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Studies of the Mechanism of Biological Calcification

II. Evidence for a Multi-Step Mechanism of Calcification by Tendon Matrix*

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The inhibitory properties of several compounds with respect to net calcification and the $^{45}Ca^{2+}$ and the $H^{32}PO^4$ exchange reactions induced by calcified bovine tendon matrix have been investigated. The inhibition of both reactions by methylenediphosphonate and a fraction derived from human urine can be decreased by increasing the amount of the bound mineral phase. Mg^{2+} does not inhibit the exchange reactions but the inhibition of net calcification by Mg^{2+} can be reversed by increasing the concentration of Ca^{2+} of the soluble phase but not by inorganic phosphate or by increasing the amount of bound mineral. Phosphonoacetate inhibits net calcification but not the exchange reactions and the inhibition is decreased neither by increasing the concentration of Ca^{2+} or HPO_4^{2-} nor by increasing the amount of bound mineral. The inhibitory effects can be explained by their interaction with the insoluble matrix, although the affinity is greater for methylene diphosphonate and the urine inhibitor than for the other compounds. A polyfunctional heterogeneous catalytic mechanism for tendon matrix calcification is proposed. According to this scheme, matrix calcification is catalyzed by a urea-sensitive macromolecular assembly that contains a minimum of three reactive sites. The first is a Mg-sensitive, Ca^{2+}-binding site that facilitates the interaction with HPO_4^{2-} of the soluble phase to form a Ca-P complex at a phosphonoacetate-sensitive second site. This Ca-P complex then reacts with a third matrix site for mineral phase initiation and crystal growth. The Ca-P aggregates present in the growth sites provide those mobile ions which participate in the ^{45}Ca and $H^{32}PO_4^{2-}$ exchange reactions and which also constitute the binding sites for methylenediphosphonate as well as the serum and urine calcification inhibitors.

Key words: Calcification — Collagen — Catalysis — Inhibition — Tendon.

Les propriétés d'inhibition de la calcification réticulaire et des réactions d'échanges de $H^{32}PO^4$ et $^{45}Ca^{2+}$, induites par la matrice calcifiée de tendon de boeuf, ont été étudiées pour divers produits. L'inhibition des deux types de réaction, par la méthylènediphosphonate et une fraction dérivée de l'urine humaine peut être diminuée en augmentant la proportion de la phase minérale liée. Mg^{2+} n'inhibe pas les réactions d'échanges, mais l'inhibition de la calcification réticulaire peut être inversée en augmentant la concentration en Ca^{2+} de la phase soluble; mais une telle réaction ne peut être obtenue ni par le phosphate inorganique, ni en augmentant la proportion du minéral lié. Le phosphono-acétate inhibe la calcification réticulaire, mais non les réactions d'échanges, et l'inhibition n'est diminuée ni en augmentant la concentration en Ca^{2+} ou HPO_4^{2-}, ni en augmentant la proportion du minéral lié. Les actions d'inhibition peuvent s'expliquer par leur interaction avec la matrice insoluble, bien que l'affinité pour le méthylènediphosphonate et l'inhibiteur urinaire soit plus grande que pour les autres produits testés. Un mécanisme catalytique hétérogène et multifonctionnel est

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proposé pour la calcification matricielle du tendon. Selon ce mécanisme, la calcification matricielle est catalysée par une entité macromoléculaire, sensible à l'urée, qui contient un minimum de trois régions actives. La première est une région sensible au Mg et capable de se lier avec Ca^{2+}, qui facilite la réaction avec HPO_4^{2-} de la phase soluble pour constituer un complexe Ca-P au niveau d'une deuxième région sensible au phosphonoacétate. Le complexe Ca-P réagit ensuite avec une troisième région matricielle pour la formation de la phase minérale et la croissance cristalline. Les agrégats Ca-P, qui sont présents au niveau des régions en voie de croissance, fournissent les ions mobiles qui participent aux réactions d'échanges ^{45}Ca et $H^{32}PO_4^{2-}$ et qui constituent aussi les zones de liaison pour le méthylènediphosphonate, ainsi que pour les inhibiteurs sériques et urinaires de la calcification.

