tc-pyrophosphate. These diseases are characterized by the accumulation of large quantities of immature collagen. Phosphate-containing enzymes which are present in large quantities in bone (e.g. alkaline phosphatase) have also been suggested to be the binding sites for $^{99m}$Tc (*11*).

A major role in the kinetics of $^{99m}$Tc-phosphate uptake in bone is played by its local vascularity, with localization of $^{99m}$Tc-phosphatase being marked at highly vascularized bone surfaces (*19*). An example for this mechanism is the high uptake of the radionuclide in the cartilage bars associated with the vascular loops of the growth plate (*20*). In addition, autoradiographic studies demonstrate that the distribution of pyrophosphate in bone reflects the distribution of the blood supply (*1,21,22*).

There is still a debate in the literature as to whether this radiopharmaceutical is transported to the bone intact, or whether it is hydrolysed during its transport to the bone. Most investigators believe that the injected radiolabeled complex is transported to the bone where it is deposited (*3,5,18,26*).

A number of articles have indicated that the radiolabeled complex is hydrolyzed into its components and that the individual components are taken up by the bone (*27–29*).

It has been demonstrated that osteogenesis after injury to the rat tibial bone could serve as a model for investigating the uptake mechanism of labeled $^{99m}$Tc-phosphates (*30*). This is a highly reproducible model of bone healing, which is triggered by ablation of the tibial bone marrow and whose stages of healing are clearly definable (*31,32*).

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In a previous study from this laboratory using this model, the incorporation of three different $^{99m}$Tc-labeled phosphates was studied and correlated with the incorporation of $^{47}$Ca and $H_3{}^{32}PO_4$. The results suggest that $^{99m}$Tc could serve as a specific marker of the anabolic phase of remodeling and that it is incorporated into the organic matrix rather than into the mineral phase (*33*). The purpose of the present study was to confirm the hypothesis that the technetium is deposited in the organic matrix and the methylene diphosphonate is deposited in the mineral phase of the bone, as two separate entities.

## MATERIALS AND METHODS

### Animal Model

A total number of 180 albino rats of the Hebrew University "Sabra" strain were used in two separate experiments. The rats weighed between 350–400 g and obtained free supply of food and water. The bone marrow injury was carried out under Ketamine (Ketalar, Parke Davis, Detroit, MI) anesthesia (33 mg/kg body weight i.p.). The proximo-medial aspect of the right tibial bone was exposed 3 mm distal to the knee joint, and a dental burr (size 5/0) rotating at 5000 RPM was used to penetrate through the cortical bone into the marrow cavity. Bone marrow was evacuated by repeated washing with saline, introduced into the intrabony space by a cannula. The skin wounds were then sutured. In sham-operated rats, tibial exposure was performed without bone penetration.

### Experimental Design

In each of the two experiments, 90 rats were used. In each experiment, three groups of 30 rats were injured 14 days, 6 days and 3 days before radionuclide administration. Each group was divided into five sub-groups of six animals each. All six animals in each sub-group were injected with one of the following radioactive compounds, $^{99m}TcO_4$, $^{99m}$Tc-MDP, Tc-MD$^{32}$P, $^{99m}$Tc-MD$^{32}$P, or MD$^{32}$P 18 hr prior to death.

### Radiochemical

Technetium-99m-pertechnitate was eluted from a molybdenum generator manufactured by Soreq Nuclear Research Center, Yavne, Israel. Phosphorus-32-methylene-diphosphonate (MD$^{32}$P) was synthesized at the Radiochemistry Department of the Nuclear Research Center-Negev, Beer-Sheva, Israel by isotopic exchange of $^{32}$P during the reaction of triisopropyl phosphate with methylene-dibromide, in the presence of $H_3{}^{32}PO_4$. This was followed by hydrolysis of the tetraalkyl ester formed with HCl, as described by Moedritzer et al. (*34*). The purity of MD$^{32}$P was established by running it on precoated silica-gel TLC plates (GoF-254; Merck, Darmstadt, Germany), in a solvent consisting of a mixture of isopropanol:$H_2O$:TCA:25% $NH_4OH$ (70 ml:50 ml:5 gr:0.5 ml). The $R_f$ of the MD$^{32}$P obtained was 0.3–0.35, which was in the range of the MDP standard used. The specific activity used in this study was 1.2 mCi/mg MDP. The "cold" MDP kit was reconstituted with $^{99m}TcO_4$ and saline, according to the manufacturer's instructions, and 0.05 mCi/rat was injected subcutaneously. Technetium-99m-pertechnitate was injected subcutaneously at a dose of 0.05 mCi/rat. Phosphorus-32-methylene-diphosphonate was mixed with cold methylene diphosphonate to yield a final concentration identical to the commercial kit.

