Effect of L- and D-Tetramisole on $^{32}$P; and $^{45}$Ca Uptake and Mineralization by Matrix Vesicle-enriched Fractions from Chicken Epiphyseal Cartilage*

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Alkaline phosphatase has been implicated in matrix vesicle-mediated calcification. We sought to clarify its role in this process by studying the effect of L-tetramisole, a strong uncompetitive inhibitor of this enzyme, and D-tetramisole, its inactive isomer, on the uptake of $^{32}$P; and $^{45}$Ca by matrix vesicle-enriched microsomes obtained from chicken epiphyseal cartilage.

Uptake of both $^{32}$P; and $^{45}$Ca was inhibited in a dosage-dependent manner by the L-isomer; however, $^{32}$P; uptake was selectively inhibited at low drug concentrations and at early time periods. With increasing incubation time, inhibition of uptake of both ions was lost. Apatite crystal growth was only weakly inhibited by both the D- and L-isomers of tetramisole.

The kinetics of ion uptake by the vesicles was complex. $^{45}$Ca/$^{32}$P; uptake ratios, taken with the selective inhibition of $^{32}$P; uptake by L-tetramisole, indicated that early phases of vesicle ion uptake might be governed by alkaline phosphatase, a known P; binding protein. Later stages were clearly controlled by apatite formation. Although inhibition of vesicle uptake of $^{32}$P; and $^{45}$Ca by graded levels of L-tetramisole paralleled inhibition of alkaline phosphatase activity, surprisingly, D-tetramisole was almost as inhibitory of ion uptake as the L-isomer. This finding, coupled with the fact that alkaline phosphatase substrates were not required for vesicle ion uptake, indicates that alkaline phosphatase hydratase activity is not directly involved in vesicle-mediated calcification under these experimental conditions.

Matrix vesicles are known to be directly associated with the initiating phase of endochondral and other types of mineralization (1-5), and several groups have studied $^{45}$Ca accumulation by isolated matrix vesicle preparations (6-9). These extracellular microstructures are notably rich in alkaline phosphatase (10-13), an enzyme long associated with the uptake of Ca; and $^{45}$Ca by matrix vesicle-enriched microsomes obtained from chicken epiphyseal cartilage.

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Experimental Procedures

Preparation of Matrix Vesicle-enriched Microsomes—MVEM1 were obtained from fractionation of homogenates of epiphyseal cartilage slices obtained from rapidly growing 8- to 10-week-old broiler strain chickens, as previously described (30). Briefly, tissue slices (21) were homogenized twice (3 ml of buffer/g, wet tissue) using a Tekmar Tissuemizer with a SDT-180EN probe in a buffer (pH 7.5) composed of 50 mM TES, 1.5 mM MgCl2, and 10% (w/w) sucrose at 0°C for 2 min each. Differential centrifugation was carried out as previously described (32). The fraction sedimenting from the final spin (100,000 x g for 45 min) was resuspended in the TES-MgCl2 buffer to a final concentration of 5 mg of protein/ml, protein being estimated by the method of Lowry et al. (33). The MVEM fraction produced by this technique was relatively heterogeneous, containing fragments of endoplasmic reticulum, mitochondria, and plasma membrane, as well as vesicles.

1 The abbreviations used are: MVEM, matrix vesicle-enriched microsomes; P; orthophosphate; TES, N-trialphosphorylmethyl)methyl-2-amino-ethanesulfonate; SCL, synthetic cartilage lymph.
as matrix vesicles (30). Despite this, previous studies using Percoll gradient fractionation of MVEM have shown that these intracellular fragments have much less ability to accumulate mineral than do the matrix vesicles under the experimental conditions used in these experiments (34).

Preparation of Synthetic Hydroxypatite—Hydroxypatite was prepared by the method of Termine et al. (34) and maintained at 4°C in the mother liquor for at least 48 h before being used. For use, the apatite, prepared as a slurry (1.24 mg/ml) in the mother liquor, was vigorously resuspended and aliquots taken for studies on the kinetics of mineral ion uptake.

