Biochemistry

Volume 10, Number 12 June 8, 1971

Studies of the Relationship of Proton Production and Calcification of Tendon Matrix *in Vitro**

R. A. Luben† and C. L. Wadkins‡

ABSTRACT: The uptake of Ca^{2+} and HPO_4^{2-} from stable solutions by a collagen-containing matrix derived from bovine tendon results in the formation of a matrix-bound mineral phase that resembles hydroxylapatite. This process is also accompanied by the liberation of H^+. The simultaneous presence in the reaction system of Ca^{2+}, HPO_4^{2-}, and an active tendon matrix is required for both processes. Low concentrations of Mg^{2+}, F^-, phosphonoacetate, methylenediphosphonate, and an acidic acid-labile substance derived from human serum and urine inhibit net calcification as well as H^+ production. The presence in the reaction system of urea or prior treatment of the tendon matrix with urea inactivates both processes. The rates of H^+ production and of net calcification are proportional to the amount of tendon matrix and those rates are also higher for calcified matrix than for uncalcified matrix. These results indicate that H^+ production is an inherent property of the calcification reaction catalyzed by the tendon matrix. Kinetic studies indicate that the initial stage of the calcification reaction is characterized by nearly equal rates of Ca^{2+} and HPO_4^{2-} uptake but no H^+ production. This stage (stage 1) of the reaction proceeds until approximately 60 μmoles of Ca^{2+} and HPO_4^{2-} per g of matrix is bound after which the ratio of the rates of Ca^{2+} uptake to HPO_4^{2-} uptake tend to approach a value of 1.5–1.7. It is during the latter stage (stage 2) that H^+ production occurs and the ratio of the rates of Ca^{2+} uptake to H^+ production during stage 2 is 1.6 which is somewhat higher than that predicted for hydroxylapatite formation. The time lapse prior to the onset of H^+ production corresponding to stage 1 is inversely proportional to the amount of the matrix-bound mineral phase. The initial rates of calcium and phosphate uptake and of H^+ production associated with stage 2 increase as the amount of matrix-bound mineral phase is increased and tend to approach limiting values at higher levels. These results suggest that the tendon matrix catalyzes calcification by a multistep process in which an acidic, matrix-bound calcium phosphate complex constitutes a rate-controlling intermediate of overall calcification which might be an acidic amorphous component of the bound mineral phase. Evidence is also presented that the calcified matrix can be induced to undergo decalcification with the consumption of H^+. The stoichiometry of this reaction corresponds to that expected for the conversion of hydroxylapatite into Ca^{2+} and HPO_4^{2-}. These results suggest that mineral-phase dissolution occurs by a mechanism different from that of the reversal of net calcification, possibly by direct dissociation of hydroxylapatite of the bound mineral phase without participation of the one or more matrix-bound intermediates that appear to characterize net calcification.

A dominant feature of several theories of the local mechanism of biological calcification is that an organic matrix functions as a nucleating substrate to induce the formation of a mineral phase from a metastable soluble phase (Glimcher, 1959; Solomons and Neuman, 1960; Posner, 1969). Microscopic examination of bone, calcified tendon, and tooth dentin reveal a specific spatial relationship between crystals of hydroxylapatite and collagen fibers (Fitton-Jackson, 1957; Glimcher, 1959). Katz (1969) proposed that reconstituted collagen fibers function as classical catalysts of heterogeneous nucleation by acting through a mechanism that reduces the surface energy and/or the number of ions present in a critical nucleus required for phase change and thereby reduces the energy barrier for hydroxylapatite formation.

Studies in this laboratory demonstrated that a collagen-containing matrix derived from beef tendon by the method of Thomas and Tomita (1967) is a very efficient substrate for heterogeneous nucleation of hydroxylapatite (Wadkins, 1968; Jethi *et al.*, 1970; Jethi and Wadkins, 1971). These studies showed that ion uptake by the tendon matrix requires the simultaneous presence of both calcium and phosphate, that

* From the Department of Biochemistry, The University of Arkansas School of Medicine, Little Rock, Arkansas. *Received November 19, 1970.* This investigation was supported by U. S. Public Health Service Grant AM-11528.

† Predoctoral fellow of the Department of Biochemistry, The University of Arkansas School of Medicine.

‡ To whom to address correspondence.