### Radionuclidic Studies

The radionuclides were administered subcutaneously in a volume of 0.3 ml, and the rats were killed 18 hr later. Tibial bones were removed, cleaned, weighed and immediately counted for $^{99m}$Tc. All bones were then demineralized in 10% trichloroacetic acid (TCA) for 6 hr, in a shaker at 60 RPM. Samples of the TCA extracts (inorganic phase) were counted, as well as the demineralized bones (organic phase). In order to confirm that demineralization with TCA removed all the calcium and phosphate that were not part of the organic matrix, a parallel experiment was carried out in which bones were ached before and after demineralization. No significant differences were found between the two methods. In order to count the $^{32}$P, bones were dissolved in 2 ml tissue solubilizer (Soluene 350, Packard), bleached with 0.2 ml of 30% $H_2O_2$ and diluted 1 + 49 with Lumax: Toluene = 1:3, before counting. All samples were counted for 1 min. Decay calculations were not necessary, as results were expressed as CPM/mg of injured over noninjured tibiae. Counting was performed using an autogamma scintillation spectrometer (Packered) and a liquid scintillation counter (Betamatic, Kontron).

### Statistical Evaluation

Radionuclide uptake was assessed by calculating the ratio of the uptake of radionuclide by treated over untreated tibia for each rat. Mean treatment/control ratio and standard error of the mean (s.e.m.) were calculated for each treatment sub-group, and the differences between groups were examined using the Wilcoxon-matched paired test. A level of $p < 0.05$ was considered significant.

## RESULTS

Bones from $^{99m}TcO_4$ injected animals did not show any significant uptake of the radionuclide above background levels, in either treated or control legs. After injection of $^{99m}$Tc-MDP, a significant increase in $^{99m}$Tc uptake (expressed as a T/C ratio of 2.04) from whole bones was observed on the 6th day after injury. At this time point there was also a significant increase in the T/C ratio in the organic phase (1.6), however no significant change in the T/C ratio was observed for the inorganic phase (1.03) (Fig. 1). When $^{99m}$Tc-MD$^{32}$P was injected into the animals and the uptake of $^{99m}$Tc followed (Fig. 2A) a similar pattern of distribution of the $^{99m}$Tc was observed. Uptake of $^{32}$P, after injecting the various labeled phosphates showed that after injection of MD$^{32}$P a significant increase in the T/C ratio was observed on the sixth and fourteenth day in the whole bone, as well as in the inorganic phase, where the T/C values ranged between 1.17–1.20. However the T/C ratios from the organic phase were significantly reduced to 0.78 and 0.72 on the sixth and fourteenth days respectively (Fig. 3). The injection of Tc-MD$^{32}$P showed a similar distribution of the isotope in the whole bone and in the inorganic phase. However the decrease in the isotope uptake in the organic phase was more marked (T/C = 0.69 and 0.57 on the sixth and fourteenth day respectively) (Fig. 4). When $^{99m}$Tc-MD$^{32}$P was injected, a significant increase in the T/C ratios for the whole bone and the inorganic phase (1.33 and 1.37 respectively) were found for $^{32}$P, only

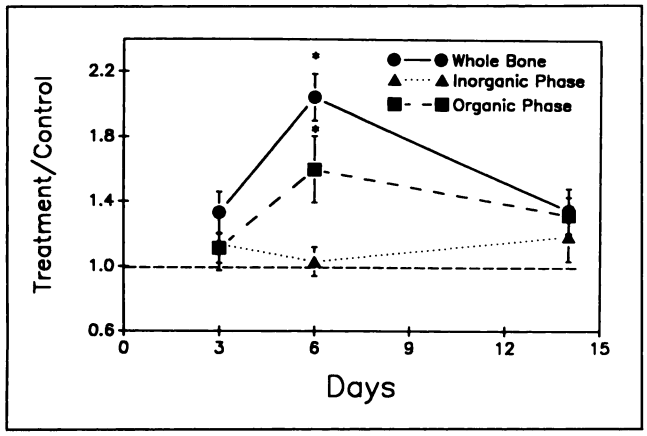


**FIGURE 1.** Uptake of $^{99m}$Tc after injection of $^{99m}$Tc-MDP at 3, 6 and 14 days of tibial bone healing. The data are expressed as mean ± s.e.m. of the treatment-to-control ratio. *Significant difference between the treated and control legs using the Wilcoxen-matched paired test ($p < 0.05$).

on the sixth day of healing, while the T/C ratio in the organic phase was 0.68 (Fig. 2B).

## DISCUSSION

The results of this study indicate that while the compound Tc-MDP has the ability to be incorporated into bone, the $^{99m}$Tc fragment is not adsorbed by it. Injection of $^{99m}$Tc-MDP leads to a significant increase in the uptake of the label by the whole bone on the sixth day after injury. At this time period, primary bone formation is the dominating process at the site of injury and no resorption is observed (*35*). This finding is in agreement with previous observations from our laboratory (*33*), and suggests that $^{99m}$Tc-MDP can provide a measure of net bone formation during healing (*33,36*) and during other pathological processes.

