Octacalcium Phosphate Carboxylates. 1. Preparation and Identification

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The formation of octacalcium phosphate carboxylates, $Ca_8(HPO_4)_m(carboxylate)_n(PO_4)_4 \cdot yH_2O$, by conversion of α-$Ca_3(PO_4)_2$ in solutions of 19 ammonium carboxylates (monocarboxylates; saturated, unsaturated, hydroxy, keto, and amino dicarboxylates; and tricarboxylates) was investigated. The various solid phases formed, depending on initial pH's and conversion times, were determined by X-ray diffraction and infrared and Raman spectroscopy. Octacalcium phosphate carboxylates containing structurally incorporated malonate, succinate, adipate, suberate, sebacate, fumarate, malate, and citrate ions were formed and identified. Octacalcium phosphate carboxylates were also formed from pyruvate and α-ketoglutarate solutions but with uncertain carboxylate ion structures. All of these identified compounds are structurally similar to octacalcium phosphate, $Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$, but have expanded a-axis unit-cell dimensions that generally increased with increasing number of carbon atoms in the carboxylate ion. Among these compounds, of special importance are those containing carboxylates that are present as intermediates in the Krebs cycle. The possible precipitation of these octacalcium phosphate carboxylates in mitochondria and their possible role as precursors in calcified tissue formation are discussed.

Introduction

Octacalcium bis(hydrogenphosphate) tetraphosphate pentahydrate, generally referred to as octacalcium phosphate (OCP, $Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$) appears to be a precursor in the formation of apatitic calcium phosphates in teeth, bones, and other biominerals.[2-4] OCP has a layer-type structure composed of alternating hydrated and apatitic layers.[5-7] Recently, Monma[8,9] prepared a series of new OCP derivatives, OCP–aliphatic dicarboxylates, in which dicarboxylate ions were incorporated instead of HPO_4^{2-} into the hydrated layer of OCP. These unusual inorganic–organic derivatives previously named carboxylate complexes[8,9] or double salts[10,11] are here named OCP–carboxylates (OCPCs).

In this work *in vitro* formation of OCPCs by α-tricalcium phosphate (α-TCP, α-$Ca_3(PO_4)_2$) conversion in solutions of 19 different carboxylates was investigated. Several of these carboxylates take part as intermediates in the Krebs cycle. The observed interactions between these physiologically active ions (calcium, phosphate, and carboxylate) are suggestive of a new and potentially highly important field in bioinorganic chemistry. The objective of this paper (part 1) is to focus attention on the preparation and identification of OCPCs that we succeeded in forming and to make note of others that failed to form. In part 2,[13] detailed characterization of six of these OCPCs is reported along with structural considerations. The structure of OCP, the parent compound of the OCPCs, is shown in part 2.[13]

Experimental Section[35]

Materials. The α-TCP was prepared by mixing and grinding a 1:1 molar mixture of $Ca_2P_2O_7$ and $CaCO_3$ in a water slurry to less than 10-μm particle size. The dried ground mixture was pressed into a pellet, heated at 1200 °C for 4 h, and then allowed to cool rapidly in air to 22 °C. The X-ray diffraction (XRD) pattern was in complete agreement with the most recent definitive data for α-TCP.[12] The chemically determined Ca/P molar ratio was 1.501 ± 0.003. The α-TCP used for hydrolysis was ground to a particle size of less than 10 μm. The $Ca_2P_2O_7$ used was prepared by heating reagent grade $CaHPO_4 \cdot 2H_2O$ (DCPD) at 900 °C for 12 h.