TABLE I: Calibration of pH-Stat.[a]

Medium	HCl Added (μequiv) (A)	Chart Deflection (mm) (B)	A/B (μequiv/mm)
H_2O	14.1	73	0.19
120 mM NaCl	8.5	42	0.20
100 mg of tendon matrix plus 120 mM NaCl	14.1	73	0.19
100 mg of tendon matrix plus 120 mM NaCl and 2.0 mM $CaCl_2$	14.7	79	0.19
100 mg of tendon matrix plus 120 mM NaCl plus 1.6 mM KH_2PO_4	14.7	77	0.19

[a] The volume of each system was 25 ml. Each was adjusted to pH at 37° prior to addition of the indicated amount of standardized HCl. The pH-Stat titrant was 0.050 N NaOH.

the reactive ion product (mM Ca^{2+} × mM HPO_4^{2-}) of the soluble phase in excess of 0.3 mM is essential, and that the calcification reaction proceeds until a soluble phase ion product of 0.3 mM^2 is attained. The initial rate of ion uptake as this limiting reactive ion product is approached is proportional to the amount of tendon matrix added. The presence of 6 M urea in the reaction system or prior exposure of the tendon matrix to urea inactivated its ability to induce ion intake. Electron microscope studies reveal the presence of crystalline structures associated with the collagen fibers of the calcified matrix.[1] These crystalline structures also manifest electron diffraction patterns comparable to those of authentic hydroxylapatite. These results constitute evidence that a macromolecular entity—possibly collagen—associated with the tendon matrix can catalyze the production of a bound form of hydroxylapatite from solutions whose activities with respect to Ca^{2+} and HPO_4^{2-} can be even lower than those of serum and of extracellular fluid associated with calcifying areas of epiphyseal cartilage (Howell *et al.*, 1968).

More recent studies (Jethi *et al.*, 1970; Jethi and Wadkins, 1971) demonstrate that following resuspension of calcified sample of the tendon matrix in a fresh calcifying medium containing $^{45}Ca^{2+}$ or [^{32}P]HPO_4^{2-}, radioisotope is incorporated into the mineral phase by net ion uptake and by exchange reactions. These studies also showed that whereas methylenediphosphonate, pyrophosphate, and an acidic, acid-labile organic compound of human serum and urine inhibit both net calcification as well as exchange, Mg^{2+}, F^-, phosphonoacetate, and urea inhibit only net calcification. These studies suggest that those compounds that inhibit the exchange reactions react directly with the matrix-bound mineral phase—possibly at crystal growth sites—whereas those compounds that do not inhibit the exchange processes react with nonmineral phase sites of the catalytic matrix.

Binding studies with methylenediphosphonate-inhibited matrix preparations revealed the presence of two such sites (Jethi and Wadkins, 1971). One of these binds only calcium ions by a phosphate-independent reaction and is inhibited competitively by Mg^{2+}. The second binds both calcium and phosphate in a 1:1 ratio and both ions are required. The interaction of calcium and phosphate at the latter site is inhibited by phosphonoacetate and by Mg^{2+} at concentrations that also inhibit net calcification. On the basis of these results, a multistep reaction pathway for net calcification of the tendon matrix was proposed.

Consideration of the ionic forms of calcium and phosphate that react at physiological pH and ionic strength to form hydroxylapatite led to the prediction that hydrogen ions should be produced during the reaction (Samachson, 1968). This was recently demonstrated for the case of spontaneous precipitation of hydroxylapatite by Francis *et al.* (1969). The potential utility of this observation for measurement of early reaction rates as well as for elucidation of the relationship of H^+ production to the catalytic process prompted this study with the tendon matrix system. This investigation has established that a quantitative relationship exists between H^+ production and net calcification as well as between H^+ consumption and decalcification and that those relationships lend support to the multistep calcification reaction pathway described above.

Methods

The calcification matrix employed in these studies was prepared by an adaptation of the method of Thomas and Tomita (1967) described by Jethi *et al.* (1970). The basic reaction system consists of 0.15 M NaCl, 2.0 mM $CaCl_2$, 2.0 mM potassium phosphate, and 50–200 mg dry weight of uncalcified or calcified matrix. The latter was prepared as described by Jethi *et al.* (1970). The final volume of the reaction system is 25 ml. The system pH was maintained at 7.4 ± 0.02 by automatic additions of 0.050 N NaOH by a Sargent Recording pH-Stat at constant temperature of 37 ± 0.25°. All components of the system, with exception of P_i were equilibrated for 2–3 min during which time the pH was adjusted to 7.4. Phosphate was added to initiate the calcification reaction. A continuous recording of base added to maintain a constant pH was then obtained. Aliquots of the soluble phase were removed at various times and analyzed for Ca^{2+} and P_i content as described previously (Wadkins, 1968). In order to minimize an artifactual pH change due to a change in the ionic strength of the system upon addition of phosphate reagent to initiate the reaction, 0.52 ml of 70 mM KH_2PO_4 which had been adjusted to pH 7.40 at 37° was added. Under these conditions a reproducible initial reaction rate of proton liberation that correlates with calcium and phosphate uptake from the reaction medium was obtained.

