Calcification of a Native Collagen Membrane

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with the technical assistance of

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Spontaneous calcification of a membrane made of native collagen has been investigated. The method permits independent variation of calcium and phosphate concentrations. With increasing phosphate concentration the precipitation of calcium-phosphate on the collogen occurs at a conspicuously lower calcium concentration as with a number of other membranes.

Introduction

The mineralization of bone is a very complex process, involving metabolism in cells, reaction in the organic matrix and the calcium phosphate concentration ratios in the tissue fluid. To investigate these processes, experiments have been performed with reconstituted acid soluble collagen, using metastable solutions of calcium phosphate [1–5]. In all cases, the metastability of the solutions limits the range of variation of calcium- and phosphate-concentrations as well as the addition of other substances.

In this study we show a method that allows greater control of the calcium- and phosphate-concentrations and their independent variation over a wide range by using a collagen membrane. The precipitation of calcium-phosphate within the collagen matrix was detected by recording the change in electrical resistance of the membrane. Parallel measurements with a variety of artificial membranes give an example of the specific properties of native collagen.

Materials and Methods

1. Production of acid soluble collagen

300 g of tendon from the neck of a calf was dissected and cut into small pieces. After adding 3 l of 0.05 M acetic acid, the tendon was homogenized with an Ultra Turrax homogenizer. The homogenate was stirred at 4 °C for two days and then centrifuged for one hour at $3000 \times g$ and 4 °C. The supernatant was isolated and filtered through a glass filter (type G 2). The filtered solution was adjusted to a concentration of 7% NaCl with addition of a 30% NaCl solution and centrifuged again 40 min at $3000 \times g$ and 4 °C. The sediment was washed with 400 ml distilled water. Sedimentation and washing were repeated three times. The sediment was diluted in 300 ml of 0.05 M acetic acid and filtered again. The protein content of the solution was estimated to be 7–8% using biuret reaction.

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2. Isolation of RNP

The ribonucleoproteid (RNP) was isolated from calf thymus and streptococci using the method of Wilhelm [6].

3. Production of membranes

The membranes were formed at room temperature by the action of RNP on the native collagen. A membrane filter (Satorius Göttingen) was impregnated with RNP and a 0.39 mm thick collagen layer was superimposed on this filter using a sledge. The RNP precipitated the collagen in a felt of collagen fibres. The collagen membrane is separated from the filter by gentle rocking in a water-filled Petri dish. The membrane has a thickness of 6 μm. Electron microscopy showed that the collagen fibres were of the native type with a 700 A period. The membranes were kept wet and thymol was added to minimize bacterial growth.

4. Solutions

The bulk solutions on both sides of the membrane contained 0.15 M NaCl and were buffered to pH 7.4. $CaCl_2$ was added to the solution on one side of the membrane and Na_2HPO_4 to the other, to give

a final concentration ranging up to 0.05 M. The solutions were buffered with Tris/HCl with a buffer concentration equal to one-tenth of the calcium or phosphate concentration. To show the effects of different buffers on the results, citric acid was used in a second series of experiments instead of HCl.

5. *The chamber*

The apparatus used is similar to one described previously [7]. Fig. 1 shows one half cell, without the concentration-change device. The half cells are machined from acrylic plastic blocks and have a volume of 210 ml each. As the collagen membranes are extremely fragile, they were held between two Monodur-Polyester nets (Vereinigte Seidenwebereien, Krefeld) with a mesh aperture of 4×10^{-4} cm^2, fixed like an embroidery frame. The net was tightened on the membrane side with a ring of Parafilm and on the other side with a silicone ring. Vigorous stirring kept each of the two bulk phases homogeneous. The membrane separated the $CaCl_2$ and Na_2HPO_4 solutions. The solution on each side was continuously pumped through a conductivity cell (Wissenschaftlich-Technische Werkstätten, Weilheim) connected to a Wayne Kerr Autobalance Bridge (Brindi, Lörrach). The conductivity was measured at a frequency of 1592 Hz. All measurements were carried out at 20 °C.