Ammonium carboxylate stock solutions were prepared from reagent grade carboxylic acids and ammonium hydroxide. A pH of approximately 5 was required to effect dissolution of the carboxylic acids. Triply distilled water was used in all procedures. The α-TCP was hydrolyzed in stirred solutions (glass-covered magnetic stirrers were used) containing 0.25 mol/L carboxylate at 37 °C under a N_2 atmosphere. The solid/solution ratio was 8–10 mg of α-TCP/mL for all carboxylates except citrate, which

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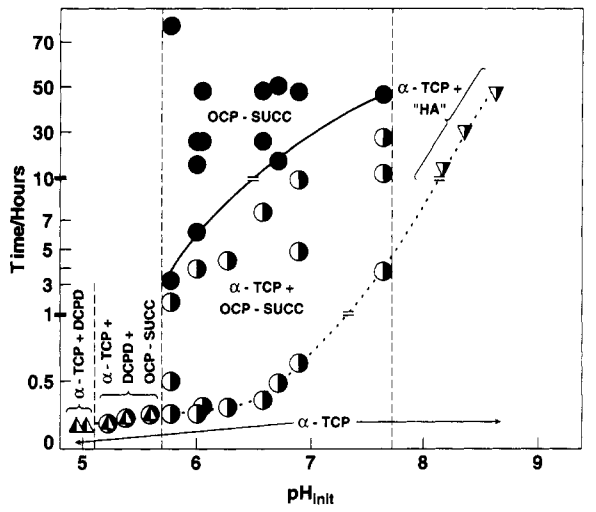

Figure 1. Conversion of α-TCP into various products in 0.25 mol/L ammonium succinate solution at 37 °C as functions of initial pH (pH_{init}) and time (note, ordinate is broken in three different linear time scales). Initial solid/solution ratio was 10 mg/L. The dotted curve defines the conditions under which the first conversions were detected. The various solid phases present in solution depending on pH_{init} are separated by vertical dashed lines in four domains: α-TCP + DCPD (▲); α-TCP + DCPD + OCP-SUCC (◮); α-TCP + OCP-SUCC (◑), and OCP-SUCC (●); and α-TCP + "HA" (▼), respectively. α-TCP + OCP-SUCC mixtures were obtained in the domain between the dotted and full curve and pure OCP-SUCC above the full curve.

was 15 mg/mL. Ammonium hydroxide was added to adjust initial pH as desired (between 4.9 and 8.7). The pH was measured with a glass/calomel electrode using commercial standard buffer solutions for calibration. Suspensions were centrifuged and then filtered through 0.22-μm Millipore filters. After being filtered and washed with approximately 150 mL of water, the resulting precipitate was dried under reduced pressure (30 kPa) at 22 °C for 16 h; prolonged drying for 48 h resulted in partial dehydration. The approximate yields were 70–90% of the theoretical values calculated from the calcium content of α-TCP.

Methods. Infrared (IR) spectra of samples in KBr pellets were used to characterize the calcium phosphate phases, DCPD, OCP, HA (hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$), and to indicate formation of the OCPC. Generally, an OCPC was identified on the basis of the carboxylate band at about 1560 cm^{-1} and changes in HPO_4^{2-} bands in the 1200–850-cm^{-1} region. Carbon microanalysis[13] was done on selected samples in which carboxylates had been detected by IR. Raman spectra were also obtained on selected samples, and these are primarily discussed in part 2[13] along with the IR and Raman spectroscopic procedures.

A principal indicator of whether or not an OCPC had formed was the presence of an overall OCP-like XRD pattern but with a d_{100} interplanar spacing larger than that of OCP, 1.868 nm. The XRD procedure and patterns are given in part 2.[13]

Results

Conversion of α-TCP in carboxylate solutions was systematically investigated as functions of intial pH and time of hydrolysis. In Figure 1, conversion of α-TCP in succinate (SUCC) solutions is shown as an example. The dotted curve represents the time at which the first appearance of the products (DCPD, OCP-SUCC, or nonstoichiometric hydroxyapatite, denoted "HA") was detected as a function of initial pH (pH_{init}). At $pH_{init} < 5.7$, α-TCP converted rapidly into DCPD or into a mixture of DCPD and OCP-SUCC, whereas at pH_{init} from 5.7 to 7.7 converted directly into OCP-SUCC. At $pH_{init} > 7.7$, α-TCP very slowly converted to "HA". The conditions for preparation of pure OCP-SUCC are indicated by the full circles (Figure 1) that represent samples in which OCP-SUCC was detected without traces of α-TCP. The time in which the conversion process was completed increased with increasing pH_{init} (Figure 1, full curve).