Die Hemmeigenschaften von verschiedenen Substanzen in bezug auf effekte Verkalkung sowie die $^{45}Ca^{2+}$ und $H^{32}PO_4^{2-}$ Austauschreaktionen, hervorgerufen durch verkalkte Rindersehnenmatrix, wurden untersucht. Die Hemmung beider Reaktionen durch Methylendiphosphonat und durch eine aus menschlichem Urin gewonnene Fraktion kann bei Erhöhung der Menge der gebundenen Mineralphase herabgesetzt werden. Mg^{2+} hemmt die Austauschreaktionen nicht; die Hemmung der effektiven Verkalkung durch Mg^{2+} kann jedoch aufgehoben werden, indem die Konzentration von Ca^{2+} in der löslichen Phase erhöht wird; dies gilt nicht, wenn anorganisches Phosphat zugefügt oder die Menge des gebundenen Minerals erhöht wird. Phosphonoacetat hemmt die effektive Verkalkung, nicht aber die Austauschreaktionen. Dabei wird die Hemmung weder durch Erhöhung der Konzentration von Ca^{2+} oder HPO_4^{2-}, noch durch Erhöhung der Menge gebundenen Minerals vermindert. Die Hemmwirkungen können durch ihre Wechselwirkung mit der unlöslichen Matrix erklärt werden, obwohl die Affinität für Methylendiphosphonat und den Urin-Hemmkörper größer ist als für die anderen Substanzen. Ein polyfunktioneller, heterogener, katalytischer Mechanismus für die Sehnenmatrixverkalkung wird vorgeschlagen. Nach diesem Schema wird die Matrixverkalkung durch einen Harnstoff-empfindlichen, makromolekulären Stoff katalysiert, der im Minimum drei aktive Zentren enthält. Das erste ist ein Mg-empfindliches, Ca^{2+}-bindendes Zentrum, welches das Zusammenwirken der löslichen Phase mit HPO_2^{2-} erleichtert, um einen Ca-P-Komplex an einem zweiten, Phosphonoacetat-empfindlichen Zentrum zu bilden. Dieser Ca-P-Komplex reagiert darauf mit einem dritten Zentrum auf der Matrix, um die Bildung der Mineralphase auszulösen und das Kristallwachstum anzuregen. Die an den Wachstumsstellen vorliegenden Ca-P-Aggregate liefern jene mobilen Ionen, welche an den ^{45}Ca und $H^{32}PO_4^{2-}$ Austauschreaktionen teilnehmen; diese bilden auch die Bindungsstellen für Methylendiphosphonat sowie für die Serum- und Urinverkalkungshemmer.

Introduction

Previous studies have demonstrated that collagen-containing fibers derived from beef tendon will induce the uptake of calcium and phosphate from stable solutions. The resulting mineral phase is matrix-bound and is characterized by a calcium to phosphate ratio of 1.5–1.8. Under certain conditions the calcified matrix will give a positive von Kossa reaction (Thomas and Tomita, 1967; Wadkins, 1968; Jethi *et al.*, 1970). Jethi *et al.* (1970) demonstrated that the calcified matrix will participate in exchange reactions with $^{45}Ca^{2+}$ or $^{32}P\text{-}HPO_4^{2-}$ of the soluble phase. The properties of these exchange reactions are similar to those described for synthetic hydroxyapatite (Weikel *et al.*, 1954; Neuman and Neuman, 1958; Pak and Bartter, 1967).

Kinetic studies of the net calcification and exchange reactions revealed that both processes are stimulated by increasing the amount of bound mineral phase (Jethi *et al.*, 1970). On the other hand, whereas both are inhibited by an acidic fraction derived from serum and urine and by methylenediphosphonate, Mg^{2+} and F^- were found to inhibit only net calcification. Neuman and Neuman (1958) have proposed that the two exchange reactions represent an interchange of

mobile surface ions of the mineral phase with the corresponding radioactive ions of the soluble phase. Our results suggest that the acidic calcification inhibitors of serum and urine as well as methylenediphosphonate react with the bound mineral phase whereas Mg^{2+} and F^- block other sites of the catalytic matrix that are involved in the overall calcification process but are not involved in the exchange reactions. This postulated mode of action of methylenediphosphonate is comparable to that proposed by Fleisch *et al.* (1969) and by Francis *et al.* (1969). It suggests that the diphosphonate compounds and the serum and urine calcification inhibitors could function by similar mechanisms.

The present communication provides additional kinetic evidence that supports this hypothesis. It also demonstrates the existence of specific matrix-bound calcium and calcium phosphate complexes that could constitute intermediates of the overall calcification process.

Methods

The collagen-containing matrix employed in these studies was prepared by an adaptation of the method of Thomas and Tomita (1967) as described by Jethi *et al.* (1970). The calcification assay was carried out in a 25 ml reaction medium composed of 15 mM barbital buffer, pH 7.4, 100 mM NaCl, 1.2 mM potassium phosphate, 1.2 mM $CaCl_2$, and 50–200 mg dry weight of either uncalcified or calcified matrix. The latter was prepared by incubating the desired amount of matrix in the above medium, except that the amounts of potassium phosphate and $CaCl_2$ were adjusted to produce the desired amount of bound mineral (cf. Jethi *et al.*, 1970). Net calcification was determined by measuring the initial rate of Ca^{2+} disappearance from the soluble phase by atomic absorption spectrophotometry and of HPO_4^{2-} disappearance by the method of Fiske and SubbaRow (1925) as previously described (Wadkins, 1968). The $^{45}Ca^{2+}$ and ^{32}P-HPO_4^{2-} exchange reactions were assayed as described by Jethi *et al.* (1970). Methylenediphosphonate was obtained from the Miles Laboratories and phosphonoacetate was obtained from Bodman Chemicals, Inc. On the basis of thin layer silica gel chromatography and titration, the purity of both compounds was estimated to be greater than 98%. The physiological calcification inhibitors employed in these studies were derived from human serum and urine by an adaptation of the purification method described previously (Howard *et al.*, 1967).

Results

Inhibition of Matrix Calcification. The results presented in Fig. 1 demonstrate that the initial rates of calcification induced by calcified and uncalcified matrices are depressed by addition of Mg^{2+} and methylenediphosphonate. One important difference is that whereas 0.2 mM Mg^{2+} produced 50% inhibition of the rate when either matrix was employed, 50 times more methylenediphosphonate was required to inhibit calcification by 50% in the calcified matrix than was necessary to produce the same degree of inhibition in the uncalcified matrix.

