ENZYMATIC INHIBITION OF DIPHOSPHONATE: A PROPOSED MECHANISM OF TISSUE UPTAKE

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Enzymes have been proposed as tissue receptors that bind ^{99m}Tc-stannous diphosphonate and its analogs. Incubation of diphosphonate with several enzymes demonstrated inhibition of acid and alkaline phosphatase activity but showed no effect on glutamic oxalacetic transaminase and lactate dehydrogenase activity. Complete reversal of the diphosphonate-induced inhibition of alkaline phosphatase activity occurred when calcium ion was added to the reaction. The specificity of calcium to induce reversal was dispelled when magnesium ion gave identical results. Diphosphonate-induced inhibition of acid phosphatase, however, was not reversed by calcium or magnesium.

The chemical adsorption of diphosphonate to hydroxyapatite crystals of bone was proposed by Francis and associates in 1969 (*1,2*). The skeletal uptake of ^{99m}Tc-stannous diphosphonate and its analogs, ^{99m}Tc-stannous polyphosphate and ^{99m}Tc-stannous pyrophosphate, is attributed to the same reaction. The process, however, fails to elucidate the mechanism of extraosseous uptake of these radiopharmaceuticals when neither calcification nor active bone formation are demonstrable. These agents have been observed to concentrate in recent infarcted heart muscle (*3*), infarcts of the cerebral cortex (*4*), and in breast carcinomas (*5*).

Our efforts to resolve this dilemma have led us to hypothesize the likely presence of tissue receptors that selectively bind the labeled phosphate radiopharmaceuticals. Support for this hypothesis and the possible character of the receptor was provided when histochemical stains of biopsied tissue showing increased ^{99m}Tc-stannous diphosphonate uptake demonstrated qualitative increased amounts of acid and alkaline phosphatase (*6*). This report is an extension of these observations and assesses the in vitro effect of stannous diphosphonate on enzyme activity.

MATERIALS AND METHODS

Enzyme kinetics. Five enzymes—acid phosphatase from wheat germ, alkaline phosphatase from Escherichia coli, alkaline phosphatase from chicken intestine, glutamic oxalacetic transaminase from porcine heart, and lactate dehydrogenase from rabbit muscle—were obtained commercially from the Worthington Biochemical Corp., Freehold, N.J. Enzymatic activity was determined by adding enzyme to the appropriate substrate and measuring the end product spectrophotometrically with a Beckman Acta III spectrophotometer. The substrates, reactions, end product, and spectrophotometer wavelengths (nm) for the enzymes are shown in Table 1.

A cuvette reaction vessel was used to study the enzyme kinetics. The total reaction volume was 1.5 ml and the activity of each enzyme was measured prior to adding the diphosphonate. Stannous diphosphonate, obtained from Diagnostic Isotopes, Upper Saddle River, N.J., was added in three different concentrations (1, 2, and 4 μmole) to the reaction vessels of each enzyme and their activity was monitored for preselected time periods to determine inhibition. Two observations were made at different times for each of the reactions. No significant variations in pH were recorded in the reaction vessel throughout the studies.

Calcium and magnesium ion effects. Calcium chloride ($CaCl_2$), in concentrations ranging from 0.025 to 2.3 μmole, was added to acid phosphatase and alkaline phosphatase systems containing stannous diphosphonate and its effect on the enzyme activity was determined. This was repeated later using the enzymes without stannous diphosphonate. The systems were monitored for either 6 or 10 min

Received Aug. 29, 1974; revision accepted Dec. 4, 1974.