"Ca and °P, Uptake—Assays of Ca and P; uptake by MVEM and hydroxylapatite were performed in a synthetic cartilage lymph containing levels of electrolytes comparable to that found in the extracellular fluid of chicken epiphyseal cartilage (35). The electrolyte levels of SCL were as follows: Ca2+, 2.0 mM; Na+, 114.3 mM; K+, 12.7 mM; Cl-, 118.5 mM; and HCO3−, 1.83 mM. The solution was buffered with 8.25 mM TES (pH 7.5) for use in an air atmosphere and contained, in addition, D-glucose, 5.55 mM; sucrose, 63.5 mM, and either 4CaCl2 or (3P)H2PO4, (carrier-free, diluted in SCL) at levels of about 1 X 106 cpm/ml. Incubations were carried out in closed 10-ml polypropylene tubes using gentle agitation in a 37°C water bath. To initiate the assay, samples of either MVEM (80 μg of protein) or hydroxylapatite (20 μg) were added to 2.0 ml of the radiolabeled SCL. At various times ranging from 20 min to 27 h, 100-μl samples were withdrawn and rapidly filtered under reduced pressure through 0.45-μm Millipore filters using a manifold vacuum chamber. Although the nominal pore size of the filters was greater than that of the vesicles (0.10-0.30 μm diameter), over 90% of the vesicle alkaline phosphatase activity was retained on the filters. Initial studies were done with 0.22-μm pore size membranes, but filtration rates were slow, and retention of vesicle phosphatase was little improved. Filters were washed twice in situ with SCL (0°C) to remove unincorporated radiolabel, and uptake was determined by liquid scintillation counting of radioactivity retained on the filter (36). To minimize nonspecific adsorption of radiolabel, the filters were presoaked at room temperature in either CaCl2 or Na2HPO4, (20 mM, pH 7.5). Background counts on filters from samples of control tubes incubated without added MVEM or apatite were subtracted from counts observed in the experimental samples.

Alkaline Phosphatase Assays—Alkaline phosphatase activity was assayed in SCL at pH 7.5 using p-nitrophenylphosphate as the substrate. Hydrolysis was monitored at 402 nm for p-nitrophenol using a molar extinction coefficient of 4500 cm−1 M−1 (18). Initial substrate concentration was set at 10 μM to minimize substrate inhibition by p-nitrophenolphosphate at pH 7.5. Assays were initiated by addition of either substrate or MVEM, the protein concentration being 40 pg/ml, as in the "Ca and °P., uptake assays.

Inhibitors—Tetramisole (D and L) was prepared as a 0.2 M aqueous stock solution (pH 7.5) and added prior to the initiation of ion uptake and enzyme assays.

Chemicals—The following were procured from Sigma; p-nitrophenolphosphate (disodium hexahydrate), p-nitrophenolphosphate standard solution, TES, and L-tetramisole. °Cac and °P, orthophosphoric acid were obtained from New England Nuclear. D-Tetramisole was provided through the courtesy of Janssen Pharmaceutica; all other chemicals were of reagent grade and were obtained from Fisher.

RESULTS

As observed in previous reports (22, 36, 37), uptake of °Ca and °P, by matrix vesicle-enriched microsomes isolated from calcifying epiphyseal cartilage followed a complex polyphasic pattern (Fig. 1). There was an initial rapid uptake of a small amount of °Ca and °P, (0.55 μmol of Ca and 0.31 μmol of P, mg of MVEM protein), usually attained by 20-min incubation in the SCL. Following a variable lag period during which little further uptake occurred, rapid accumulation of both °Ca and °P, ensued, lasting for a period of about 2-3 h. The rate of accumulation then slowed considerably, continuing at a gradually decreasing rate throughout the remainder of the 24-h incubation period.