The possibility that the potency of some calcification inhibitors depends on the extent of matrix calcification while other inhibitors are not, was investigated and the results are summarized in Table 1. Of the several compounds studied, it is apparent that Mg^{2+}, F^-, and phosphonoacetate are just as effective with calcified, as well as uncalcified, matrix whereas higher levels of methylenediphosphonate and the acidic fractions derived from human urine and serum are required to inhibit the calcified matrix 50% as compared to uncalcified matrix. Table 1 also demonstrates that those inhibitors that are mineral-phase dependent are also potent inhibitors of the $^{45}Ca^{2+}$ and ^{32}P-HPO_4^{2-} exchange reactions while

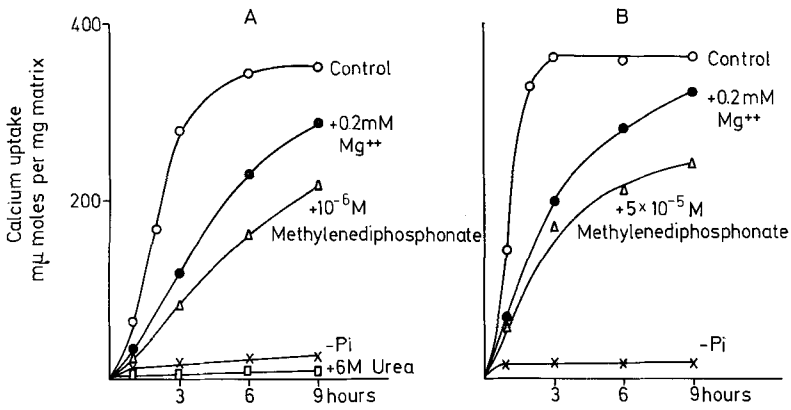

Fig. 1. Inhibition of calcification of calcified and uncalcified matrix. All assays were carried out as described in Methods above. Example A contained 100 mg dry weight of matrix that had not been previously exposed to the calcification medium and example B contained 100 mg that had been exposed to the standard calcification medium for 10 h during which time 48 μmoles of Ca^{2+} and 28 μmoles Pi (inorganic phosphate) were removed from the soluble phase and bound to the matrix

Table 1. *Inhibition of Net Calcification and Exchange*

Inhibitor	Concentration required for 50% inhibition		
	Net calcification		^{45}Ca-Exchange
	Uncalcified matrix	Calcified matrix	
Mg^{2+}	0.2 mM	0.2 mM	[a]
F^-	$5 \cdot 10^{-5}$ M	$5 \cdot 10^{-5}$ M	[b]
Phosphonacetate	$8 \cdot 10^{-5}$ M	$8 \cdot 10^{-5}$ M	[c]
Methylenediphosphonate	$1 \cdot 10^{-6}$ M	$5 \cdot 10^{-5}$ M	$5 \cdot 10^{-5}$ M
Urine inhibitor A[d]	0.2 ml	5.0 ml	5.0 ml
Serum inhibitor A[d]	0.5 ml	8.0 ml	8.0 ml

The standard assay system described in Methods above was employed. For these studies 100 mg tendon matrix was used to which was bound 50 μmoles of Ca^{2+} and 30 μmoles of Pi.

[a] No inhibition observed at 8 mM.

[b] No inhibition observed at 1 mM.

[c] No inhibition observed at 1 mM.

[d] Values indicate the volume-equivalent of unfractionated human serum or urine.

those that are not mineral-phase dependent do not inhibit the exchange reactions at even ten-fold higher concentrations. The present results provide additional evidence that methylenediphosphonate and the serum and urine inhibitors influence net calcification and the exchange reactions by interaction with the mineral phase. On the other hand Mg^{2+}, F^-, and phosphonoacetate, which do not inhibit the exchange reactions, act at matrix sites outside the mineral phase that are involved in net calcification but not in the exchange reactions.

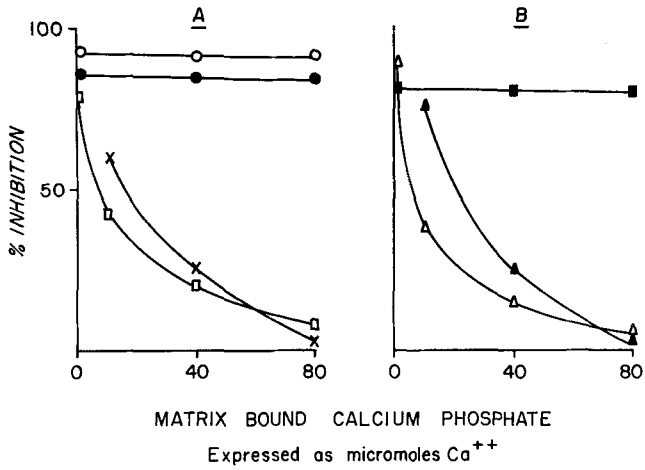

Fig. 2. Percentage inhibition of calcification and the $^{45}Ca^{2+}$-exchange with calcified matrix. The percentage inhibition of the initial reaction rate of net calcification and of the $^{45}Ca^{2+}$-exchange by several calcification inhibitors was determined as described in Fig. 1, in Methods above, and by Jethi *et al.* (1970). ○–○–○ net calcification with 5×10^{-5} M F^-; ●–●–● net calcification with 1.0 mM Mg^{2++}; □–□–□ net calcification with 5×10^{-6} M methylenediphosphonate; ×–×–× ^{45}Ca-exchange with 5×10^{-6} M methylenediphosphonate; ■–■–■ net calcification with 2×10^{-4} M phosphonoacetate; △–△–△ net calcification with urine inhibitor A ≏ 1.0 ml whole urine; ▲–▲–▲ ^{45}Ca exchange with urine inhibitor A ≏ 1.0 ml whole urine