Our previous findings show preferential incorporation of $^{99m}$Tc into the organic phase of the injured leg, not into the inorganic phase, suggesting that there is a separation of the $^{99m}$Tc-MDP into its $^{99m}$Tc and MDP components prior to incorporation of the phosphate into the mineral phase. To provide further support for this hypothesis, we traced the incorporation into the bone of $^{32}$P injected into rats as MD$^{32}$P and $^{99m}$Tc-MD$^{32}$P. In both situations the $^{32}$P was preferentially incorporated into the inorganic phase of the injured bone, because it was the nucleus of the phosphate component. On the other hand, incorporation of $^{32}$P into the organic phase of the injured bone was significantly less than in the control leg. These results indicate that MDP is adsorbed preferentially onto the mineral phase of bone hydroxyapatite, a phenomenon that has been shown to occur in vitro (*15,16*).

The finding that $^{99m}$Tc and $^{32}$P are found in the organic and inorganic phases respectively when $^{99m}$Tc-MD$^{32}$P is injected, indicates that Tc-MDP is hydrolyzed into its technetium and phosphate components. The results of this

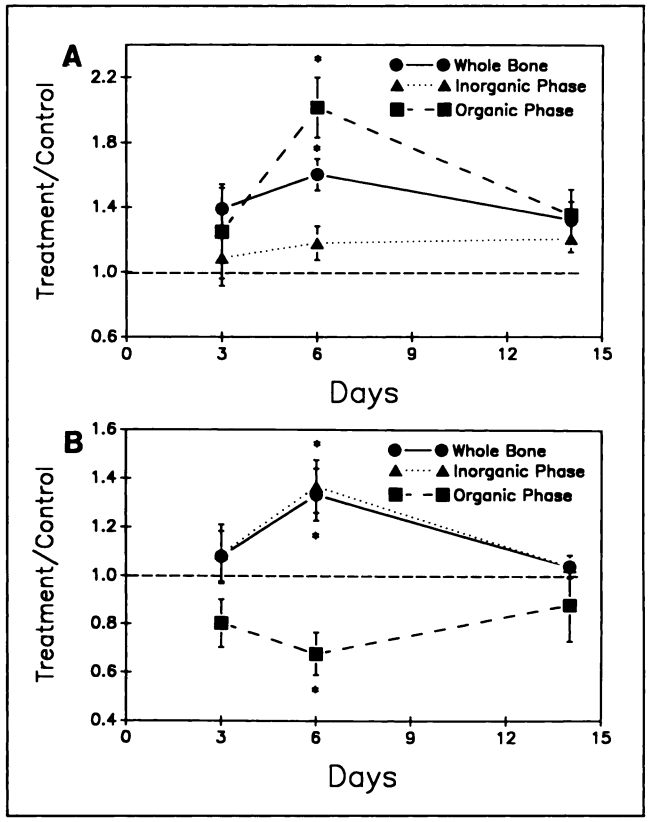


**FIGURE 2.** (A) Uptake of $^{99m}$Tc after injection of $^{99m}$Tc-MD$^{32}$P at 3, 6 and 14 days of tibial bone healing. The data are expressed as mean ± s.e.m. of the treatment-to-control ratio. *Significant difference between the treated and control legs using the Wilcoxen matched paired test ($p < 0.05$). (B) Uptake of MD$^{32}$P after injection of $^{99m}$Tc-MD$^{32}$P at 3, 6 and 14 days of tibial bone healing. The data are expressed as mean ± s.e.m. of the treatment-to-control ratio. *Significant difference between the treated and control legs using the Wilcoxen-matched paired test ($p < 0.05$).

study support the hypothesis that when $^{99m}$Tc-labeled phosphate compounds are used as a tracer of bone pathology in clinical nuclear medicine, it is specific for bone osteoid formation (*12,17,22,37*). This hypothesis explains the increased uptake found in osteomalacia, Paget's disease of bone and hyperparathyroidism, diseases characterized by the accumulation of large quantities of osteoid.

The in vivo and in vitro studies suggesting that $^{99m}$Tc-MDP is a marker of mineralization (*13–16*) are open to criticism. The in vivo (*13,14*) studies are based on autoradiography in which the differentiation between the morphologically superimposed organic and inorganic phases of the bone is difficult. In the in vitro studies (*15,16*), hydrolysis of the $^{99m}$Tc-MDP into its components can not occur because of lack of any enzymatic activity, and therefore the technetium must follow the MDP absorption onto the hydroxyapatite.