The ratio of °Ca and °P, uptake varied substantially with time (Fig. 2A). During the initial limited uptake phase, °Ca/°P, uptake ratios were high relative to apatite (1.93 ± 0.25); during the phase of most rapid accumulation (1-2 h of incubation), Ca/P, uptake ratios declined sharply to values approaching 1.0. As the rate of accumulation began to slow (about 3 h), uptake ratios increased to about 1.5 and then gradually increased to 1.64 during the long period of steady accumulation that followed.

Graded levels of L-tetramisole (1.0 to 5.0 mM) when added to the incubation medium caused progressive decrease in the
Inhibition of Matrix Vesicle Calcification

rate of mineral ion accumulation (Fig. 1). This effect can be more clearly seen in Fig. 3A where it is evident that inhibition of $^{32}\text{P}$ uptake increased proportionally with increasing L-tetramisole levels, up to 2.5 mM. Beyond that concentration up to 10 mM, little further inhibition was evident. In contrast, the response of $^{45}\text{Ca}$ uptake to increasing L-tetramisole was polyphasic. Little inhibition of $^{45}\text{Ca}$ uptake occurred up to 1.0 mM, but 2.5 mM caused substantial inhibition (about 30%). Higher concentrations of the drug caused further inhibition, down to about 50% of the control value with 10 mM.

The effect of the drug varied with time (Fig. 3B). Inhibition of $^{32}\text{P}$ uptake was greatest during the initial uptake period (up to 1 h) and was least during the period when the rapid rate of accumulation of $^{32}\text{P}$ began to abate (about 3 h). Beyond 5 h, inhibition gradually decreased and was reduced to a minimum by 24 h. The effect of L-tetramisole on $^{45}\text{Ca}$ uptake was different. Early effects of the drug on $^{45}\text{Ca}$ uptake were small, but increased rapidly with time, reaching a maximum shortly after 5 h. Thereafter, inhibition paralleled that of $^{32}\text{P}$ uptake, gradually declining to minimal effect by 24 h.

The net inhibitory effect of increasing concentrations of L-tetramisole on the $^{45}\text{Ca}/^{32}\text{P}$ uptake ratios at different stages of mineralization is shown in Fig. 4. Here the dosage-response effect on $^{45}\text{Ca}/^{32}\text{P}$ uptake ratios shows that the highest degree of selective inhibition of $^{45}\text{P}$ uptake was attained with 2.5 mM L-tetramisole, at nearly all stages of mineralization. At very low levels of mineral ion uptake (Fig. 4A), progressive inhibition of $^{32}\text{P}$ versus $^{45}\text{Ca}$ uptake was seen with increasing drug concentration, up to 2.5 mM. Beyond that concentration the selective inhibition of $^{45}\text{P}$ uptake was progressively lost. Examination of the effects of L-tetramisole at successive increasing stages of mineralization revealed that maximal selective inhibition of $^{32}\text{P}$ uptake occurred during the induction phase of heavy mineralization, i.e., between 1 and 2.5% uptake of $^{32}\text{P}$ (Fig. 4B) or up to about 1.5 h of incubation (Fig. 4B). At later stages of mineral ion accumulation, the selective inhibition of $^{45}\text{P}$ uptake was progressively lost, and by 24 h (45–50% $^{32}\text{P}$ uptake) almost no selectivity in inhibition was seen, regardless of the drug concentration.

In an attempt to sort out the complex effects of L-tetramisole on mineralization induced by the cartilage vesicles, studies were made on the effects of graded levels of the drug on ion deposition in a hydroxylapatite-seeded SCL system. Fig. 5A and B, shows the uptake of $^{45}\text{Ca}$ and $^{32}\text{P}$ by this apatite-seeded system. In contrast to the cartilage vesicles, uptake of both $^{45}\text{Ca}$ and $^{32}\text{P}$ began immediately upon addition of apatite to the system. The most rapid rate of uptake occurred during the first hour, after which it progressively decreased with time, attaining uptake levels of about 48–50% of the total solution $^{45}\text{Ca}$ and 40–45% of the solution $^{32}\text{P}$, by 24 h.