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TABLE 1. TECHNIQUE TO DETERMINE ENZYME ACTIVITY

Enzyme	Substrate	Reaction	End product measured	Absorbance (nm)
Acid phosphatase (wheat germ)	o-Carboxyphenylphosphate	o-Carboxyphenylphosphate → salicylic acid + phosphate	Salicylic acid	300
Alkaline phosphatase (E. coli + chicken intestine)	p-Nitrophenylphosphate	p-Nitrophenylphosphate → p-Nitrophenol + phosphate	p-Nitrophenol	405
Glutamic oxalacetic transaminase (porcine heart muscle)	α-Ketoglutarate	α-Ketoglutarate oxalacetate + NADH → malate + NAD	NADH disappearance	340
Lactate dehydrogenase (rabbit muscle)	Lactate	Lactate + NAD pyruvate + NADH	NADH	340

to determine enzyme activity. The procedures were repeated using magnesium chloride ($MgCl_2 \cdot 6H_2O$) in a concentration of 1.2 μmole in both acid and alkaline phosphatase systems. The results of each reaction were plotted on linear graph paper with absorbance in nanometers on the ordinate and time in seconds on the abscissa.

RESULTS

The effect of diphosphonate on the enzyme systems is shown in Figs. 1 and 2. Figure 1A describes the linearity of the acid phosphatase activity over a 10-min period and the effective decrease (inhibition) of the enzymatic activity during the same time interval with the addition of increasing amounts of stannous diphosphonate. One micromole of stannous diphosphonate caused 26%, 2 μmole 28%, and 4 μmole 29% inhibition of acid phosphatase activity 10 min after initiating the reaction. The results are only qualitative and show no stoichiometric relationship to the diphosphonate concentration. Adding stannous chloride to the acid phosphatase system did not change the linearity of the enzyme activity.

Figure 1B demonstrates the effect of diphosphonate on alkaline phosphatase (chicken intestine) activity. The enzyme shows significant and proportional inhibition with increasing concentrations of diphosphonate. Seventy-seven percent, 83%, and 89% inhibition were recorded for 1, 2, and 4 μmole of diphosphonate, respectively, 6 min after initiating the reaction. The results were identical for alkaline phosphatase from E. coli.

Figure 2 graphs the effect of diphosphonate on the activity of glutamic oxalacetic transaminase and lactate dehydrogenase, respectively. Both enzymes showed no inhibition of their activity with increasing diphosphonate concentrations.

Adding calcium ion to the enzyme system was predicated on the assumption that its presence was important, if not essential, to skeletal uptake of ^{99m}Tc-stannous diphosphonate and its analogs. Calcium ion alone did not alter the linear activity of either acid or alkaline phosphatase (Fig. 3). However, when calcium was added to the diphosphonate-inhibited alkaline phosphatase systems, the inhibition was completely reversed (Fig. 3A). The

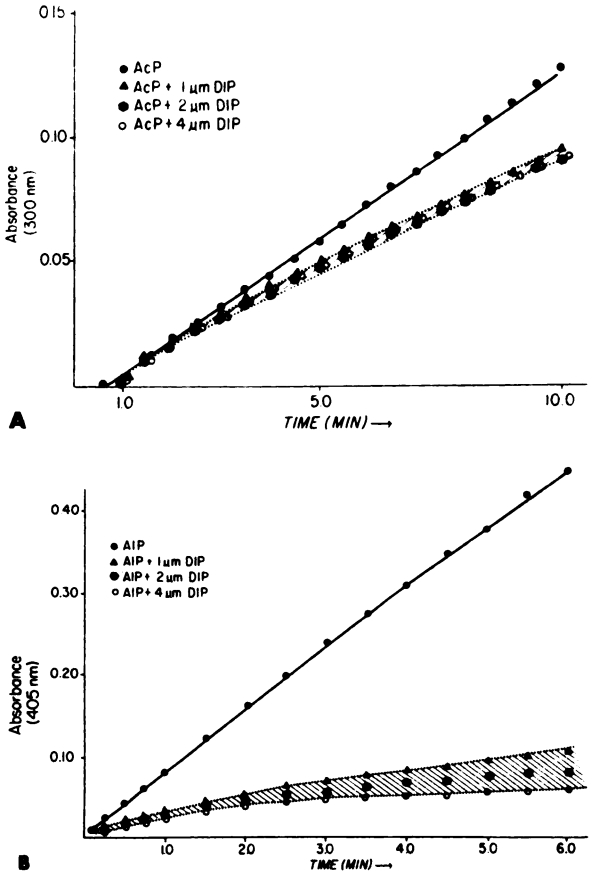

FIG. 1. Effect of diphosphonate (DiP) on acid phosphatase (AcP) activity (A) and alkaline phosphatase (AlP) activity (B).