The recording pH-Stat was calibrated by measuring the recorder deflection following addition of known amounts of HCl to the various systems described in Table I. From these results it can be seen that a nearly constant recorder deflection per equivalent of acid added was observed for all systems, that the basic calcification system does not constitute a significant buffering system, and that quantitative estimations of H^+ production by the reaction system during calcification is possible by this method. Replicate determinations of pH-Stat response (millimeters per microequivalent) over a range of standard acid or base present in the reaction system showed a relative standard deviation [$(\sigma)/\bar{x} \times 100$] of 5%.

Methylenediphosphonate was obtained from 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

[1] Unpublished observations of R. A. Luben.

TABLE II: Calcification of Tendon Matrix.[a]

Expt	Reaction System	$-\Delta Ca^{2+}$ (μmoles)	$-\Delta P_i$	$+\Delta H^+$ (μequiv)
1	Complete	37.3	22.4	14.0
	Minus matrix	0.1	0.1	<0.5
	Minus Ca^{2+}		0.3	<0.5
	Minus P_i	1.0		<0.5
2	Complete	41.3	24.2	19.0
	Minus P_i	0.3		<0.5
	Plus 6 M urea	0.2	0.2	<0.5
	Urea-treated matrix	0.1	0.2	<0.5
	NaCl-washed matrix	38.7	23.8	18.0

[a] The standard reaction system described in Methods was employed. Each reaction system contained 100 mg of tendon matrix. Urea-treated matrix was prepared by exposing 100 mg of matrix to freshly prepared 6 M urea, filtered, followed by resuspension of the matrix in 150 mM NaCl and filtration. The washing procedure was repeated two times. NaCl-washed matrix was prepared as described above except that 150 mM NaCl was substituted for urea. The data presented constitute the Ca^{2+} and P_i uptake from the soluble phase and H^+ produced during the 60-min reaction period.

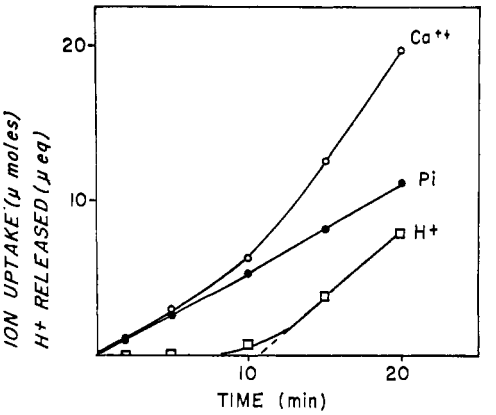

FIGURE 1: Relationship of Ca^{2+} and P_i uptake and H^+ production during calcification of tendon matrix. The standard reaction conditions described in Methods were employed. The reaction system contained 200 mg of tendon matrix, 2.0 mM Ca^{2+}, and 1.7 mM P_i.

estimated to be greater than 98%. The serum and urine calcification inhibitors employed in these studies were derived from human sources by an adaptation of the ion-exchange and gel filtration purification method described previously (Howard *et al.*, 1967).

Results

Proton Production during Calcification. The data presented in Table II show that uptake of calcium and phosphate during a 1-hr incubation period is accompanied by net proton production and that these three phenomena require the simultaneous presence in the reaction system of Ca^{2+}, HPO_4^{2-}, and the tendon matrix. The fact that no disappearance of Ca^{2+} of HPO_4^{2-} and only minimal production of H^+ were detected in the absence of the tendon matrix is consistent with the absence of any visible or filtrable precipitate and attests to the stability of the basic reaction system in the absence of the tendon matrix. Similarly, the absence of any detectable ion uptake and H^+ production when either Ca^{2+} or HPO_4^{2-} was omitted indicates that endogenous ion content of the tendon matrix is not sufficient to activate net calcification.

Experiment 2 demonstrates that the presence in the reaction system of 6 M urea or prior treatment of the matrix with 6 M urea followed by washing with 150 mM NaCl markedly depresses the ability of that matrix to induce calcification and H^+ production whereas simple washing with the NaCl solution had only minimal effect. These results indicate that calcification and H^+ production are most likely induced by a macromolecular structure present within the tendon matrix.