Again, in contrast to the cartilage vesicles, $^{45}\text{Ca}/^{32}\text{P}$ uptake ratios by the apatite-seeded system were essentially constant with time, ranging in value from 1.59 to 1.62 (Fig. 6A), indicating that a slightly Ca-deficient apatite was being formed. Examining the effect of L-tetramisole on uptake of $^{45}\text{Ca}$ and $^{32}\text{P}$, by apatite (Fig. 7A), it was evident that much less inhibition was produced than with the vesicles. Only 11 and 16% inhibition of $^{45}\text{Ca}$ and $^{32}\text{P}$ uptake, respectively, were produced by 5.0 mM L-tetramisole with apatite, as opposed to 36 and 47% inhibition, respectively, with the matrix vesicles. While there was some selectivity in the inhibition of $^{32}\text{P}$ versus $^{45}\text{Ca}$ uptake by apatite, these differences were much less than noted with the cartilage vesicles and occurred later rather than early in the incubation period (Fig. 7B).

The general lack of dosage-dependent effect of the drug on

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**Fig. 3. Comparative effect of L-tetramisole on $^{32}\text{P}$ and $^{45}\text{Ca}$ uptake by epiphyseal cartilage matrix vesicle-enriched microsomes.** A, dosage-response to increasing L-tetramisole concentrations; B, change in response with incubation time. C, $^{32}\text{P}$ uptake; D, $^{45}\text{Ca}$ uptake. Values in A are the mean ± S.E. of all time points; those in B are the mean ± S.E. of all L-tetramisole concentrations. The number of samples analyzed at each point indicated ranged from 8 to 22. In A, note the selective inhibition of $^{32}\text{P}$ uptake at low L-tetramisole concentrations, with nearly maximal effect seen at 2.5 mM. In B, note that the selective inhibition of $^{32}\text{P}$ uptake occurred only during the early period of vesicle incubation, whereas with $^{45}\text{Ca}$ uptake, inhibition increased with time, up to 5 h.

**Fig. 4. Dosage-response to L-tetramisole of $^{45}\text{Ca}/^{32}\text{P}$ uptake ratios at various stages of P loading by epiphyseal cartilage vesicles.** A, 1.0%; B, 1.0–2.5%; C, 5–15%; D, 15–25%; E, 20–35%; and F, 45–50% uptake of the total P, in the incubation medium. Values are the mean ± S.E. of from 3 to 10 samples each. The effect of the various drug concentrations is shown at successive stages of mineral ion loading, rather than at successive time points, to emphasize the selective effect of the drug at different stages of mineralization. Note the markedly different ion-selective responses to the drug at the different stages of mineralization.
Inhibition of Matrix Vesicle Calcification

**Fig. 5.** Effect of varying L-tetramisole concentrations on the time course of $^{45}$Ca and $^{32}$P uptake by synthetic hydroxyapatite. A, $^{45}$Ca uptake; B, $^{32}$P uptake. Apatite (20 μg) was incubated for the indicated times in 2.0 ml of synthetic cartilage lymph, as described for the vesicle fraction (see under "Experimental Procedures"). C—O, controls; ●—●, 2.5 mM; △—△, 5.0 mM; and ■—■, 10.0 mM L-tetramisole. Values are the mean ± S.E. of 4 samples at each drug concentration. Uptake of both $^{45}$Ca and $^{32}$P is expressed as a percentage of the total of each originally present in the incubation medium. Note the relatively minor inhibition of L-tetramisole on uptake of $^{45}$Ca and $^{32}$P, compared to its effect on the vesicle preparations (Fig. 1).