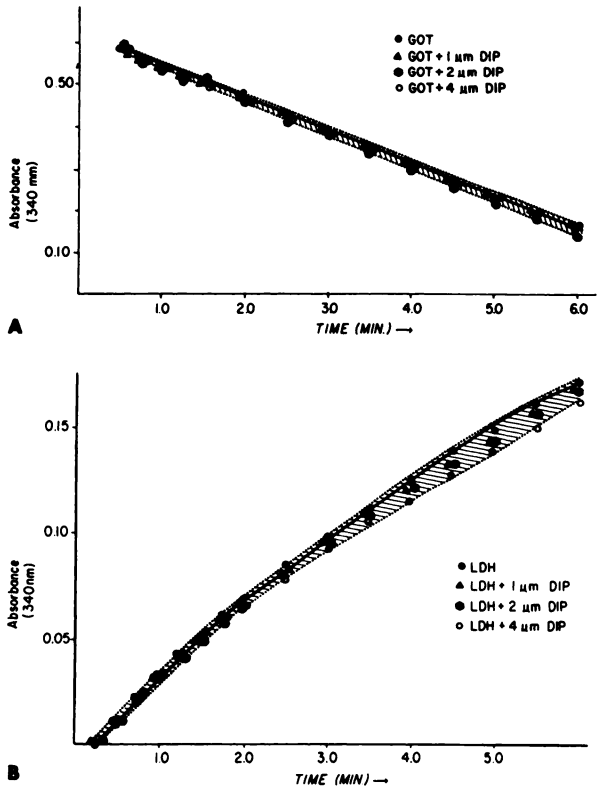

FIG. 2. Effect of diphosphonate (DiP) on glutamic oxalacetic transaminase (GOT) activity (A) and lactate dehydrogenase (LDH) activity (B).

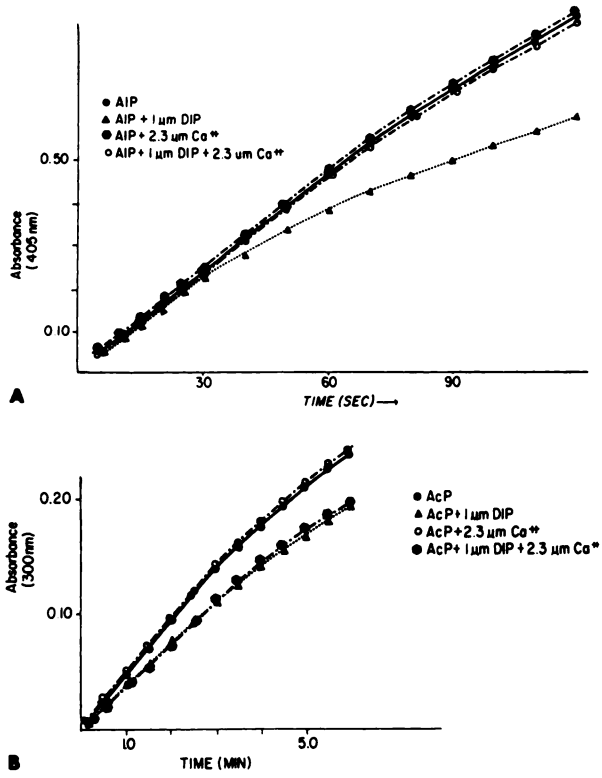

FIG. 3. Effect of calcium ion on diphosphonate-induced alkaline phosphatase inhibition (A) and acid phosphatase inhibition (B).

diphosphonate inhibition of acid phosphatase, on the other hand, was not reversed or altered by the calcium ion (Fig. 3B). The concentration of calcium ion required to initiate reversal of 1 µmole of diphosphonate-induced inhibition of alkaline phosphatase was 1.2 µmole and is shown in Fig. 4.