The higher specificity of $^{99m}$Tc-labeled MDP compared to $^{32}$P labeled MDP as a tracer of bone pathology is obvious from the quantitative difference noted in this study between the uptake ratio of $^{99m}$Tc-MDP (1.60–2.04) and Tc-

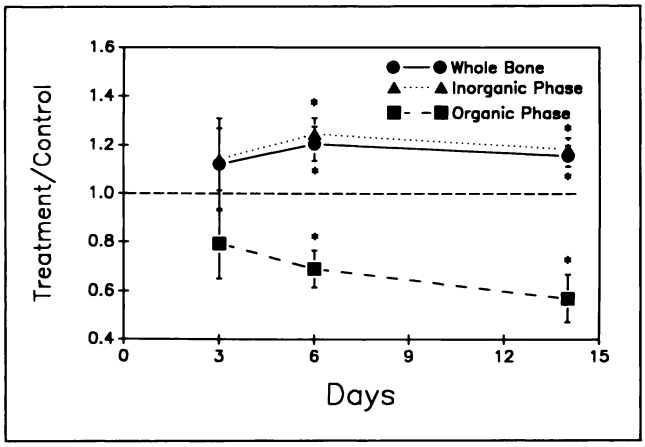


**FIGURE 3.** Uptake of MD$^{32}$P after injection of MD$^{32}$P at 3, 6 and 14 days of tibial bone healing. The data are expressed as mean ± s.e.m. of the treatment-to-control ratio. *Significant difference between the treated and control legs using the Wilcoxen-matched paired test ($p < 0.05$).

MD$^{32}$P (1.17%–1.20%). Both $^{32}$P-labeled methylene diphosphonate and $^{99m}$Tc-MDP show significantly higher uptake by healing bones as compared to the controls at 6 and 14 days post-injury. This period is the most active bone-forming period. However, there is a differential uptake between $^{99m}$Tc-labeled MDP and $^{32}$P-labeled MDP: $^{99m}$T cuptake is significant only on the sixth day, while $^{32}$P provides significant label on both 6 and 14 days post-injury. This difference may reflect the time differential between matrix production which proceeds mineralization during bone forming.

An interesting question that arises from the study is the site at which the hydrolysis of Tc-MDP occurs. The fact that $^{99m}TcO_4$ alone is not taken up by bone indicates that the $^{99m}$Tc-MDP must arrive at the bone site intact in order for the label to be detected in it. Hygeian et al. (*37*) have further suggested that hydrolysis of Tc-MDP in blood and interstitial fluid can only be minor, based on the transition time of Tc-MDP in these tissues (*38*) and therefore it is most likely that the hydrolysis occurs in the bone tissue.

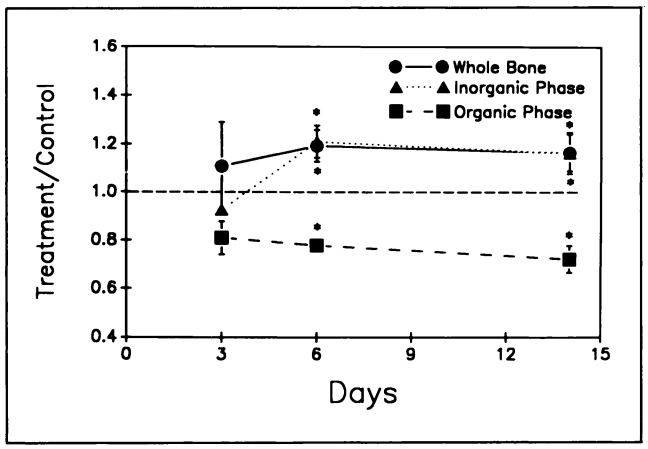


**FIGURE 4.** Uptake of MD$^{32}$P after injection of Tc-MD$^{32}$P at 3, 6 and 14 days of tibial bone healing. The data are expressed as mean ± s.e.m. of the treatment-to-control ratio. *Significant difference between the treated and control legs using the Wilcoxen-matched paired test ($p < 0.05$).

Although increased $^{32}$P uptake is specific to the inorganic phase of the healing leg, its uptake into the organic phase is lower in the healing leg than in the control one, as reflected by a T/C ratio significantly less than one. This could be explained by reutilization of $^{32}$P-labeled molecules that, after their initial incorporation, are released from the inorganic phase and are reutilized preferentially by the actively calcifying site, thus resulting in its redistribution. This does not occur with the $^{99m}$Tc moiety. In conclusion, these studies support the hypothesis that the increase of $^{99m}$Tc uptake at sites of bone remodeling is associated with bone matrix formation and not with calcification. It also suggests that the use of dual-labeled $^{99m}$Tc-MD$^{32}$P may be used to study the regulation of osteoid formation and calcification simultaneously.

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