**Fig. 6.** Effect of graded L-tetramisole concentrations on $^{46}$Ca/$^{32}$P uptake ratios by hydroxyapatite with incubation time. A, control; B, 2.5 mM; C, 5.0 mM; and D, 10.0 mM L-tetramisole. Values are means ± S.E. of 4 samples each. Note the almost constant $^{46}$Ca/$^{32}$P uptake ratios by the control apatite incubation with time and the minimal effect of the drug on the uptake ratios, except at the highest drug concentration (10 mM).

$^{46}$Ca/$^{32}$P uptake ratios by apatite can be seen in Fig. 8, A–D. This contrasted with the effect of L-tetramisole on the cartilage microsomal vesicles (Fig. 4, A–F), where marked dosage-dependent selective inhibition of $^{32}$P uptake was seen.

Comparison of the effect of graded levels of L-tetramisole on alkaline phosphatase activity toward p-nitrophenylphosphate as a substrate at physiological pH and ion concentrations and its effect on $^{32}$P and $^{46}$Ca uptake by cartilage vesicles and by apatite are shown in Fig. 9. At low drug levels (1.0–2.5 mM), inhibition of enzyme activity quite closely paralleled inhibition of $^{32}$P uptake by the vesicles; at higher drug levels, inhibition of the enzyme broadly paralleled that of $^{46}$Ca uptake. At the two higher drug concentrations (5 and 10 mM), the inhibitory effect on alkaline phosphatase activity was 1.5 to 1.8 times greater than its effect on ion uptake by the cartilage vesicles and 3 to 6 times greater than its effect on ion uptake by apatite.

To gain further insight into the relationship between the inhibitory effect of L-tetramisole on MVEM ion uptake and its effect on alkaline phosphatase activity, parallel experiments were conducted in which both D- and L-isomers of tetramisole were studied for their effect on uptake of both $^{32}$P and $^{46}$Ca by the MVEM. Since only the L-isomer was active.

**Fig. 7.** Comparative effect of L-tetramisole on $^{32}$P and $^{46}$Ca uptake by hydroxyapatite. A, dosage-response to increasing L-tetramisole concentration; B, change in response with incubation time. Values in A are the mean ± S.E. of 8–10 samples each; those in B are means ± S.E. of all L-tetramisole concentrations (6–7 samples each). Note in A, the slight selective inhibition of $^{32}$P uptake by the lower concentrations of the drug, and in B, that selective inhibition of $^{32}$P uptake occurred only after prolonged incubation. ●, $^{46}$Ca uptake; ○, $^{32}$P uptake.

**Fig. 8.** Dosage-response to L-tetramisole of $^{46}$Ca/$^{32}$P uptake ratios by hydroxyapatite at various stages of P_i deposition. A, 11–18%; B, 19–26%; C, 22–30%; and D, 52–43% uptake of the total P_i in the incubation medium. Values are means ± S.E. of 3 samples each. Note, in contrast to the effect on microsomal vesicle uptake (Fig. 4), that there was relatively little perturbation of $^{46}$Ca/$^{32}$P uptake ratios by the varying concentrations of the drug at the various stages of apatite mineral deposition.
Inhibition of Matrix Vesicle Calcification

as an inhibitor of alkaline phosphatase activity, any significant reduction in inhibition of MVEM ion uptake by D-tetramisole, as compared to the L-isomer, could indicate participation of alkaline phosphatase. As can be seen in Fig. 10, both forms of the drug caused a progressive dosage-dependent inhibition of 32P, and 45Ca uptake. Further, the differences between the effects of the D- and L-isomers were not great. At concentrations higher than 2.5 mM and at certain times during the incubation (3 and 5 h), the inhibition of 32P uptake by D-tetramisole was somewhat less than that of L-tetramisole. A similar effect was seen on 45Ca uptake over the full range of drug concentrations studied.

Comparison of Fig. 11 with Fig. 10 shows that the stereoselective inhibition by L-tetramisole versus D-tetramisole occurred during the period of most rapid ion accumulation. Since three separate kinetic studies on apatite crystal growth showed indistinguishable weak inhibition by D-versus L-tetramisole (data not shown), this indicates that the rapid ion uptake phase of MVEM was not governed by apatite formation.