The uniqueness of calcium ion reversal of diphosphonate-induced inhibition of alkaline phosphatase activity was tested by substituting magnesium ion for calcium. Figure 5A describes the complete reversal of diphosphonate-induced alkaline phosphatase inhibition with magnesium whereas Fig. 5B shows that the magnesium had no effect on reversing diphosphonate-induced acid phosphatase inhibition. The molar relationship of magnesium to diphosphonate in the reaction was approximately 1:1.

DISCUSSION

Our contention that enzymatic complexing of ^{99m}Tc-stannous diphosphonate or its analogs (^{99m}Tc-stannous polyphosphate and ^{99m}Tc-stannous pyrophosphate) accounts for their uptake by body tissues is based on two observations. First, for more than half a century active bone formation (osteogenesis) has been closely linked to alkaline phosphatase activity (*7*) and the most intense areas of ^{99m}Tc-stannous diphosphonate accumulation in the human skeleton are at the sites of active osteogenesis. Osteoblastic cells are known to contain alkaline phosphatase and in instances where the number of osteoblasts are increased, as in Paget's disease of the bone, the concentration of serum alkaline phosphatase is proportionally increased and parallels the intensity of ^{99m}Tc-stannous diphosphonate uptake at the disease sites on the radionuclide skeletal image. Second, investigators from our laboratory (*6*) have demonstrated by histochemical staining the presence in increased amounts of acid and alkaline phosphatase in biopsied breast tissue of women showing breast uptake of ^{99m}Tc-stannous polyphosphate and diphosphonate. These factors formed the basis of our decision to study the phosphatase enzymes.

Both acid and alkaline phosphatase are widely distributed throughout the body with the highest concentrations of acid phosphatase appearing in the prostate and the highest concentrations of alkaline phosphatase residing in bone and liver (*7,8*). Acid phosphatase is also found in osteoclastic cells of bone and has been demonstrated in variable concentrations in breast cancers (*9*). The cellular localization of alkaline phosphatase shows a predilection for tissue membranes and may require zinc for its enzymatic activity (*10,11*). The principal function of both enzymes is to hydrolyze (catalyze) phosphoryl substrates to produce inorganic phos-

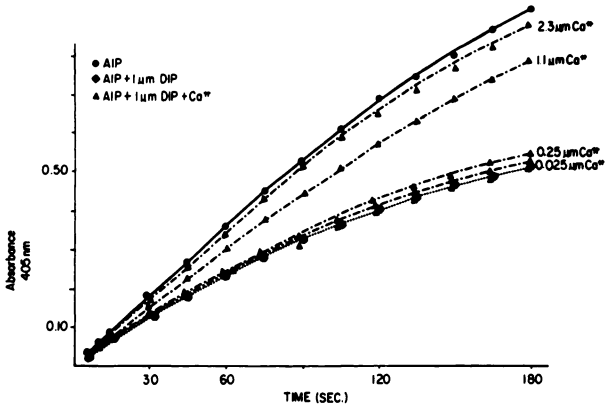

FIG. 4. Effect of increasing calcium concentrations on diphosphonate-induced alkaline phosphatase.