Effect of Calcification Inhibitors. Several substances previously shown to inhibit net Ca^{2+} and phosphate uptake by uncalcified and calcified matrices and the $^{45}Ca^{2+}$- and [^{32}P]-HPO_4^{2-}-exchange reactions induced by the latter (Jethi *et al.*, 1970) were investigated for their effects of H^+ production. The results shown in Table III demonstrate that Mg^{2+}, fluoride, the calcification inhibitor derived from human serum and urine, methylenediphosphonate, and phosphonoacetate produce nearly identical inhibitory effects on calcium and phosphate uptake and on H^+ production. Since previous studies indicate that methylenediphosphonate and the serum and urine calcification inhibitors function by interaction with specific sites of the bound mineral phase whereas Mg^{2+}, F^-, and phosphonoacetate interact with nonmineral-phase matrix sites (Jethi *et al.*, 1970; Jethi and Wadkins, 1971), the results presented here suggest that both kinds of reactive matrix sites are involved in H^+ production.

Proton Production Induced by Uncalcified Matrix. The results presented in Figure 1 compare the rates of calcium phosphate uptake and H^+ production when uncalcified matrix was employed. These data indicate that the calcification process is composed of two stages. The initial reaction period of stage 1 is characterized by similar rates of calcium and phosphate uptake without detectable H^+ production. This lag period in H^+ production lasts until approximately 6 μmoles of calcium and 6 μmoles of phosphate are removed from the soluble phase. The subsequent period or stage 2 is characterized by an increased rate of calcium uptake and the ratio of calcium uptake to phosphate uptake is 1.5–1.7 and the ratio of calcium uptake to H^+ formation is 1.6–1.8. Thus it is during stage 2 that the calcium and phosphate uptake and H^+ production expected for apatite formation are observed. These results suggest that a matrix-bound calcium phosphate complex—herein referred to as CaP_i—is produced during stage 1 and that bulk mineral-phase formation associated with H^+ liberation occurs exclusively during stage 2.

Effect of Matrix Concentration and Its Mineralized State on Proton Production. The data presented in Figure 2 show that the initial rate of proton production during stage 2 of the calcification reaction is proportional to the amount of tendon matrix added to the reaction system. These results also show that the rate of proton production is increased when an equal amount of calcified matrix was employed.

Previous studies in this laboratory (Wadkins, 1968; Jethi *et al.*, 1970) demonstrated that similar results are obtained under these conditions when the rates of calcium and phosphate uptake from the soluble phase were measured. These results were interpreted to indicate that a component of the tendon matrix catalyzes the calcification process and it ap-

TABLE III: Inhibition of Tendon Matrix Calcification.[a]

Expt	Additions	Ratio	$-\Delta Ca/-\Delta P_i$	% Inhibn Ca^{2+}	% Inhibn H^+
1	Control		1.5		
	NaF (1×10^{-5} M)		1.6	32	35
	NaF (5×10^{-5} M)		1.5	58	60
	$MgCl_2$ (0.2 mM)		1.6	53	49
	$MgCl_2$ (1.0 mM)		1.5	91	92
2	Control		1.7		
	Calcification inhibitor ≏ 0.5 ml of urine		1.6	58	59
	Calcification inhibitor ≏ 5.0 ml of urine		1.5	98	100
	Calcification inhibitor ≏ 8.0 ml of serum		1.6	70	71
3	Control		1.5		
	Methylenediphosphonate				
	2×10^{-7} M		1.6	54	57
	1×10^{-6} M		1.7	89	89
	Phosphonoacetate				
	1×10^{-6} M		1.5	62	58
	1×10^{-5} M		1.6	100	100

[a] The standard reaction system described in Methods was employed. Each system contained 100 mg of tendon matrix. Per cent inhibition and rate ratios were calculated from maximum linear rates after initiation of H^+ production and the ΔCa^{2+} and ΔP_i values during the corresponding time interval.

pears reasonable to apply the same interpretation for net proton production. Furthermore, it would appear that a rate-limiting process that controls net calcification and proton production under these conditions is related to the amount of the matrix-bound mineral phase.

Proton Production Accompanying Ion Uptake by Calcified Matrix. The results presented in Figure 3 demonstrate that the calcification reaction induced by previously calcified tendon matrix differs in two important aspects from that of uncalcified matrix. The first difference is that H^+ production begins immediately after initiation of ion uptake. The second difference is that the ratios of calcium uptake to phosphate uptake and of calcium uptake to H^+ production are essentially constant throughout the reaction period and are quantitatively the same as those observed during stage 2 with uncalcified matrix.