DISCUSSION

The kinetics and stoichiometry of 45Ca and 32P uptake by the matrix vesicle fractions during the initial phase of incubation in the synthetic cartilage lymph (i.e. the high Ca/P; uptake ratios, Figs. 2 and 11) show that Ca2+ is selectively accumulated during the early incubation. This finding is in agreement with our earlier studies (22, 30, 35, 36) and supports the conclusions of electron microprobe studies by Ali et al. (38, 39) and others (40).

Although the nature of the uptake process of 45Ca and 32P by the MVEM fraction has not been fully defined, the rapid limited uptake of both ions which occurred upon initiation of the incubations appears to represent an exchange with pre-existing ion stores. For example, before incubation the MVEM contained approximately 1.37 μmol of Ca and 0.85 μmol of P/mg of protein, whereas the rapid uptake of labeled ions was in the range of 0.55 μmol of Ca and 0.31 μmol of P/mg of protein. Thus, the rapid incorporation of these levels of 45Ca and 32P could have occurred by exchange with little net accumulation. Since there was no requirement of high energy substrates, there is no evidence that the uptake process was active.

Based on data shown in Fig. 11, it is evident that something more than a simple exchange process was taking place during the lag phase. The rate of initial incorporation of 45Ca was distinctly slower than that of 32P, resulting in low 45Ca/32P uptake ratios. However, during this lag period, 45Ca accumulation steadily accelerated, whereas, 32P uptake remained relatively constant. This is evident from the rapid linear rise in 45Ca/32P uptake ratios during this period of the incubation. The reason for this selective incorporation of 45Ca is not certain but may be due to binding to vesicle membrane proteins and/or phospholipids. Uptake at this stage clearly was not associated with apatite formation.

When the burst of ion incorporation occurred following the lag period, the Ca/P; uptake ratios rapidly declined, and during the period of accelerating mineral deposition, normal vesicle loading was usually characterized by very low Ca/P, uptake ratios (1.0-1.3 Figs. 2A and 4B). Uptake at this critical stage of vesicle-mediated mineralization appears to be governed by formation of some type of noncrystalline, or very poorly crystalline, calcium acid phosphate, not by apatite. This interpretation is supported by our previous observations that vesicle mineral formed during the early stages of induction had a low Ca/P, ratio and was essentially amorphous (22, 36). The first crystaline phase formed appears to be a low Ca/P ratio octacalcium phosphate-like mineral. However, several lines of evidence indicate that formation of apatite becomes the controlling factor at later stages of vesicle incubation. First, at the end of the period of massive ion uptake, 45Ca/32P uptake ratios of the control vesicle (Ca/P = 1.64 ± 0.06) closely approximated that of theoretical apatite. Second, with synthetic apatite-seeded incubations, 45Ca/32P uptake ratios remained almost constant at apatite-like ratios throughout the 24-h incubation period, showing that the synthetic lymph readily supported apatite crystal growth.

During the early stages of vesicle incubation, L-tetramisole inhibited 32P uptake more than 45Ca (Fig. 3B). In the presence of 2.5 mM drug, less than 1 mol of P than for every 3 mol of Ca during this initial phase (Fig. 4A). Later, during the initial phase of rapid mineral deposition which was usually characterized by very low Ca/P, uptake ratios (Figs. 24 and 4B), the selective inhibition of 32P, versus 45Ca uptake by the drug (relative to the control) was maximal. This suggests that tetramisole in some manner specifically inhibits P, uptake during this critical uptake period. In later stages of vesicle incubation, both the selectivity and the inhibitory effect of the drug were largely overcome (Fig. 4, E and F). This finding supports the interpretation that apatite formation is the controlling factor in later stages of vesicle mineralization, inasmuch as the drug exerted only minor inhibition of uptake of mineral ions in apatite-seeded systems (Fig. 5, A and B) and had minimal effect on 45Ca/32P uptake ratios (Fig. 6B). These findings, taken with previous x-ray diffraction studies of vesicle-induced mineralization showing the presence of apatite at later stages of vesicle incubation (22, 36, 41), almost certainly establish that apatite is formed by matrix vesicles in cartilage lymph incubation systems.

