Calcification and Decalcification Rates *in Vitro* A New Technique[1] (37441)

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In vitro calcification and decalification experiments are often carried out to serve as model processes, so that individual factors, involved in the more complex processes of biological calcification and decalcification, can be controlled and studied. The many methods available for following the extent of calcification and decalcification can be divided into chemical and physical methods (1). The chemical methods include monitoring the calcium and phosphorus concentrations by direct analyses, either by precipitation, color reactions, or spectroscopy (2, 3), while among the physical methods that of X-ray diffraction is the most important (4). The disadvantages of all these methods are that the former often involve tedious isolation and long procedures, while the latter lack sensitivity.

This paper describes a new technique in which the extent of calcification and decalcification can be followed readily and quantitatively by observing the sedimentation velocities of bone and dentin specimens.

Materials and Methods. Intact and compact hydrated cortical bone of the human adult has a density of 1.70–2.05, while hydrated collagen fibrils have a density of 1.10 (5). This rather large density difference can be utilized to observe the rate of calcification and decalcification by following changes in the rate of sedimentation, *i.e.*, measuring the rate of fall of a small section of bone through calcifying and decalcifying solutions.

The method utilizes a precision diameter glass column (obtained by special order from ACE Glass Inc., Vineland, NJ) with a guiding channel on one end (Fig. 1). The column can be filled free of air bubbles, aligned vertically, and can be rotated inside an air thermostat. The time for consecutive periods of fall between bench marks is determined by a stopwatch to within 0.1 sec.

For the decalcification studies, the bone specimens were prepared as follows: the middle portion of compact femoral bone was cut into blocks of approximately 1 cm in length and 0.5 cm in width. The blocks were placed in a milling press and milled into slabs of approximate thicknessess from 0.4–1 mm. The slabs were cut into rectangular rods with a saw blade mounted in a lathe. Finally, the rods were soaked in water in a petri dish placed under a dissecting microscope. With a pair of forceps and a very sharp scalpel, the rods were cut into approximately equal cubes of about 0.6 mm.

After drying in a desiccator, the bone cubes were weighed on a Cahn electrobalance capable of weighing to 0.01 mg. Since the cubes were cut from rods of similar shape, it was assumed that bone cubes of about equal weight would have approximately the same surface area. For the calcification studies, the "bone" samples were cut from rachitic rat cartilage (6). They were again handled under a dissecting microscope, preparing cubes of approximately the same size, as above.

Two types of reagents were used for decalcification, *i.e.*, an acid, and a Ca-complexing agent. As the former, we chose lactic acid (0.1–0.3 *M*, pH 4.20), and as the latter, EDTA (0.15–0.7 *M*, pH 7.40). The calcifying solutions were prepared according to the

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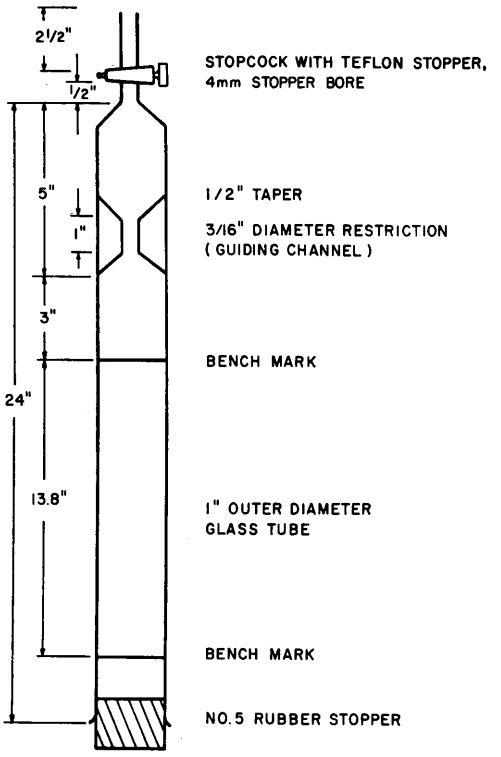

FIG. 1. Schematic drawing of sedimentation column.

method of Sobel and co-workers (7, 8), and consisted of the following ion concentrations (mmoles/liter): Na, 95.0; Cl, 80.0; HCO_3, 22.0; K, 5.00; Ca, 2.25; P, 1.61.

Results and Discussion. Typical results, on a plot of the time of fall against the duration of the decalcification process, show an S-shaped rise of the times, in the end leveling off sharply to a final value. At each time interval, an average of 3 or 4 readings was taken. The plot of the velocity of fall against the time of decalcification is shown in Fig. 2. The effects of exposing the bone to various agents prior to decalcification are shown in Fig. 3. It can be seen that, of the chemicals studied, stannous fluoride inhibits decalcification, while other stannous halides enhance decalcification. Chemicals which have no effect reproduce the control curve. For clarity, their points are not shown. The results for decalcification with lactic acid are shown in Fig. 4, while Fig. 5 shows a calcification run using rachitic rat cartilage.