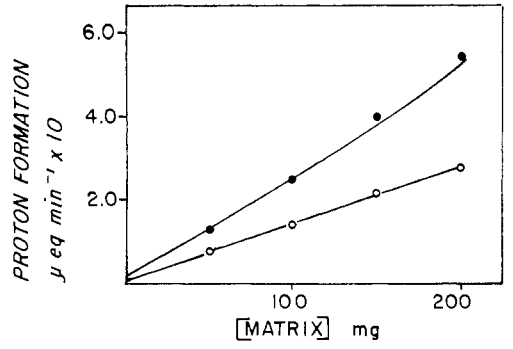

FIGURE 2: Relationship between amount of tendon matrix and maximum rate of H^+ production. All reactions carried out as described in Methods. (○) Uncalcified matrix; (●) calcified matrix to which was bound 28 μmoles of Ca^{2+} and 18 μmoles of P_i per 100 mg. The initial concentration of Ca^{2+} and P_i of the soluble phase was 2.0 and 1.7 mM, respectively. The reaction rate was calculated from the maximum initial rate of H^+ production.

Many studies in which the amount of tendon matrix, reaction temperature, and pH were varied showed changes in reaction rate but no marked influence on the stoichiometry of the overall reaction. The effects of these and other reaction conditions on the rates and extent of the calcification process will be described in a subsequent communication.

Effect of the Amount of Matrix-Bound Mineral Phase on Rates of Calcification and H^+ Production. The results presented in Figure 4 demonstrate that the amount of the bound mineral phase has a significant effect on the rate of calcium uptake, the duration of the H^+ lag period and on the rate of H^+ production. When the amount of the tendon matrix was held constant and the amount of bound mineral, expressed as micromoles of bound Ca^{2+}, is increased from 0 to 20, the rate of Ca^{2+} uptake during stage 2 calcification and the corresponding rate of H^+ liberation change very little. The rates of both processes were markedly stimulated when 25–35

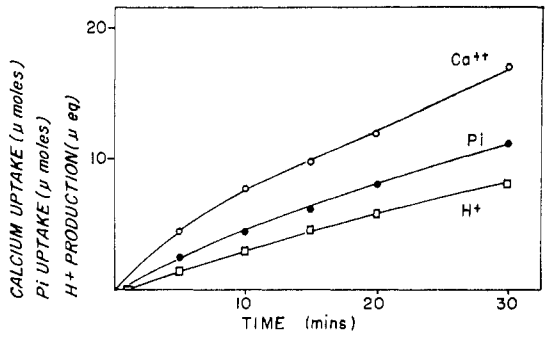

FIGURE 3: Rate of calcification and H^+ production by calcified tendon matrix. 100 mg of tendon matrix to which was bound 42 μmoles of Ca^{2+} and 25 μmoles of P_i was employed. The reaction system was as described in Methods and contained 2.0 mM Ca^{2+} and 1.9 mM P_i.

TABLE IV: Decalcification of Calcified Matrix.[a]

Time (min)	Ca Released (μmoles)	$+\Delta Ca^{2+}/+\Delta P_i$	$+\Delta Ca^{2+}/-\Delta H^+$
2	5.2	1.7	1.3
5	12.6	1.7	1.2
10	22.8	1.6	1.2
15	28.2	1.8	1.2
20	33.6	1.6	1.3
300	35.0	1.6	1.3

[a] The reaction was carried out at 37° by the pH-Stat method described in Methods except that 0.05 N HCl was employed to maintain constant pH. Aliquots of the reaction system were withdrawn at indicated times, filtered to remove matrix, and analyzed for calcium and phosphate as described in Methods. The reaction system was composed of 150 mM NaCl, 100 mg dry weight of tendon matrix which had been previously calcified so as to have 61 μmoles of bound calcium and 36 μmoles of bound phosphate. The reaction system volume was 25 ml, pH 6.0. For this system the pH-Stat was calibrated essentially as described in Table I except that graded amounts of standard NaOH were added to the reaction system.

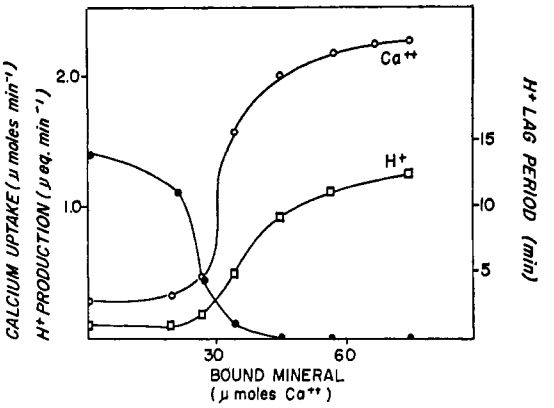

FIGURE 4: Effect of matrix-bound mineral on calcification and H^+ production rates and on H^+ lag period. 200 mg of tendon matrix was calcified so as to contain the indicated amounts of bound calcium and phosphate. The NaCl-washed matrix samples were then incubated in standard reaction system described in Methods containing 2.0 mM Ca^{2+} and 1.9 mM P_i. Maximum rates of H^+ production, (□) and the corresponding rates of Ca^{2+} uptake during the same reaction period (○) were determined as described in Methods. The H^+ lag period (●) was determined by extrapolating the maximum proton production recording to base line as demonstrated in Figure 1.