Control of Inhibition. The effect of the level of the bound mineral phase together with other reaction conditions were investigated. As shown in Figs. 2A and 2B, when the amounts of inhibitor and matrix were constant but the amount of bound mineral increased, inhibition of net calcification as well as the $^{45}Ca^{2+}$ exchange reaction decreased in the case of methylenediphosphonate and the urine inhibitor but inhibition of calcification by Mg^{2+}, F^-, and phosphonoacetate remained the same.

Table 2 suggests that the inhibition of net calcification by Mg^{2+} can be reversed by increasing the soluble phase concentration of Ca^{2+} but not of phosphate. In addition, F^- inhibition can be reversed by increasing the concentration of either calcium or phosphate, whereas inhibition by phosphonoacetate, methylenediphosphonate and the urine inhibitor was not decreased by increasing the calcium or phosphate concentration of the soluble phase. These results suggest that the matrix has at least three types of reactive sites involved in the calcification process. The first is a Ca^{2+} binding site that can be blocked by Mg^{2+}, the second is an intermediate site that is blocked by phosphonoacetate and a third is related to the developing mineral phase. Growth sites or surface Ca-P aggregates of the mineral phase could constitute the mobile ions that participate in the $^{45}Ca^{2+}$ and $^{32}P\text{-}HPO_4^{2-}$ exchange reaction. They might also serve as binding sites of methylenediphosphonate and of the serum calcification inhibitors.

Interaction of Inhibitors with the Catalytic Matrix. In order to investigate the possibility that these inhibitors might function by interaction with specific

Table 2. *Reversal of Inhibition*

Exp.	Reaction system	Reactant concentration in soluble phase (μmoles)							
		Ca 42	Pi 43	Ca 85	Pi 43	Ca 51	Pi 40	Ca 51	Pi 80
		Rate of calcium uptake (mμmoles/mg matrix/15 min)							
1	Control	81		160		70		128	
	+ Mg^{2+} (1.0 mM)	36		158		24		63	
2	Control	65		136		—		104	
	+ F^- (8×10^{-6} M)	35		134		—		108	
3	Control	44		180		—		94	
	+ Phosphonoacetate (8×10^{-5} M)	20		70		—		40	
4	Control	60		335		—		220	
	+ methylenediphosphonate (5×10^{-5} M)	25		158		—		100	
5	Control	45		218		—		150	
	+ urine fraction A≏ 4 ml urine	6		40		—		24	
		Rate of $^{45}Ca^{2+}$ exchange (mμmoles/mg/15 min)							
6	Control	49		60		—		53	
	+ urine fraction A≏ 4 ml urine	17		24		—		21	
7	Control	17		60		—		19	
	+ phosphonoacetate (4×10^{-4} M)	18		23		—		19	
8	Control	35		40		—		41	
	+ methylenediphosphonate (5×10^{-5} M)	12		11		—		8	

The rates of ion uptake were measured as described in Methods. The matrix employed in all experiments was 100 mg but Exp. 1, 2, 4, 6 and 8 contained 60 μmoles of bound Ca^{2+} and 33 μmoles bound Pi, and Exp. 3, 4, 7 contained 40 μmoles bound Ca^{2+} and 27 μmoles bound Pi.

catalytic sites of the matrix or that they might react with complexes of the reacting ions in the soluble phase, studies were carried out as described in the protocols in Tables 3 and 4. Table 3 shows that the inhibitory effects of methylenediphosphonate and the urine inhibitor are not decreased by washing inhibitor-treated samples of the calcified matrix. On the other hand, the inhibitory effects of phosphonoacetate, Mg^{2+}, and F^- are reversed by washing. Table 4 shows that the rate of net calcification and the $^{45}Ca^{2+}$ exchange reaction induced by the washed, inhibitor-treated matrix were not decreased when the matrix was resuspended in the soluble phase derived from the original incubation system. Since the inhibitor was not detected in the soluble phase nor was it bound to the washed matrix, it must have been removed from the matrix during the washing procedure. From these results, we conclude that all of the calcification inhibitors exert their influence after reacting with the calcified matrix and that Mg^{2+}, F^-, and phosphonoacetate have a lower affinity for that matrix than do methylenediphosphonate and the urine inhibitor.