The velocity changes of sedimentation of the bone specimens level off on approaching the end point of decalcification. Correspondingly declining decalcification rates were also demonstrated by X-ray diffraction and chemical analyses. By contrast, it was observed during calcification that the sedimentation velocities of the bone samples (Fig. 5) rose with time at a declining rate, *i.e.*, started to level off above about 50% of full calcification.

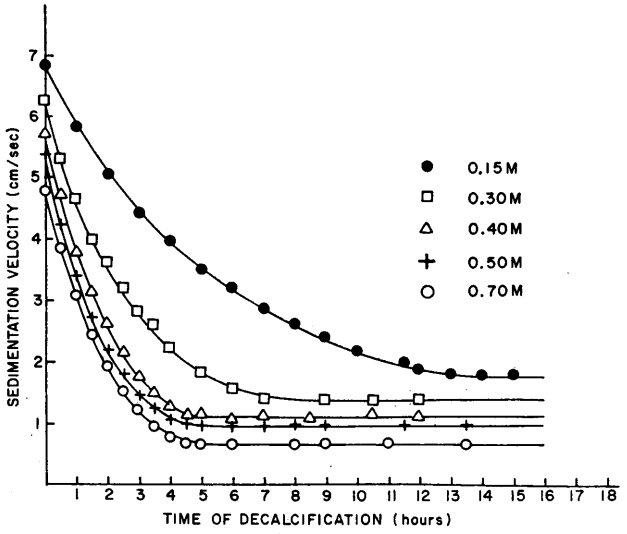

FIG. 2. Effect of EDTA concentration on sedimentation velocity; 0.25 mg bone, pH 7.40, 37°.

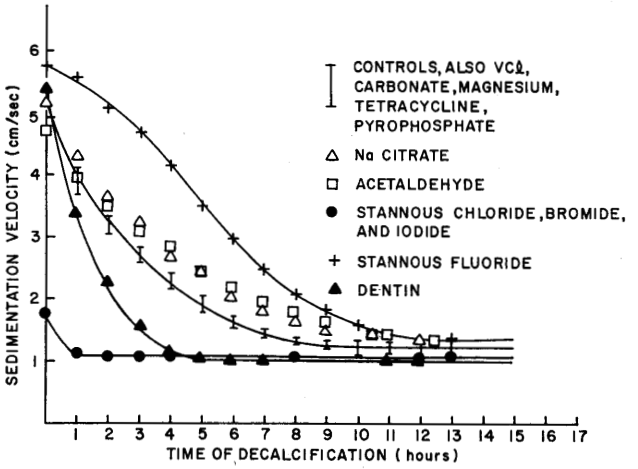

FIG. 3. Effect of pretreatment of bone on decalcification; 0.15 *M* EDTA, 0.15 mg bone, pH 7.40, 37°.

The final sedimentation velocities of the decalcified bones were different for individual specimens and for different EDTA concentrations (Fig. 2). This is probably the result of minor differences in bone shape and in the density of the solutions. The density effect was checked by performing an experiment in which a bone specimen, decalcified by 0.15 *M* EDTA, was placed into a column containing 0.7 *M* EDTA. Allowing for differences in shape factor, the sedimentation velocity was the same as that of the bone specimen of originally the same weight, but decalcified all the way in 0.7 *M* EDTA to the same degree.

In order to follow decalcification in the column independently and directly, bone specimens of approximately the same size were decalcified in the columns for specific periods of time, then removed from the columns and analyzed for calcium and phosphorus content (9, 10). Figure 6 shows a plot of velocity of fall of the specimens versus the amount of calcium and phosphorus remaining in the specimens. A high correlation coefficient ($r = 0.97$) indicates the validity of the assumption of tying changes in fall velocity to changes in the amounts of calcium

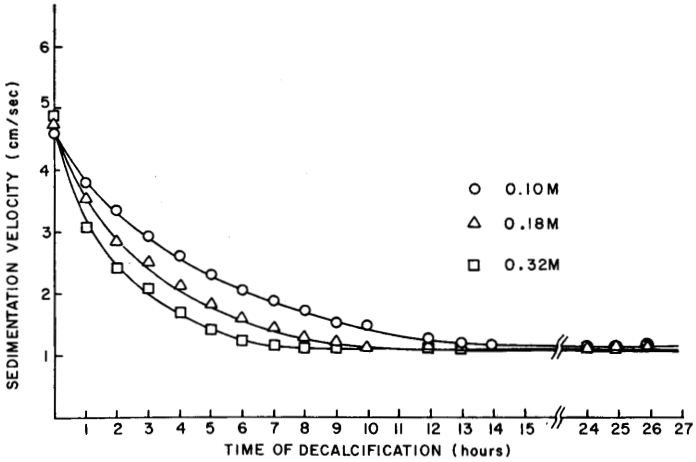

FIG. 4. Effect of lactic acid concentration on sedimentation velocity; 0.14 mg bone, pH 4.30, 37°.