The inhibitory effect of L-tetramisole on vesicle ion uptake was selective toward P, especially at lower drug concentrations, and at critical early stages of vesicle ion loading. This suggests that at least part of the effect of the drug may have resulted from its well known inhibitory effect on alkaline phosphatase (12, 24-29). However, data presented in Fig. 9 show that alkaline phosphatase hydrolase activity is more...
Inhibition of Matrix Vesicle Calcification

Fig. 10. Comparative effect of graded concentrations of D- and L-tetramisole on $^{45}$Ca uptake (A) and $^{32}$P$_1$ uptake (B) by matrix vesicle-enriched microsomes. Incubation times are indicated in each panel. O—O, D-tetramisole; •—•, L-tetramisole. Values are the mean ± S.E. of duplicate samples at each drug concentration. Relative uptake is expressed as a fraction of the mean uptake of all samples assayed at each time point. Note at early times the progressive inhibition of both $^{45}$Ca and $^{32}$P$_1$ uptake by both isomers of the drug and the loss of selective inhibition at longer incubation periods. At 1 h note that L-tetramisole was more inhibitory of $^{32}$P$_1$ uptake than the D-isomer; and at intermediate times, the L-form was more inhibitory of both $^{45}$Ca and $^{32}$P$_1$ uptake than was D-tetramisole.

Fig. 11. Time course of $^{45}$Ca and $^{32}$P$_1$ uptake and $^{45}$Ca/$^{32}$P$_1$ uptake ratios of samples shown in Fig. 10. Values are the mean ± S.E. of 20 samples at each time point. These were used to calculate the relative ion uptake shown in Fig. 10. •—•, $^{45}$Ca; O—O, $^{32}$P$_1$ uptake; ■—■, $^{45}$Ca/$^{32}$P$_1$ uptake ratios.

sensitive to L-tetramisole than is ion uptake by the MVEM, even under incubation conditions employed for vesicle mineralization. Further, both the D- and L-isomers of tetramisole produced similar dosage-related inhibition of ion uptake by matrix vesicles, and to a smaller extent, by apatite-incubated systems (data not shown). Since only the L-isomer was inhibitory toward the phosphohydrolase activity of the enzyme, this shows that the effect of L-tetramisole on the vesicles cannot be explained simply by its effect on alkaline phosphatase.

In fact, the D-tetramisole data would seem to rule out any specific effect of the drug on alkaline phosphatase activity in its inhibition of matrix vesicle-induced mineralization. Such conclusions have been reached from some other studies using the two stereoisomers of the drug (24, 25); however, in another study, Fallon et al. (29) reported selective inhibition with the L-isomer using unusually high concentrations of the drugs. A large number of studies have shown that the L-form of the drug is a strong uncompetitive inhibitor of alkaline phosphatase activity. This type of inhibition indicates that the drug acts by forming a stable enzyme-product-inhibitor ternary complex. Detailed kinetic studies by Cyboron et al. (18) have shown that L-tetramisole forms a complex with the enzyme-phosphate, rather than with the enzyme-alcohol product. Since cartilage membrane alkaline phosphatase exhibits high affinity for P at physiological pH ($K_i = 20-50$ μM (18)), it is possible that one of the roles of the enzyme in matrix vesicles may be as a P$_1$-binding protein or as part of the P transport system in the vesicle membrane. If so, it may be that both the D- and L-forms of tetramisole interfere with P binding by the protein, but only the L-form inhibits its
catalytic activity. This might explain the selective early inhibition by the drug of P, uptake by the vesicles. However, this remains to be determined. The possibility that the hydro-
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