Table 3. *Inhibition of Calcification and Exchange*

Inhibitor	Ca uptake		Pi uptake		$^{45}Ca^{2+}$ exchange	
	% Inhibition					
	A	B	A	B	A	B
Methylenediphosphonate (1×10^{-5} M)	49	51	48	50	80	70
Urine Fr. A 4.0 ml urine	63	61	70	66	84	81
Phosphonoacetate (1.6×10^{-4} M)	10	68	0	74	0	0
Mg^{2+} (0.2 mM)	8	48	0	47	0	0
F^- (8×10^{-5} M)	7	30	7	33	0	0

Two samples of matrix of 100 mg each—A and B—were incubated in standard calcification medium containing 2.7 mM Ca^{2+} and 2.3 mM Pi for 20 h. The amount of mineral bound corresponded to 60 μmoles Ca^{2+} and 38 μm Pi. A was incubated with indicated amounts of inhibitors for 1 h, B was incubated with the same volume of water. Both systems were filtered and the matrix washed two times with NaCl-barbital buffer. Each matrix was then suspended in the standard assay medium (cf. Methods) and the rates of calcification and exchange determined. During the latter incubation, B contained the indicated amounts of inhibitors. Percentage inhibition was estimated from the values determined above as compared to those derived from an identically treated matrix which was not exposed at any time to inhibitors.

Table 4. *Inhibition of Calcification and Exchange*

Inhibitor	Ca^{2+} uptake		Pi uptake		$^{45}Ca^{2+}$ exchange	
	% Inhibition					
	A	B	A	B	A	B
Phosphonoacetate (1.6×10^{-4} M)	12	74	17	76	0	0
Mg^{2+} (4×10^{-4} M)	7	85	6	86	0	0
F^- (8×10^{-5} M)	0	26	0	30	0	0

Two samples of matrix—A and B—of 100 mg each were calcified as described in Table 3. Sample A was then exposed to the indicated concentration of inhibitors for one hour and B was exposed to the same volume of water. Both systems were then filtered and the matrix from each was washed two times with the NaCl-barbital buffer. The Ca^{2+} and Pi concentration of each original filtrate (soluble reaction phase) was determined and adjusted togive 2.0 mM Ca^{2+} and 1.6 mM Pi. The amount of inhibitors indicated above were added to filtrate B. Both filtrates were then recombined with the corresponding washed matrix and the rates of calcification and the $^{45}Ca^{2+}$ exchange rates measured as described in Methods. Percentage inhibition was determined as described in Table 3.

Direct Demonstration of Extramineral Phase Calcium and Phosphate Binding Sites. It was frequently observed that whereas elevated concentrations of methylenediphosphonate produced almost complete inhibition of the $^{45}Ca^{2+}$ and the $^{32}P\text{-}HPO_4^{2-}$ exchange reactions of calcified matrix, maximum inhibition of the initial reaction rate of net calcification was always less than 100% of the control reaction rate. These findings suggested that methylenediphosphonate-insensitive binding of Ca^{2+} and HPO_4^{2-} by the matrix can take place. The results presented in Table 5 demonstrate that a previously calcified matrix treated subsequently

Table 5. *Methylenediphosphonate-insensitive Ion Uptake*

Exp.	Conditions	Ion uptake (μmoles)	
		$^{45}Ca^{2+}$	^{32}Pi
1	Complete	5.5	4.5
2	Complete, not washed	0.2	0.2
3	Minus Pi	2.5	0.2
4	Plus phosphonoacetate (8×10^{-4} M)	2.3	0.2
5	Minus Pi plus phosphonoacetate (8×10^{-4} M)	2.3	0.2
6	Plus Mg^{2+} (1.6 mM)	0.2	0.2
7	Not treated with methylene-diphosphonate (1 h assay)	30.0	19.0

100 mg tendon matrix was calcified under conditions resulting in 58 μmoles Ca^{2+} and 40 μmoles Pi bound. The calcified matrix was then incubated with 1.2 mM Methylene-diphosphonate for 1 h, separated from soluble phase, washed with NaCl-barbital buffer and incubated in standard assay medium (cf. Methods) containing $^{45}Ca^{2+}$-$CaCl_2$ (300000 cpm) or ^{32}P-HPO_4^{-} (300000 cpm) as indicated. Ion uptake was measured as the disappearance of radioactivity from soluble phase and by spectrophotometric methods described previously (Wadkins, 1968). For all assays (Exp. 1–6), ion uptake was measured at 6 min, 30 min, 1 h and 20 h and the same values as those presented above were obtained. Exp. 7 shows that the incubation and washing procedure did not alter normal calcification ability.

with methylenediphosphonate and washed to remove loosely-bound ions will bind an additional 55 μmoles of Ca^{2+} and 45 μmoles of phosphate per g of matrix when incubated in the standard reaction system (Exp. 1). The amount of methylene-diphosphonate employed was sufficient to completely inhibit the $^{45}Ca^{2+}$ and ^{32}P-HPO_4^{2-} exchange reactions. No incorporation was detected when the washing step was omitted (Exp. 2) indicating that the matrix binding sites detected in Exp. 1 were already associated with unlabeled ions which did not exchange with the labeled ions of the soluble phase during the subsequent incubation.

The ratio of the calcium to phosphate bound in Exp. 1 is somewhat greater than 1.0 but is consistently lower than the 1.5–1.8 observed for net calcification in the absence of methylenediphosphonate (Exp. 7). Furthermore, the uptake of calcium and phosphate demonstrated in Exp. 1 is almost complete within 5 min. These facts suggest that the ion uptake of Exp. 1 is not due to incomplete inhibition of mineral phase accretion or of the exchange reactions by the bound methylenediphosphonate, but is more likely due to ion binding at other reactive matrix sites.