In Tables I and II data on 45 selected samples from 71 preparations are given. In Table I are listed preparative conditions (initial and final pH, and reaction time), carbon content (% C), and IR and XRD data for the solid phases formed in solutions of monocarboxylates and saturated and unsaturated dicarboxylates. The d_{100} interplanar spacings for the OCPCs are also given.

Formates and acetates did not form OCPCs; depending on pH_{init}, α-TCP hydrolyzed into DCPD (samples 1 and 3) or into a poorly crystallized OCP-like compound, denoted "OCP" (samples 2 and 4).

Saturated dicarboxylates, listed in order of increasing number of carbon atoms in the ion, could be divided into two subgroups: with odd (malonate, azelate) and even number of carbon atoms (succinate, adipate, suberate, and sebacate). In malonate (MALON) solutions, α-TCP converted partially into OCP-MALON (samples 5 and 6), whereas in azelate solutions no OCPC formed (samples 29 and 30). All investigated dicarboxylates with an even number of carbons in the chain-formed OCPCs. The contents of carbon indicated that approximately one HPO_4^{2-} ion in the OCP formula unit was substituted by one dicarboxylate (see part 2[13]). It is important to point out that OCPCs slowly converted with time into "HA" that was easily detectible after extended ageing time (samples 24 and 26). The OCP-sebacate (OCP-SEB) was obtained in low yield mixed with "OCP", "HA", and/or calcium sebacate monohydrate[14] (CSM), samples 31 and 33. OCP-SEB transformed with time into "HA" faster than the other OCPCs.

Investigation of the geometrical isomers of butenedioic acid showed that the *trans* isomer (fumarate) formed an OCPC; the *cis* isomer (maleate) did not.

For the six of the ten carboxylates listed in Table I which formed OCPCs the d_{100} values corresponding to the *a*-axis dimension of OCP-dicarboxylates generally increased with increasing number of carbon atoms in the dicarboxylate ion.

Table II gives the results obtained for α-TCP conversion in solutions of carboxylates containing additional functional groups: hydroxy, keto, and amino.

Conversion of α-TCP in L-malate solution was very slow; after 96 h OCP-MALATE was formed but only as a minor solid phase (~20%) mixed with "HA". L-Malate showed a tendency to be adsorbed at the "HA" surface, as based on the following. The IR carboxylate band intensity was much too high to be derived from a minor phase of OCP-MALATE, as indicated by the weak d_{100} XRD line intensity. Consequently, the high carbon content (Table II, sample 42), in excess for OCP-MALATE present (~100%), may be accounted for by adsorbed malate. The solid phase formed in citrate (CIT) solutions showed an IR spectrum typical of poorly crystallized OCP and the presence of carboxylate ions. The XRD pattern showed a shift of the d_{100} line, indicating that the OCP-CIT was formed.

From the group of ketocarboxylates, only two, pyruvate (PYR) and α-ketoglutarate (α-KETOGLU), formed OCPCs but mixed with "HA". Unexpectedly, IR and Raman spectra of these compounds did not show the presence of a keto group. In addition, these two OCP-carboxylates have very

Table I. Preparation and Identification of OCP–Carboxylates Formed by Hydrolysis of α-TCP in Carboxylate Solutions