μmoles of bound mineral was present whereas both progress toward limiting values when higher amounts of the mineral phase were present. The comparable effects on the rates of Ca^{2+} uptake (and also on phosphate uptake) and on H^+ production provide additional evedence that H^+ production is related to the mechanism of the calcification process.

These results also demonstrate that the length of the lag period associated with the onset of H^+ production or the duration of stage 1 of the calcification reaction is decreased as the amount of the bound mineral is increased. It is apparent from these results that the range of bound mineral that produces the marked increase in the rates of calcium uptake and H^+ production corresponds to that which produces the greatest relative decrease of the H^+ lag period. These results indicate that the amount of the reaction product, the bound mineral phase, has a marked influence on the kinetic properties of both stages 1 and 2 of the subsequent calcification reaction.

Decalcification of Previously Calcified Matrix. The calcified tendon matrix is quite stable when exposed to a calcium- and/or phosphate-free medium at pH 7.5 or above. At pH 7.4 and 37°, the calcified matrix will release calcium and phosphate to the soluble phase until the ion product (mM Ca^{2+} × mM HPO_4^{2-} attains a value of 0.3 mM^2. At lower pH values, the final ion product is higher. It will be recalled that previous studies in this laboratory (Wadkins, 1968) showed that net calcification of the tendon matrix will occur only if the reactive ion product exceeds 0.3 mM^2.

Decalcification of the tendon matrix is associated with H^+ consumption. The results presented in Table IV show that when previously calcified matrix is added to a calcium- and phosphate-free medium composed of 150 mM NaCl and maintained at pH 6.0 at 37° by the pH-Stat method, more than 50% of the bound calcium was released to the soluble phase at equilibrium. Furthermore, these data show that the ratio of calcium released to phosphate released and of calcium released to H^+ consumed were essentially constant at 1.7 and 1.25, respectively. The reactive ion product at which equilibrium was attained is seen to be 1.1 mM^2 at this pH. When the reaction was investigated by using the same conditions except that the reactive ion product of the soluble phase was initially adjusted to 1.1 mM^2, no increase of the calcium or phosphate concentration of the soluble phase was observed nor was H^+ consumption detected. The magnitude of the ratios observed during the decalcification reaction is identical to that expected for dissolution of hydroxylapatite.

Recent studies have shown that the decalcification reaction is inhibited by concentrations of methylenediphosphonate and the acid-labile serum component that also inhibit net calcification. These results will be presented in detail in a later communication.

Discussion

The studies described in this communication demonstrate that H^+ production accompanies Ca^{2+} and P_i uptake by tendon matrix, that the simultaneous presence of Ca^{2+}, P_i, and matrix is required, that the rate of H^+ production is proportional to the amount of matrix added to the system, and that all three processes—Ca^{2+} and P_i uptake and H^+ production—are similarly inhibited by Mg^{2+}, F^-, phosphonoacetate, methylenediphosphonate, the urine calcification inhibitor, and urea. On the basis of these results we have concluded that H^+ production is an inherent property of the calcification process catalyzed by the tendon matrix expected for the formation of matrix-bound apatite represented by the reaction

$$5Ca^{2+} + 3HPO_4^{2-} + H_2O \rightarrow Ca_5(PO_4)_3(OH) + 4H^+ \quad (1)$$

Our studies show, however, that the ratio of calcium uptake associated with stage 2 calcification induced by the uncalcified matrix as well as during the entire reaction period induced by previously calcified matrix is reproducibly in the range 1.5–1.7 but is more often lower than 1.67 predicted by eq 1. The ratio of calcium uptake to H^+ production associated with those two systems is reproducibly 1.6–1.8 which is higher than the

value of 1.25 predicted by eq 1. A possible explanation of the differences between the properties presented here and those predicted by eq 1 is that part of the matrix-bound mineral phase might be a form of calcium phosphate that is less basic than hydroxylapatite and also characterized by a lower calcium to phosphate ratio. If the production of that form of calcium phosphate were associated with no H^+ production, then the calcium uptake to H^+ production ratios observed here suggest that as much as 20% of the bound mineral phase could be this hypothetical acidic form. Posner (1969) demonstrated the presence of an amorphous form of calcium phosphate in bone mineral and suggested that it is less basic than hydroxylapatite, has a Ca/P content of 1.45–1.55, and that it might be a precursor of bone mineral *in vivo*. Vatassery *et al.* (1970) reported that the hydroxyl group content of bone mineral is approximately 25% less than expected on the basis of its calcium and phosphate content and concluded that this difference might correspond to the acidic amorphous calcium phosphate component described by Posner (1969). We have not made direct estimates of the relative amounts of hydroxylapatite and amorphous calcium phosphate present in the matrix-bound mineral phase described here, but these studies are in progress.