Exp. 3 of Table 5 indicates that in the absence of added HPO_4^{2-}, no ^{32}P HPO_4^{2-} was bound to the matrix and that the amount of bound Ca^{2+} was reduced by approximately 60%. These results suggest that the calcium bound during Exp. 1 consists of two components—a phosphate-dependent and a phosphate-independent component. Exp. 4 and 5 demonstrate that phosphate binding and the phosphate-dependent binding of Ca^{2+} are prevented by the addition of phosphonoacetate. Furthermore, both Ca^{2+} components as well as phosphate binding are prevented by Mg^{2+} (Exp. 6). It is important to note that the concentrations of Mg^{2+} and phosphonoacetate employed here are those that also produce complete

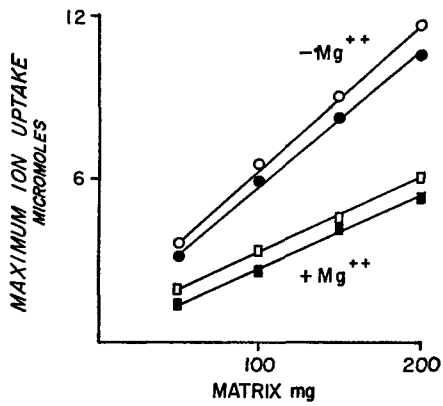

Fig. 3. Methylenediphosphonate-insensitive calcium and phosphate binding by calcified matrix. The indicated amounts of calcified matrix containing 38 μmoles Ca^{2+} and 24 μmoles Pi were each treated with 1.0 mM methylenediphosphonate, washed twice with NaCl-barbital buffer and then exposed to the standard reaction medium as described in Methods above containing 100000 cpm ^{45}Ca, or ^{32}P-HPO_4^{2-} and incubated for 20 h at 30°. Aliquots of soluble phase were removed at 0, 10, 20, and 60 min and at 20 h, and the ion uptake determined from disappearance of radioactivity. The net uptake of Ca^{2+} from the medium was also measured by atomic absorption spectrophotometry (Wadkins, 1968). Net uptake by both methods was identical at all time periods studied. Under these conditions, after treatment with 1 mM methylenediphosphonate, exchange activity was not detectable. ○–○–○ net Ca^{2+} uptake minus Mg^{2+}; ●–●–● net Pi uptake minus Mg^{2+}; □–□–□ net Ca^{2+} uptake plus 0.2 mM Mg^{2+}; ■–■–■ net Pi uptake plus 0.2 mM Mg^{2+}

inhibition of Ca^{2+} and HPO_4^{2-} into the bound mineral phase (cf. Table 1). The results presented in Fig. 3 demonstrate that the amount of calcium and phosphate bound as described in Exp. 1 of Table 5 is proportional to the amount of tendon matrix employed. Several experiments have shown that the maximum ion uptake under these conditions corresponds to 55–65 μmoles of Ca^{2+} and 35–45 μmoles of phosphate per g dry weight of matrix. Of that amount of Ca^{2+} uptake, approximately 60% is phosphate-dependent and the remainder is phosphate-independent.

The results presented in Fig. 3 also show that the methylenediphosphonate-insensitive binding of Ca^{2+} and HPO_4^{2-} is inhibited by approximately 50% over the entire range of tendon matrix by the concentration of Mg^{2+} that also inhibits the rate of net calcification by 50%. This finding added to the effects noted with phosphonoacetate (cf. Tables 1 and 5) strongly suggest that the matrix-bound calcium phosphate complexes described here could be intermediates in the overall calcification process.

Discussion

The studies described above suggest that the calcification process catalyzed by the bovine tendon matrix is inhibited by a group of compounds that can be categorized into three general classes. Class 1 consists of those agents that inhibit net calcification but not the $^{45}Ca^{2+}$, nor the ^{32}P-HPO_4^{2-}, exchange reactions. This class includes Mg^{2+} and F^-. Their inhibitory effects can be reversed by

increasing the concentration of Ca^{2+} or HPO_4^{2-} in the soluble phase but are not decreased by increasing the amount of the matrix-bound mineral phase. Class 2, represented by substances such as phosphonoacetic acid, inhibits net calcification but neither exchange reaction. Its inhibitory effect is not reversed by increasing the concentration of Ca^{2+} or HPO_4^{2-} in the soluble phase nor by increasing the amount of the matrix-bound mineral phase. Class 3 comprises methylenediphosphonate and the urine calcification inhibitors (cf. Methods). These substances do inhibit the two exchange reactions as well as net calcification and their inhibitory effects can be decreased by increasing the amount of the matrix bound mineral phase but not by increasing the Ca^{2+} or HPO_4^{2-} concentration of the soluble phase.

From the data presented here (cf. Tables 3 and 4) we conclude that all of these substances inhibit net calcification—and the two exchange reactions for Class 3—by interaction with the catalytic matrix and not by formation of unreactive soluble complexes of calcium phosphate. The data do show that Class 1 and 2 inhibitors have a lower affinity for the matrix than do those of Class 3. However, the differential inhibitory properties suggest that these substances react at different sites in the calcified matrix and that the overall calcification process could be a multi-step process. Accordingly, the heterogeneous phase transformation mediated by the tendon matrix might consist of the interaction of ionic calcium with a specific Ca^{2+} binding site of the matrix, which would then facilitate the interaction of inorganic phosphate to produce a matrix bound Ca-P complex that would ultimately be incorporated into the mineral phase bound at a third matrix site.

