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Calcium Phosphate Deposition from Balanced Salt Solutions

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Minimal levels of calcium and phosphate ions necessary to induce mineral deposition within 24 hours at 37° from balanced salt solutions buffered in the physiological pH range are described. It is concluded that most physiological fluids apparently have the potential to form calcium phosphate *de novo* in the absence of any nucleating catalyst or inhibitory agent. The possible effects that external factors under cellular control may have upon this process are discussed.

Key words: Mineral — Formation — Physiological — Solution.

Introduction

Although physiological fluids are considered to be supersaturated with respect to pre-existing apatite crystals, they are generally thought to contain concentrations of calcium and phosphate much lower than those required to produce mineral deposition *de novo* (Neuman and Neuman, 1958). Thus, physiological fluids have the ability to promote continued mineral accumulation by crystal growth mechanisms, but have not previously been considered to have the potential to form new mineral in the absence of external catalysts (Fleisch and Neuman, 1961). It is the purpose of this communication to describe minimal levels of calcium and phosphate ions necessary to induce mineral deposition in the physiological pH range within a finite period of time (24 h) at 37° from balanced salt solutions free of added inhibitory or nucleating agents.

Materials and Methods

The precipitating media used in this study were modified Earle's balanced salt solutions suitable for use in tissue culture experiments. After final mixing, they contained NaCl (91.0 mM), KCl (5.4 mM), $MgSO_4 \cdot 7H_2O$ (0.8 mM), $NaHCO_3$ (26.0 mM), glucose (1.0 g/l), HEPES (N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid) buffer (25.0 mM), $CaCl_2$ (0–3 mM) and Na_2HPO_4 (0–2 mM). HEPES buffer has a pK_a of 7.31 at 37° and does not bind mineral ions over its operative pH range (CRC Handbook of Biochemistry, H. A. Sober, ed., 1970). Iso-pH experiments in the absence of buffer indicated no HEPES-related nucleating or inhibitory activity toward calcium phosphate formation.

All mixing solutions (see below) were adjusted to either pH 7.20, 7.40 or 7.60 with NaOH or HCl at 37°.

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Table 1. Minimum Ca × P product at which mineral deposition occurred within 24 h of incubation at 37° in buffered balanced salt solutions[a]

Solution (pH)	Minimum Ca × P product[b] (mM^2)
7.20	4.2 ± 0.3
7.40	2.0 ± 0.2
7.60	0.9 ± 0.1

[a] Ca/P molar mixing ratio = 1.46.
[b] Means ± std. deviations.

Precise precipitating ion concentrations were obtained prior to final mixing by diluting stock solutions whose exact calcium or phosphate contents were determined by chemical analysis (Termine and Eanes, 1972) with solutions containing all the constituents of the stock save calcium or phosphate. All diluting and stock solutions were brought to 37° prior to use. NaCl (91.0 mM), KCl (5.4 mM), glucose (1 g/l) and HEPES buffer (25.0 mM) were common to all stock and diluting solutions. In addition, the stock calcium solution contained $CaCl_2$ (10.75 mM) and $MgSO_4 \cdot 7\,H_2O$ (1.6 mM) while the stock phosphate solution contained Na_2HPO_4 (7.35 mM) and $NaHCO_3$ (52.0 mM). [The "diluting calcium" solution added only $MgSO_4 \cdot 7H_2O$ (1.6 mM) to the four common reagents while the "diluting phosphate" solution only added $NaHCO_3$ (52.0 mM).] Upon appropriate dilution, the final calcium solution was mixed with the final phosphate solution and stored without further stirring under a 95% O_2–5% CO_2 atmosphere in a tightly stoppered vessel at 37° for 24 h. Solution pH was found to be maintained over this period. To avoid extraneous nucleation effects, stock and diluting solutions were Millipore-filtered prior to use and double-cleaned vessels were employed throughout.

Precipitation was assessed by UV turbidimetry (Termine and Posner, 1970) at 225 nm in a Zeiss PMQ II Spectrophotometer using a 0.5 mm slit and by transmission electron microscopy (TEM) of drop-drained specimens (Eanes *et al.*, 1973) using an AEI-6B electron microscope at 60 kV. As a spot check, one of the "non-visible", but UV- and TEM-detectible precipitates (pH 7.40, CaxP=2.1 mM^2), was isolated by pressure Millipore filtration (25 nm filters), freeze-dried and then examined by X-ray diffraction and infrared spectroscopy (Termine and Eanes, 1972) in order to confirm that the precipitate was a calcium phosphate. The secondary ion content (Mg^{2+}, CO_3^{2-}, etc.) of this precipitate was not determined.

Reagent grade chemicals from the same lot number were employed throughout.

Results

Table 1 shows the minimum Ca × P products at which calcium phosphate formation was detected in the balanced salt solutions described above. In each case, positive results indicating precipitation were obtained using both UV turbidity and TEM criteria. At Ca × P products lower than those listed in this table, precipitation was often induced by prolonged incubation at 37° (48–120 h).