The studies with uncalcified tendon matrix demonstrate that an early stage of the calcification process is characterized by the binding of calcium and phosphate by a reaction that is not associated with proton production. The ratio of calcium phosphate bound during this period is 1.0–1.2. This reaction proceeds until 55–65 μmoles of calcium and phosphate is bound per g of matrix after which H^+ production is initiated and an elevated rate of calcification ensues which is characterized by a Ca/P rate ratio of 1.5–1.7. Previous studies (Jethi and Wadkins, 1971) demonstrated that the calcium bound during the initial stage is phosphate dependent and that phosphate uptake is calcium dependent. Furthermore, the binding of both ions during this stage was inhibited by Mg^{2+} and phosphonoacetate at concentrations that also inhibit net calcification and proton production. Thus the evidence suggests that the matrix-bound form of calcium and phosphate produced during the initial phase could be a calcium phosphate complex that constitutes a bound intermediate of the overall calcification process. This hypothetical complex will be referred to in this discussion of CaP_1.

Previous studies (Jethi and Wadkins, 1971) also demonstrated that CaP_1 is formed by a methylenediphosphonate-insensitive reaction although this compound is a potent inhibitor of net calcification and of proton production (*cf.* Table II). Fleisch *et al.* (1969) showed that several diphosphonate compounds inhibit spontaneous precipitation of hydroxylapatite *in vitro* as well as the associated proton production, but did not under the same conditions prevent the formation of amorphous calcium phosphate nor its associated proton production. The possibility exists therefore that the CaP_1 complex proposed here could constitute a specific intermediate of a bound form of amorphous calcium phosphate which after subsequent proton release could be incorporated into the matrix-bound mineral phase. This possibility is currently being investigated.

The studies described here and in previous communications (Jethi *et al.*, 1970; Jethi and Wadkins, 1971) demonstrate that the amount of the matrix-bound mineral phase exerts significant control over the kinetic properties of the overall calcification process. Thus matrix calcification that most closely resembles hydroxylapatite formation is observed under two kinds of reaction conditions. One is with uncalcified matrix that has been incubated with Ca^{2+} and HPO_4^{2-} sufficiently long that the bound CaP_1 complex attains a value of 50–60 μmoles/g of matrix. The other is with previously calcified matrix that contains greater than 200 μmoles of calcium (and approximately 125 μmoles of phosphate) bound as the mineral phase. Lower amounts of the latter increased the duration of the stage 1 lag period. A possible interpretation of these results is that the level of CaP_1 controls the duration of the stage 1 H^+ lag period and that CaP_1 can be formed from the bound mineral phase or from the Ca^{2+} and HPO_4^{2-} of the soluble phase. An alternative explanation is that the level of the bound mineral phase can influence structural diffusion barriers imposed by the catalytic matrix and thereby increase the rate by which the optimal CaP_1 level is attained or possibly decrease the amount of bound CaP_1 required to fully activate stage 2 calcification. Thus, important considerations for more complete characterization of this system will be to determine if the level of bound CaP_1 can be directly demonstrated to influence the several kinetic properties described above.

The data presented here and in previous communications (Jethi *et al.*, 1970; Jethi and Wadkins, 1971) provide evidence that calcification process induced by the tendon matrix can be explained on the basis of a linear sequence of obligatory reaction steps. This model is conceptually comparable to polyfunctional heterogeneous catalysis with minimal desorption of intermediates and product. The prime utility of this model is that it allows for the differential effects of the several calcification inhibitors as well as the notable efficiency of the matrix. As emphasized earlier, this particular matrix will induce the formation of a bound product that closely resembles hydroxylapatite from solutions whose calcium and phosphate concentrations are even lower than those of serum and the fluid phase ambient to calcifying epiphyseal cartilage (Howell *et al.*, 1968) and much lower than those required in the absence of an inducing substrate (Solomons and Neuman, 1960). This model which invokes multiple binding of specific intermediates in addition to the mineral-phase product provides a considerable entropic advantage for the system.

The studies presented here also demonstrated that the mineral phase of previously calcified matrix will undergo dissolution at pH 6.0 and that H^+ are consumed in the process. The stoichiometry of the reaction was found to coincide with that predicted by reversal of eq 1. These results plus the observation that at pH 6.0 the reaction tends to approach an apparent equilibrium corresponding to a reactive ion product of the soluble phase of 1.1 mM[2] suggests that the calcification reaction is a reversible process. However, this correlation with eq 1, as contrasted to the significant differences noted previously for net calcification, suggests different mechanisms for the two processes. Thus it is possible that whereas net calcification proceeds through specific matrix-bound intermediates, dissolution involves direct dissociation of the mineral phase. These relationships are currently being investigated.