Kinetic studies described in a previous communication (Jethi *et al.*, 1970) suggest a linear increase of the initial reaction rates of net HPO_4^{2-} uptake and of the $^{45}Ca^{2-}$ and the ^{32}P-HPO_4^{2-} exchange reactions as the amount of bound mineral was increased while the amount of tendon matrix was held constant. The initial rate of Ca^{2+} uptake did increase at lower levels but tended to approach a limiting value at higher levels of bound mineral. These results indicate the involvement of a matrix binding site outside the mineral phase for Ca^{2+} that is rate limiting in the calcification process at higher levels of the mineral phase and is not involved in the $^{45}Ca^{2+}$ exchange reaction. The present data demonstrate that the inhibition of calcification by Mg^{2+} can be reversed by increasing the Ca^{2+} concentration of the soluble phase but cannot be reversed by increasing the phosphate concentration. These data provide additional support for a catalytic matrix site with divalent cation specificity. Fluoride inhibition, on the other hand, can be reversed by increasing the concentration of either Ca^{2+} or HPO_4^{2-}. The precise mechanism of fluoride inhibition is not readily apparent, but since the concentration of fluoride that inhibits 50% is only 1% that of Ca^{2+} and HPO_4^{2-} of the reaction medium, and fluoride appears to inhibit by interaction with the catalytic matrix, it is possible that fluoride reacts reversibly to form a soluble calcium fluoride phosphate complex which might inhibit a functional matrix site.

Previous studies reported that uncalcified matrix does not induce the $^{45}Ca^{2+}$ and ^{32}P-HPO_4^{2-} exchange reactions, whereas calcified matrix does (Jethi *et al.*, 1970). The increase of the initial reaction rate of net calcification, as well as both exchange reactions, as the amount of mineral phase was increased was interpreted

as an increased number of crystal growth sites of the mineral phase which under the conditions were rate limiting for both exchange and net calcification. The studies described here (cf. Table 1 and Figs. 1 and 2) demonstrate that the degree of inhibition of net calcification and exchange by Class 3 compounds is greater when the level of the mineral matrix is low. In fact, the inhibition of both reactions by methylenediphosphonate can be alleviated completely by increasing the amount of the bound mineral phase. We have interpreted these results to mean that the Class 3 inhibitors react with a bound Ca-P aggregate that constitutes the postulated growth sites of the bound mineral phase and thereby decreases the rate of net calcification as well as the two exchange reactions. Thus, at constant levels of methylenediphosphonate as well as of the urine calcification inhibitor, a greater extent of inhibition would be expected with lower amounts of the bound mineral phase than with greater amounts. The ability of these substances to depress the rate of calcification by previously uncalcified matrix could mean that small amounts of Ca-P aggregates or nuclei are already present at the matrix mineral phase growth site. Another possibility is that in the presence of the inhibitor, the calcification process does occur until the bound Ca-P aggregate which reacts with the inhibitor is formed at the mineral phase binding site and that subsequent growth of that aggregate is depressed. In this case the amount of Ca^{2+} and HPO_4^{2-} present in the aggregate would be small compared to the amount bound to the mineral phase matrix site of the uninhibited system.

The inhibition produced by phosphonoacetate is distinctive because it is reversed neither by increasing the Ca^{2+} or HPO_4^{2-} concentration of the soluble phase, nor by increasing the amount of the bound mineral phase. According to the concept developed here, phosphonoacetate does not react with the cationic site because Ca^{2+} does not reverse its effect, nor does phosphonoacetate react with the mineral site since it does not influence the $^{45}Ca^{2+}$ and ^{32}P-HPO_4^{2-} exchange reactions. We suggest therefore that this inhibitor reacts with a matrix site different from the two described above. The function of that postulated site might be to produce a calcium phosphate complex that eventually interacts with the mineral phase bound at the third site.

This suggested mode of action of phosphonoacetate receives additional support from the data presented in Table 5 and Fig. 3. These results show that the calcified matrix that had been treated with sufficient methylenediphosphonate to inhibit the net growth of the mineral phase as well as both exchange reactions and then washed to remove adventitious Ca^{2+} and HPO_4^{2-} will bind an additional, but small, amount of Ca^{2+} and HPO_4^{2-}. Approximately two-thirds of that Ca^{2+} uptake is HPO_4^{2-}-dependent and is approximately the same on a molar basis as the amount of HPO_4^{2-} bound. The uptake of both kinds of bound Ca^{2-} (i.e., phosphate-dependent and phosphate-independent) was inhibited by the concentration of Mg^{2+} that also inhibits net calcification. Phosphonoacetate, on the other hand, prevented the uptake of only the phosphate-dependent form of bound Ca^{2+} as well as the uptake of HPO_4^{2-}. The concentration of phosphonoacetate effective in this case also corresponds to that which inhibits net calcification. The data presented in Fig. 3 demonstrates that the methylenediphosphonate-insensitive Ca^{2+} uptake is proportional to the amount of matrix employed and corresponds to approximately 60 μmoles/g of matrix. From the results presented in Table 5,

it appears that the Mg^{2+}-sensitive site will bind approximately 20 μm Ca^{2+} and no HPO_4^{2-}, whereas the phosphonoacetate-sensitive site will bind approximately 40 μmoles of Ca^{2+}, and 40 μmoles of HPO_4^{2-}, per g of matrix. The ability of Mg^{2+} to prevent Ca^{2+} and HPO_4^{2-} uptake at both sites at concentrations that will also inhibit net calcification is consistent with an obligatory mechanism of calcification taking place in sequence at reactive sites. Similarly, the ability of phosphonoacetate to prevent Ca^{2+} and HPO_4^{2-} uptake at the second site postulated above as well as net calcification supports the concept that a Ca-P complex constitutes an intermediate of the overall calcification process.