For all Ca × P products tested, the initial mineral phase which deposited had the properties of an amorphous calcium phosphate (ACP). After 24 h of incubation at 37°, crystal formation was observed only at relatively high Ca × P products associated with "visible" precipitates, i. e., at Ca × P products greater than or equal to 5, 4 and 3 mM^2 for pH values of 7.2, 7.4 and 7.6, respectively, indicating greater stability for amorphous precipitates formed at lower products. An electron micrograph illustrating these ACP precipitates at lower Ca × P product is shown in Fig. 1. The particles depicted ranged from 80–700 Å in diameter. The upper limit of this particle size range was less than one-half of that observed at higher Ca × P products (~1700 Å).

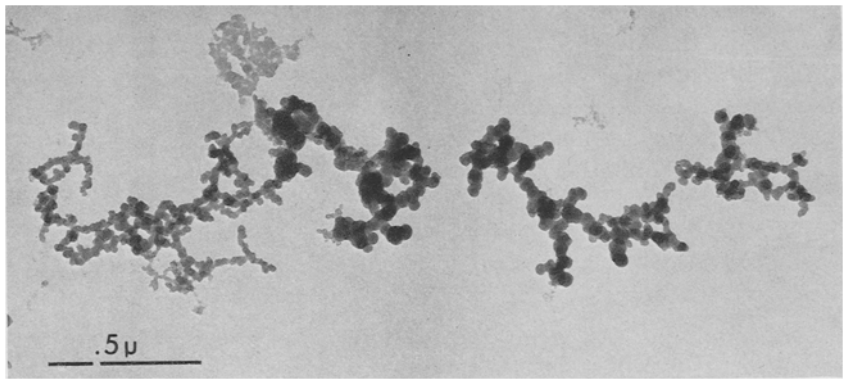

Fig. 1. Transmission electron micrograph of a drop-drained grid specimen of amorphous calcium phosphate from a 24 hr old pH 7.4 (37°) preparation initially 2.1 mM^2 in CaxP molar product, × 40000

Discussion

Working with a synthetic lymph fluid allowed to reach pH 7.4 or 7.6 at time zero, Howell *et al.* (1969) observed calcium phosphate formation within 20 h using a calcium concentration of 2.00 mM and a phosphate concentration of 1.14 mM. Under the iso-pH conditions used in the present study, mineral deposition occurred within 24 h from balanced salt solutions containing as little as 1.71 mM calcium and 1.18 mM phosphate at pH 7.40 and from solutions containing only 1.14 mM calcium and 0.78 mM phosphate at pH 7.60. These ionic concentrations are only slightly above (pH 7.4 values) or well within (pH 7.6 values) those found in serum, rat dorsal interstitial fluid (Rasmussen, 1972) and rat epiphyseal cartilage fluid (Howell *et al.*, 1968). Prolonged incubation (48–120 h) at 37° and at pH 7.4 induced precipitation at calcium and phosphate ion concentrations even lower than the physiological levels. In addition, it has previously been reported that small amounts of added heavy metal ions can induce precipitation at low Ca × P products (Fleisch *et al.*, 1965; Bachra and van Harskamp, 1970). All these data indicate that physiological fluids do have the potential to induce mineral formation *de novo* in the absence of any catalyst or inhibitory agent. Since physiological fluids do not normally calcify, the action of inorganic (Russell and Fleisch, 1970) and macromolecular (Howell *et al.*, 1969) inhibitors of calcification would appear to be absolutely critical in this regard.

In the absence of nucleating or inhibitory agents, both the rate at which mineral is formed and the actual amount of calcium phosphate that is precipitated are quite low at decreasing Ca × P products (Termine and Posner, 1970). For example, at pH 7.4 and a Ca × P product of 2.1 mM^2, only 3–5% of the total calcium and phosphate ions present actually precipitated within 24 h under the conditions employed in this study. Approximately 100–200 l of such a solution would be needed to attain 50 wt-% mineralization of 1 g of organic matrix. Also, a flow rate of ~80–130 ml/min would be required to achieve a 50 wt-% mineral content at a calcification site within 24 h using these solution concentrations in a volume-restricted, continuous flow system.

Thus, it would appear that a more accelerated rate of calcium phosphate formation than that described above would be desirable for efficient hard tissue mineralization. Once calcification inhibitors are removed, macro-molecular nucleating catalysts or extracellular organelles might help accomplish this as well as spatially control the mineralization process. In addition, it is known that calcium phosphate formation rates increase at higher Ca/P mixing ratios (Termine, 1972) and thus, cellular control of the levels of precipitating ions at the calcification site could also accelerate mineral formation from physiological solutions. Finally, local increases in calcifying site pH (Cuervo *et al.*, 1971) would tend to greatly accelerate mineral deposition at any given Ca $\times$ P product as shown above and elsewhere (Termine, 1972).

In summary, most physiological fluids appear to have the potential to form calcium phosphate *de novo*. Once this process is initiated, apatite formation would proceed through normal physicochemical mechanisms. In active bone or tooth calcification, however, it is most probable that the initiation of calcium phosphate formation, the spatial arrangement of mineral within the organic matrix and the rate at which the total mineralization sequence proceeds are all under cellular control.

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Note added in proof: The particles shown in Fig. 1 were absent from all starting solutions, exhibited TEM beam damage and converted to apatite crystals upon prolonged incubation at 37°.