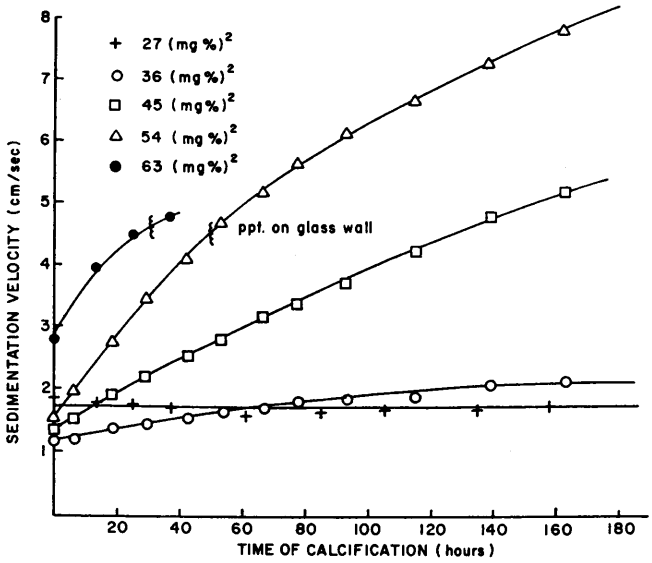

FIG. 5. Effect of concentration of calcifying solution on sedimentation velocity (Ca/P = 1.40).

and phosphorus in the bone. Parallel evidence of the course of decalcification was also derived from X-ray diffraction and density measurements.

Thus, the settling tube apparatus provides a quick and useful method for the study of calcification and decalcification processes. The system is completely enclosed and, once all reagents are introduced into the column, the action of various factors that influence calcification and decalcification can be easily studied without disturbing the system. The bone samples required for each run are very small (0.25 mg), so that one small section from the same sample is enough for a series of runs, avoiding individual variations. Even

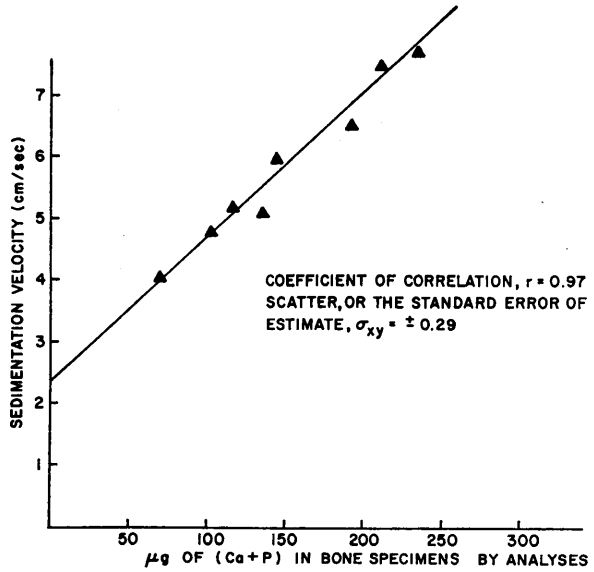

FIG. 6. Correlation plot of sedimentation velocity with the amount of calcium and phosphorus in the bone specimens.

biopsis specimens could be used. If required, the experimental setup can be automated by installing a motor to turn the column, and a light source with detector to follow the fall of the bone specimen.

The swelling behavior of bone and tendon collagen has been studied by Glimcher and Katz (11). It was shown that the Achilles tendon showed slight swelling in water, but swelled to a jelly mass when placed in 0.5 *M* acetic acid. However, the behavior of decalcified chicken bone was very different. No swelling effect was observed in water or acetic acid. In view of this, tests were carried out in this study, during which pictures of bone specimens immersed in the liquid medium were taken before and after decalcification. No dimensional changes in the bone specimens were observed. Our assumption that the shape of bone specimens did not change noticeably as a result of our calcification or decalcification procedure was therefore reasonable. There remains, though, the possibility that the friction factor may change somewhat when going from the calcified to the decalcified specimens, see below.

The size of the bone specimens used in the experiment was insignificant compared with the volume of the solutions (approx. 220 ml) in the columns. The amount of mineral dissolved in, or removed from, the solution was thus quite small during each run. Therefore, it was justifiable to assume that the density, or viscosity, of the solution remained constant during each decalcification or calcification cycle.