Phosphonoacetate and methylenediphosphonate possess similar chemical structures. Both compounds would be expected to exist in anionic forms at pH 7.5, although the diphosphonate probably has a higher net negative charge. Models of both compounds indicate that the linear distance between charge centers could be as much as 2–3 Å longer for the diphosphonate. These considerations suggest remarkable steric and/or ionic requirements of the reactive sites of the catalytic matrix.

The calcification model proposed here, consisting of a linear sequence of three obligatory steps in the formation of a matrix-bound mineral phase is comparable in principle to polyfunctional heterogeneous catalysis characterized by minimal desorption of intermediates (Thomas and Thomas, 1967). It differs from a single nucleation-growth site model involving the construction of the apatite lattice by the stepwise addition of individual ions to a single nucleation site followed by crystal growth *in situ*. It is not readily apparent how the data presented here could be rationalized in terms of the single site model.

The basic utility of the multi-step model proposed here is that it allows for the differential effects of the several calcification inhibitors and the existence of the demonstrated matrix-bound Ca^{2+} and calcium phosphate complexes as well as the marked efficiency that characterizes this catalytic substrate. As pointed out earlier (Jethi *et al.*, 1970) the tendon matrix employed in these studies will induce the formation of a bound mineral phase that in several respects resembles apatite, from solutions whose $(Ca^{2+}) \times (HPO_4^{2-})$ product is as low as 0.5–1.0 mM^2. This value is lower than that reported for serum as well as the fluid phase ambient to the calcifying sites of epiphyseal cartilage (Howell *et al.*, 1968) and considerably lower than that required for apatite formation in the absence of an inducing substrate (Solomons and Neuman, 1960). Thus, the involvement of specific matrix sites that could achieve the selective formation and binding of critical intermediates as well as binding of the mineral phase product could provide a considerable entropic advantage to the system.

References

Fiske, C. H., SubbaRow, Y.: The colorimetric determination of phosphorus. J. biol. Chem. **66**, 375–380 (1925).

Fleisch, H., Russell, R. G. G., Francis, M. D.: Diphosphonates inhibit hydroxyapatite dissolution *in vitro* and bone resorption in tissue culture *in vivo*. Science **165**, 1262–1264 (1969).

— — Straumann, F.: Effect of pyrophosphate on hydroxyapatite and its implications in calcium homeostasis. Nature (Lond.) **212**, 901–903 (1966).

Francis, M. D., Russell, R. G. G., Fleisch, H.: Diphosphates inhibit formation of calcium phosphate crystals *in vitro* and pathological calcification *in vivo*. Science **165**, 1264–1266 (1969).

Howard, J. E., Thomas, W. C., Jr., Barker, L. M., Smith, L. H., Wadkins, C. L.: The recognition and isolation from urine and serum of a peptide inhibitor to calcification. Johns Hopk. Medical J. **120**, 237–243 (1967).

Howell, D. C., Pita, J. C., Marquez, J. F., Madruga, J. E.: Partition of calcium, phosphate, and protein in the fluid phase aspirated at calcifying sites in epiphyseal cartilage. J. clin. Invest. **47**, 1121–1132 (1968).

Jethi, R. K., Inlow, C. W., Wadkins, C. L.: Studies of the mechanism of biological calcification. I. Kinetic properties of the *in vitro* calcification of collagen-containing matrix. Calc. Tiss. Res. **6**, 81–92 (1971).

Neuman, W. F., Neuman, M. W.: The chemical dynamics of bone mineral. Chicago: Univ. Chicago Press 1958.

Pak, C. Y. C., Bartter, F.: Ionic interaction with bone mineral—Evidence for an isoionic calcium exchange with hydroxyapatite. Biochim. biophys. Acta (Amst.) **141**, 401–409 (1967).

Solomons, C. C., Neumann, W. F.: On the mechanisms of calcification: the remineralization of dentin. J. biol. Chem. **235**, 2502–2506 (1960).

Thomas, J. M., Thomas, W. J.: Introduction to the principles of heterogeneous catalysis, p. 338. New York: Academic Press 1967.

Thomas, W. C., Jr., Tomita, A.: Mineralization of human and bovine tissue *in vitro*. Amer. J. Path. **51**, 621–628 (1967).

Wadkins, C. L.: Experimental factors that influence collagen calcification *in vitro*. Calc. Tiss. Res. **2**, 214–228 (1968).

Weikel, J. H., Jr., Neuman, W. F., Feldman, I.: The surface chemistry of bone. VIII. On the mechanism of ionic exchange. J. Amer. chem. Soc. **76**, 5202–5207 (1954).