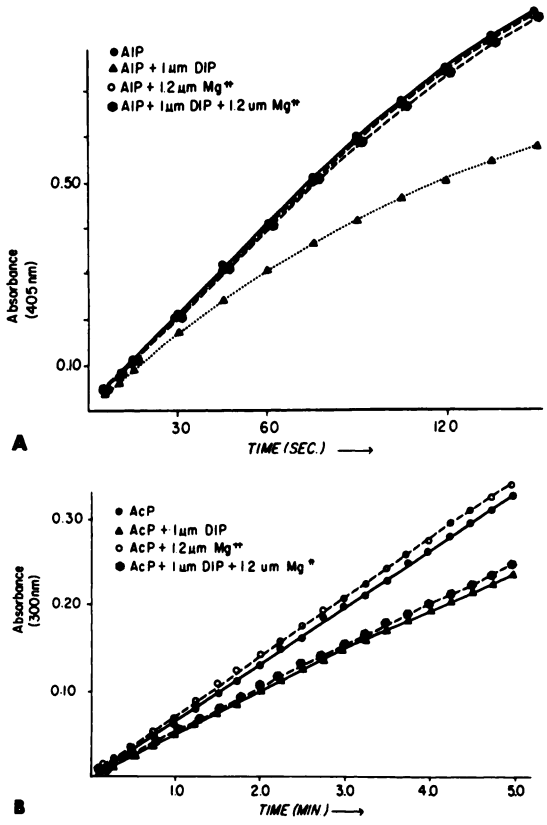

FIG. 5. Effect of magnesium ion on diphosphonate-induced alkaline phosphatase inhibition (A) and diphosphonate-induced acid phosphatase inhibition (B).

phate. At their respective pHs an intermediate reaction forms a phosphatase-substrate complex and its rate of hydrolysis (cleavage) is regulated by the chemical structure of the substrate (*12*). Alkaline phosphatase also serves as a phosphotransferase by transferring phosphoryl groups directly to an acceptor molecule.

The diphosphonate-induced in vitro inhibition of acid and alkaline phosphatase activity probably results from the diphosphonate forming a complex with the enzymes. Apparently the P-C-P bond of diphosphonate resists hydrolysis by the phosphatases and may assume the role of an inhibitor forming an enzyme-inhibitor-substrate complex (*13*). Since P-O-P bonds are readily cleaved by the phosphatases and their structure is common to both polyphosphate and pyrophosphate, it is likely that the stability of their enzyme complex depends on the nature and availability of other substrates.

Reversing diphosphonate-induced alkaline phosphatase inhibition with calcium and magnesium ions does not exclude the specificity of the reaction since metabolically the two cations react similarly. It is possible that the cations mediate the reversal of inhibition by inactivating the diphosphonate or by stimulating a diphosphonate-depressed enzyme activator system (i.e., adenosinetriphosphate and zinc metalloenzyme). The inability of calcium and magnesium to reverse diphosphonate-induced acid phosphatase inhibition was an unexpected finding and could be explained by the differences in pH of the reactions or species variations of the acid phosphatase.

The mechanism involved in localizing ^{99m}Tc-stannous diphosphonate and pyrophosphate in recent myocardial infarcts (*3*) could also be explained by their complexing with myocardial enzyme [creatine phosphokinase (CPK), glutamic oxalacetic transaminase (GOT) or lactate dehydrogenase (LDH)] released in the infarcted areas. The affinity of ^{99m}Tc-stannous diphosphonate and ^{99m}Tc-pyrophosphate to concentrate in the infarcted myocardium parallels the early serum elevation of these enzymes and shows proportionally less uptake as the enzymes return to normal levels. The lack of diphosphonate inhibition of porcine heart glutamic oxaloacetic transaminase activity, however, raises questions concerning the specificity of our hypothesis. Although the role of calcium is unknown in these reactions, it does appear in increased concentrations in the areas of infarcted myocardium (*14,15*). It is obvious that further studies on these enzymes, particularly CPK, are needed to support or refute our hypothesis. In addition, the answer to whether qualitative or quantitative differences exist between the diphosphonate and pyrophosphate molecules in inhibiting the enzyme systems would help in establishing their individual clinical usefulness.

ACKNOWLEDGMENT

The authors would like to thank G. H. Schmitt for his advice in this study. This paper was presented at the 21st Annual Meeting of the Society of Nuclear Medicine in San Diego, June 11–14, 1974.

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