For an interpretation of our data, it is desirable to analyze the relation between changes of bone density and velocity of sedimentation in greater detail so as to be able to formulate reasonable mechanisms of the decalcification and calcification processes.

For the initial, solvent penetrated (slightly swollen) bone, we can formulate the overall density as

$$\bar{\rho}_{\text{initial}} = (V_A\rho_A + V_C\rho_C + V_S\rho_S)/V, \quad (1)$$

where V is the total bone volume, and V_A, V_C, V_S, ρ_A, ρ_C, ρ_S refer to the volume portions and densities of (hydroxy) apatite (HAP), collagen, and solution (here in the bone), respectively. If after time t, part of the apatite disappears and is replaced by solution, the density of the bone at this time $\bar{\rho}_t$, will be

$$\bar{\rho}_t = \frac{V_{A_t}\rho_A + V_C\rho_C + V_S\rho_S + (V - V_C + V_S + V_{A_t})\rho_S}{V}, \quad (2)$$

where V_{A_t} is the volume of HAP at time t. The final bone density, with all HAP replaced by the solution, will be

$$\bar{\rho}_{\text{final}} = (V_C\rho_C + V_S\rho_S + V_A\rho_S)/V. \quad (3)$$

The sedimentation velocity v, can be written as

$$v = K(\bar{\rho}_t - \rho_S), \quad (4)$$

where $K = Vg/f$, g is the acceleration due to gravity, and f is the frictional coefficient. The rate of velocity change is then

$$-(dv/dt) = -[d(K\bar{\rho}_t)/dt]. \quad (5)$$

Differentiating Eq. (2) with respect to time, all the constant additive terms drop out, and we obtain

$$-\frac{dv}{dt} = -K'\frac{d(V_{A_t})}{dt} = -K\frac{d(\Delta\bar{\rho}_t)}{dt}, \quad (6)$$

where $K' = (K/V)(\rho_A - \rho_S)$. Since V_A is directly proportional to the mass of apatite [HAP], at any time, the proportionality between $-(dv/dt)$ and $-(d[HAP]/dt)$ is established (see Fig. 6).

Our experimental data indicate, however, one difficulty. Since the changes in velocity and thus the rates of HAP disappearance, according to the above assumptions and derivation should be a function of the density changes alone, the relative decreases in sedimentation velocity should equal the relative decreases in density, *i.e.*,

$$(v_t - v_\infty)/(v_i - v_\infty) = (\bar{\rho}_t - \bar{\rho}_\infty)/(\bar{\rho}_i - \bar{\rho}_\infty), \quad (7a)$$

or also:

$$v_i/v_\infty = \Delta\bar{\rho}_i/\Delta\bar{\rho}_\infty, \quad (7b)$$

where $\Delta\bar{\rho}_i$ and $\Delta\bar{\rho}_\infty$ are the density differences at $t = 0$ and $t = \infty$, respectively.

This derivation assumes that the friction

coefficient, of the bone samples, remains unaffected by the processes of decalcification and by the length of exposure to EDTA solution. If that were strictly true, the ratio of settling velocities of original to decalcified bone should indeed be equal to the ratio of the density difference between bone and solution and between the decalcified bone and solution [Eq. (7b)]. If these two ratios are compared, the velocity ratios are in the range 4–5, but the ratios of the density differences can only be estimated. The densities of compact bone, decalcified bone, and of 0.15 *M* EDTA solution, as measured in the present investigation are 1.895, 1.127, and 1.024, respectively, but while the density of the solutions can be measured with high precision, and those of native bone and wet cartilage are also well known (approx. 1.89 and 1.21, respectively), the values required here, *i.e.*, of EDTA swollen bone and cartilage are not easy to come by. The latter was measured picnometrically in EDTA solutions and, *e.g.*, for 0.15 *M* solution of $\rho = 1.024$, was found to be 1.127. The bone density at the beginning of the experiment may be as low as 1.6 because of swelling. The density ratios would then be approximately 5–6. This nearly equals, but not quite, the velocity ratios. The remaining difference, of about 20%, is likely to be due to a change in f, the shape factor, since it can hardly be expected that an anisotropic entity like bone should swell isodiametrically.

The kinetics of calcification and decalcification will be discussed elsewhere.

Summary. A sedimentation column apparatus was developed in which the rates of calcification and decalcification of bone and dentin could be followed readily and quantitatively. The method utilizes a long column with guiding channel and can be rotated inside an air thermostat. The rates of calcification and decalcification are measured by following the sedimentation velocities of the specimen in the column of calcifying or decalcifying solutions with time. The bone specimens used are very small. Typical results obtained in decalcification with EDTA and lactic acid, and of calcification rates of rachitic cartilage are presented and discussed.

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