		preparative conditions				identification of solid phases				
						infrared		X-ray diffraction		
carboxylic acid	sample no.	pH init	pH final	time (h)	C (%)	calcium phosphate	–COO group	minor	major	d_{100}[a] (nm)
monocarboxylic										
formic (HCOOH)	1	5.40	5.55	24		DCPD	nd	nd	DCPD	
	2	6.04	6.38	24		"OCP"	nd	nd	"OCP"	1.88
acetic (CH_3COOH)	3	5.32	5.51	24		DCPD	nd	nd	DCPD	
	4	6.11	6.42	24		"OCP"	nd	nd	"OCP"	1.86
saturated dicarboxylic										
malonic ($HOOCCH_2COOH$)	5	6.21	7.41	7		α-TCP	d	α-TCP	OCP-MALON	2.01
	6	6.18	6.70	24	2.45	α-TCP	d	α-TCP	OCP-MALON	1.96
succinic ($HOOC(CH_2)_2COOH$)	8	5.21	5.52	72		DCPD, OCPC	d	DCPD	OCP-SUCC	2.15
	11	5.90	6.37	24	4.44	OCPC	d	nd	OCP-SUCC	2.14
	12	5.90	6.26	48	4.62	OCPC	d	nd	OCP-SUCC	2.14
	13	6.28	6.94	24	4.19	OCPC	d	nd	OCP-SUCC	2.14
	14	6.28	6.92	48	4.25	OCPC	d	nd	OCP-SUCC	2.13
	15	6.60	6.73	24	4.79	OCPC	d	nd	OCP-SUCC	2.12
	16	6.69	7.39	48	4.08	OCPC	d	nd	OCP-SUCC	2.09
	20	8.61	8.62	48		α-TCP, "HA"	nd	"HA"	α-TCP	
adipic ($HOOC(CH_2)_4COOH$)	21	6.13	7.05	24	5.59	OCPC	d	nd	OCP-ADIP	2.37
	24	6.34	6.87	120	6.10	OCPC	d	"HA"	OCP-ADIP	2.37
suberic ($HOOC(CH_2)_6COOH$)	25	6.87	7.44	48	6.78	OCPC	d	nd	OCP-SUB	2.62
	26	6.34	6.94	120	7.46	OCPC	d	"HA"	OCP-SUB	2.61
azelaic ($HOOC(CH_2)_7COOH$)	29	6.27	7.03	24		"OCP"	nd	"HA"	"OCP"	1.87
	30	6.50	6.60	120		"OCP", "HA"	nd	"OCP"	"HA"	
sebacic ($HOOC(CH_2)_8COOH$)	31	6.03	7.01	6	5.72	OCPC	d	"OCP", "HA", CSM	OCP-SEB	2.58
	33	6.42	7.27	24	4.33	OCPC	d	"HA", "OCP", CSM	OCP-SEB	2.60
unsaturated dicarboxylic										
maleic (HOOCCH=CHCOOH)	35	6.70	6.83	24		α-TCP, "OCP"	nd	α-TCP	"OCP"	1.89
	36	6.83	7.13	48		α-TCP, "OCP"	nd	α-TCP	"OCP"	1.88
fumaric (HOOCCH=CHCOOH)	37	6.82	7.31	24	3.87	OCPC	d	nd	OCP-FUM	2.15
	38	6.46	7.08	48	3.60	OCPC	d	nd	OCP-FUM	2.15

[a] Measured interplanar spacings for OCP and OCP-carboxylates when present as major phases, α-TCP = α-$Ca_3(PO_4)_2$, DCPD = $CaHPO_4·2H_2O$, HA = $Ca_{10}(PO_4)_6(OH)_2$, OCP = $Ca_8(HPO_4)_2(PO_4)_4·5H_2O$, quotation marks for HA and OCP indicate poorly crystallized materials. OCPC = $Ca_8(HPO_4)_m(carboxylate)_n(PO_4)_4·yH_2O$, for specific carboxylates incorporated, i.e., malonate, succinate, etc., abbreviations are OCP-MALON, OCP-SUCC, etc. CSM = $Ca(CH_2)_8(COO)_2·H_2O$. d = detected, nd = not detected.