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Isolation and Identification of Cytokinins Located in the Transfer Ribonucleic Acid of Tobacco Callus Grown in the Presence of 6-Benzylaminopurine*

W. J. Burrows,† F. Skoog,† and N. J. Leonard‡

ABSTRACT: From the tRNA of tobacco callus grown in the presence of the synthetic cytokinin 6-benzylaminopurine, four cytokinin-active ribonucleosides have been isolated. Of these, the two ribonucleosides present in largest amount were identified conclusively by their chromatographic properties, ultraviolet spectra, and low- and high-resolution mass spectra as the natural tRNA components 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine and 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine. A third ribonucleoside, present in smaller amount, was indicated as another natural cytokinin, 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine, on the basis of the data obtainable.

The fourth cytokinin-active ribonucleoside was identified conclusively as 6-benzylamino-9-β-D-ribofuranosylpurine, normally not a natural tRNA component. Thus, we have shown that in the presence of an unnatural cytokinin, 6-benzylaminopurine, tobacco callus tissue generates natural cytokinin-active tRNA components and in addition the related *unnatural* ribonucleoside.

Evidence for and against the incorporation of the cytokinin 6-benzylaminopurine (**1**) into tRNA has been reported (Fox, 1966; Fox and Chen, 1967; Kende and Tavares, 1968; Richmond *et al.*, 1970). It was argued that this molecule must be incorporated into tRNA if cytokinins exert their growth regulatory action as constituents of tRNA. The problem of incorporation is only part of a broader problem which had not been attacked experimentally until now, namely, the question of what cytokinin-active ribonucleosides, natural or unnatural, are actually present in the tRNA of cytokinin-dependent plant tissue grown on a defined basal medium supplemented with a synthetic cytokinin. In order to answer this question, tobacco callus dependent on exogenous cytokinin was grown on a medium with added 6-benzylaminopurine (**1**), and the tRNA was isolated, purified, and hydrolyzed to identify the constituent cytokinins.[1] We are now able to report the isolation and identification of four cytokinin-active ribonucleosides in the tRNA of cytokinin-dependent tobacco callus supplied with 6-benzylaminopurine.

Experimental Section

Extraction of tRNA. Tobacco callus which had previously been grown on basal medium (Linsmaier and Skoog, 1965) supplemented with 1 μg/l. of 6-benzylaminopurine (**1**), BAP[2] (Shell development Company SD4901) was propagated on the same medium containing 10 μg/l. or 100 μg/l. of BAP. The tissue was harvested after approximately 21-days growth and stored at −20°. The frozen tissue (47 kg) was homogenized in 0.3 volume of 0.4 M Tris-HCl buffer (pH 7.3) and 0.5 vol-

* From the Institute of Plant Development, Birge Hall, University of Wisconsin, Madison, Wisconsin 53706 (W. J. B. and F. S.), and the School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801 (N. J. L.). *Received December 18, 1970.* Supported at the University of Wisconsin by National Science Foundation Research Grants GB-6994X and GB-25812 and by the Research Committee of the Graduate School with funds from the Wisconsin Alumni Research Foundation. Supported at the University of Illinois by National Institutes of Health Research Grants GM-05829, GM-16864, and CA-11388. These results were first presented (by F. S.) at the Symposium on Control Mechanisms of Growth and Differentiation, Society of Experimental Biology, Sept 1970.

† Present address: Shell Research Limited, Woodstock Agricultural Research Centre, Sittingbourne, Kent, England.

‡ To whom to address correspondence.

[1] We were encouraged in undertaking the cultivation of tobacco callus and preparation of tRNA on the large scale required for the identification of cytokinins by the results of preliminary experiments by P. K. Evans, N. Murai, and J. J. McDonald in these laboratories relating to the possible incorporation of 6-(3-methyl-2-butenylamino)purine, double labeled with ^{3}H and ^{14}C, and of 6-benzylaminopurine into tRNA. These experiments indicated the presence of cytokinin activity in tRNA ribonucleosides of tobacco callus cultured on media with a single exogenous cytokinin, and, although they failed to give conclusive evidence for the incorporation of the exogenous cytokinin into tRNA, this possibility was not excluded.

[2] Abbreviations used are: BAP, 6-benzylaminopurine (**1**): BAPA, 6-benzylamino-9-β-D-ribofuranosylpurine (**5**).