Table II. Preparation and Identification of OCP-Carboxylates Formed by Hydrolysis of α-TCP in Hydroxy-, Keto-, and Aminocarboxylate Solutions

		preparative conditions				identification of solid phases				
						infrared		X-ray diffraction		
carboxylic acid	sample no.	pH init	pH final	time (h)	C (%)	calcium phosphate	–COO group	minor	major	d_{100}[a] (nm)
hydroxycarboxylic										
L-malic ($HOOCCH_2CH(OH)COOH$)	41	5.88	7.06	24		α-TCP, "HA"	nd	"HA"	α-TCP	
	42	5.78	6.52	96	4.06	"HA"	d	OCP-MAL	"HA"	2.10
citric ($HOOCCH_2C(OH)(COOH)CH_2COOH$)	44	6.45	6.95	96		OCPC	d	nd	OCP-CIT	2.10
	46	6.64	7.28	120	2.69	OCPC	d	nd	OCP-CIT	2.05
	48	6.53	7.54	340	2.91	OCPC	d	nd	OCP-CIT	2.06
ketocarboxylic										
pyruvic ($CH_3COCOOH$)	51	5.09	6.89	168	1.46	OCPC	d	"HA"	OCPC	1.96
	52	6.10	7.04	168		OCPC	d	"HA"	OCPC	2.01
oxaloacetic ($HOOCCH_2COCOOH$)	55	6.21	6.32	24		α-TCP	nd	"HA"	α-TCP	
	56	5.95	6.42	120	5.02	"HA"	d	α-TCP	"HA"	
α-ketoglutaric ($HOOC(CH_2)_2COCOOH$)	61	5.92	6.80	96	1.44	"HA"	d	"HA"	OCPC	1.96
	62	6.48	7.33	500		"HA"	d	"HA"	OCPC	2.01
β-ketoglutaric ($HOOCCH_2COCH_2COOH$)	63	5.92	6.38	96		"HA"	nd	nd	"HA"	
	64	6.18	6.45	500		"HA"	nd	nd	"HA"	
aminocarboxylic										
glycine ($CH_2(NH_2)COOH$)	65	6.09	6.51	96		"HA"	nd	nd	"HA"	
	66	6.59	6.20	500	0.67	"HA"	nd	nd	"HA"	
aspartic ($HOOCCH_2CH(NH_2)COOH$)	67	5.83	7.07	48		α-TCP	nd	nd	α-TCP	
	68	4.92	6.46	72		α-TCP	nd	nd	α-TCP	
glutamic ($HOOCCH_2CH_2CH(NH_2)COOH$)	70	4.97	5.73	96		"OCP"	nd	nd	"OCP"	1.87
	71	5.51	6.59	216	1.24	"OCP"	d	nd	"OCP"	1.89

[a] Measured interplanar spacings for OCP and OCP-carboxylates. Meanings of abbreviations are the same as in Table I.

similar d_{100} values (~1.97 nm) although the number of carbons in pyruvate and α-ketoglutarate is different, 3 and 5, respectively. These findings, leading to uncertainty in carboxylate ion structure, are the reason these salts were denoted as OCPC rather than OCP-PYR and OCP-α-KETOGLU (Table II). In oxaloacetate and β-ketoglutarate solutions, α-TCP converted into "HA". The carbon component of the 120 h product in oxaloacetate and the IR carboxylate band (sample 56) are attributed to adsorbed anions.

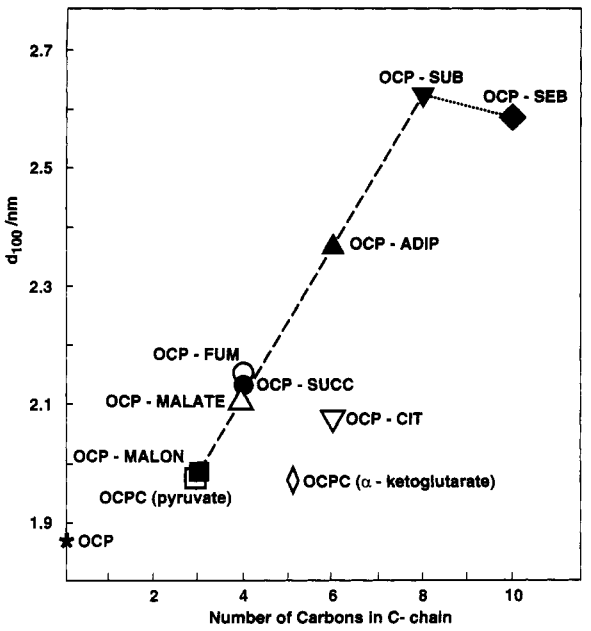

Figure 2. d_{100} values of different OCP-carboxylates as a function of number of carbon atoms corresponding to length of C chain. The d_{100} values of OCPCs with saturated dicarboxylates (filled symbols) show linear expansion with number of carbons up to eight (dashed line) and no further expansion above eight (dotted line). See text for discussion of other OCPCs denoted by unfilled symbols.

Aminodicarboxylates (aspartate and glutamate) inhibited transformation of α-TCP into OCP. There was no indication of OCPC formation; the presence of the IR carboxylate band was attributed to adsorption of glutamate on the OCP surface.

Discussion

Results on converion of α-TCP in ammonium succinate solutions in pH_{init} range from 4.9 to 8.7 (Figure 1) are compared with findings of Monma et al.[15] They studied hydrolysis of α-TCP at various pHs in water solutions containing additives that did not incorporate into the hydrolytic products. Monma et al.[15] found DCPD at pH_{init} < 5.5, OCP at pH_{init} from 5.5 to 7.5, and HA at pH_{init} > 7.5. In the present work, in succinate solutions, the pH_{init} range of DCPD and "HA" formation is very similar to that of the Monma et al. study, while in the pH_{init} range from 5.7 to 7.7, OCP-SUCC (rather than "OCP") was formed in the succinate solutions (Figure 1). From Figure 1 (full curve) it is evident that the best conditions for preparation of OCP-SUCC are at pH_{init} from 5.7 (~3 h) to 7.7 (~48 h). In Monma's subsequent studies[9] of α-TCP conversion in solutions containing aliphatic dicarboxylates, the OCPCs, including OCP-SUCC, were all prepared at pH_{init} ~ 6 and a reaction time of ~3 h. The longer time (~6 h) required for conversion under similar conditions in this work may be due to larger α-TCP particle sizes that caused a slower dissolution rate.

In Figure 2 average d_{100} values for OCPCs calculated from listed (Tables I and II) and unlisted samples are given as a function of number of carbon atoms in the carboxylate ion incorporated. The d_{100} values of OCPCs with saturated dicarboxylates (filled symbols) show linear dependence with the number of carbons, up to eight. This is in accordance with Monma's[9] findings. The newly prepared OCP-SEB, containing saturated dicarboxylate with 10 carbon atoms, does not follow this linear correlation; the d_{100} value of OCP-SEB (~2.59 nm) is very close to that of OCP-SUB (2.62 nm). It can be concluded that the upper limit of a-axis expansion for the OCP-carboxylates is about 1.4 times that of the OCP a axis.

Some of the new OCP-carboxylates described here show positive linear correlations of d_{100} values versus the number of carbons. The d_{100} values for OCP-fumarate (OCP-FUM) and OCP-MALATE, structures with unsaturated dicarboxylate and hydroxydicarboxylate, respectively, are both close to that of OCP-SUCC. All three of these ions contain four carbon atoms in the C chain (Tables I and II).

The d_{100} value for OCP-CIT is displaced from the straight line (Figure 2). The value of OCP-CIT (d_{100} = 2.07 ± 0.03 nm) corresponds to the d_{100} values for OCPCs containing dicarboxylates with four carbons in the C chain, whereas citrate contains a total of six carbon atoms. This correspondence to a four-carbon chain can be explained by the presence of two four-carbon chains in the branched six-carbon chain of the citrate ion. The OCPCs formed in pyruvate and α-ketoglutarate solution showed a d_{100} spacing of a dicarboxylate with three carbons (OCP-MALON). This correspondence is difficult to explain both for pyruvate and α-ketoglutarate, principally because pyruvate is a monocarboxylate and α-ketoglutarate has a chain of five carbons. The absence of keto group in IR and Raman spectra indicated that the ketocarboxylate had converted to an enol tautomer or some other transformation had taken place. The modified α-ketoglutarate ion may be tilted in the lattice causing a reduced interplanar spacing, or it could disproportionate into a three-carbon chain ion by cleavage of a carbon–carbon bond. Elucidation of the composition and structure of these modified ketocarboxylates in OCPCs requires further investigation.

Nonincorporation of some investigated carboxylates into the OCP structure may be due to incompatible anion charge and geometry for bridging pairs of Ca ions across the hydrated layer of OCP (see part 2[13] for details). For example, monocarboxylates (formate, acetate) and aminomonocarboxylate (glycine) can reach only a negative charge of −1 and also lack the second carboxylate group to bridge Ca ions across the hydrated layer in order to repeat the OCPC structure in the a-axis direction. In the experiments with the long-chain azelate ion, having an odd number of carbons, no OCPC formed. This is explained on the basis that the HPO_4^{2-} sites in OCP occupied by the carboxylate ions in OCPCs are essentially centrosymmetric. Molecules with an odd number of carbon atoms violate the symmetry requirement of the site and, therefore, do not fit as well. This explains why the azelate ion (nine carbons in the C chain) did not form a OCPC whereas sebacate (10 carbons in the C chain) did. This is in accordance with Monma's[9] observation that the OCPCs with an even number of carbons in the C chain were easier to prepare in higher yield than those with an odd number (three, five, and seven) of carbons.

An OCPC was not formed with maleate ions which have carboxylate groups in the *cis* position separated by a distance of about 0.24 nm. The only plausible orientation of the maleate ion in the OCP structure that could bridge calcium ions along the a axis (Ca–Ca distance in OCP ~0.59 nm) would have the maleate C=C bond approx-

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imately parallel to the *a* axis and calcium–oxygen bonds approximately perpendicular to the –COO planes; this geometry is apparently unsuitable for bonding. Nonincorporation of oxaloacetate, β-ketoglutarate, aspartate, and glutamate may be due to steric factors and hydrogen bond interactions introduced by the keto and amino groups.

Among the carboxylates that form OCPCs, the physiologically relevant molecules are of special importance. Six of the OCPCs prepared and identified in this work were formed from carboxylates that are involved in the respiratory processes: pyruvate, malate, fumarate, succinate, α-ketoglutarate, and citrate. Previous studies[16–18] reported that mitochondria can concentrate calcium and phosphate ions by means of respiratory energy to levels greatly exceeding the solubility products of all known calcium phosphate salts. Intramitochondrial precipitation of electron-dense mineral granules, possibly in the form of amorphous calcium phosphate, has also been detected *in vivo*.[19–23] On the basis of these findings and the *in vitro* formation of OCPCs described herein, it is hypothesized that this amorphous precipitate in mitochondria may contain OCP-carboxylate components in which the carboxylate ions come from the Krebs cycle. OCPCs in mitochondria could be a means for storage of intermediates from the Krebs cycle and during particle dissolution replenish the carboxylates required to drive the citric acid cycle. The dissolution and formation of OCPCs would occur depending on the concentration of calcium, phosphate, and carboxylate ions.

Moreover, in postulated biomineralization pathways,[18,24,25] OCPCs may act as precursors to apatite in calcified tissues by nucleating in extracellular fluids, mitochondria, and/or matrix vesicles. The possible formation of OCPC precursors, specifically OCP-CIT, is suggested by the presence of citrate ions in bone, dentin and in renal and prostatic calculi.[26–28] In addition, citrate incorporation by epitaxial growth of OCP-CIT on the surfaces of mature biological apatite crystals is plausible because of the structural similarity between OCP and apatite.[29] Previous *in vitro* experiments have suggested adsorption of citrate from extracellular fluid on the surface of apatitic crystals[30–32] and/or substitution of PO_4^{3-} by citrate on the apatitic crystal surface.[33,34]

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(35) Certain commercial materials and equipment are identified in this paper to specify the experimental procedure. In no instance does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, the National Institutes of Health, or the ADA Health Foundation or that the material or equipment identified is necessarily the best available for the purpose.