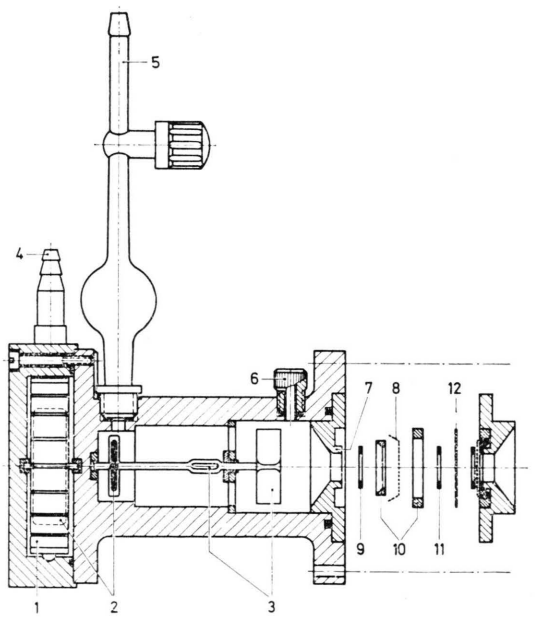

Fig. 1. One half cell of the basic chamber. Numbered parts are: (1) stirring blade wheel; (2) magnetic coupling; (3) stirring blades; (4) water inlet to move stirring wheel; (5) filling cap; (6) screwed squeezing joint to connect with concentration change device; (7) membrane support; (8) polyester net; (9) silicone ring; (10) "embroidery frame"; (11) Parafilm ring; (12) membrane.

6. *Concentration-change device*

It is possible to change the concentration in each half-cell continuously with a system which is roughly sketched in Fig. 2. The main compartment B is connected with two additional compartments A and C. At the beginning of the experiment, A and B are filled with different solutions, the empty lower compartment C is connected with A via a valve V. If the valve is open, gravity causes a flow of solution from A to B and from B to C. The air from compartment C is driven out and streams from the bottom towards the top into A. This process stops as soon as the valve V is closed. Thus the concentration in B can be changed by any amount desired. In our case B is identical with the chamber described in 5.

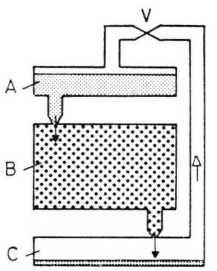

Fig. 2. Principle of concentration change in compartment B.
A, upper compartment;
C, empty, lower compartment;
V, valve.

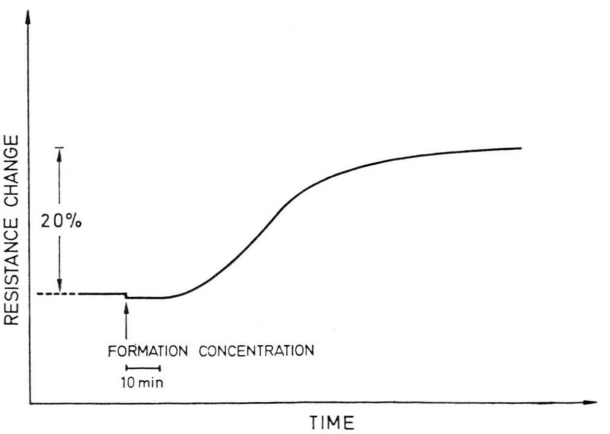

Fig. 3. Change of membrane resistance due to precipitation of calcium-phosphate.

Results

The precipitation of calcium-phosphate in the membrane changes the resistance of the membrane as calcium-phosphate crystals accumulate at the collagen fibres and close the pores of the membrane. As conductivity can be measured with an accuracy of 0.1%, the occurence of calcification may be determined with very high sensitivity. After changing the concentration on one side of the membrane, the resistance is monitored for 15 – 20 min. If there is no precipitation, the resistance undergoes a small change due to the change of concentration but otherwise remains constant. As soon as the "formation-concentration" is reached, calcification occurs and the membrane resistance increases. Fig. 3 gives an

Fig. 4. Deposition of calcium-phosphate needles to collagen fibres.

Fig. 5. Calcium-phosphate needles in a membrane section.

impression of the order of magnitude of these resistance changes observed in our set-up.

The initial precipitation of amorphous calcium-phosphate could not be shown by electron microscopy, but after about ten minutes small needles of calcium-phosphate deposited on the collagen fibres (Fig. 4) growing to a length of about 1000 A after further calcification took place (Fig. 5). To show the specific response between collagen and phosphate, we carried out experiments with a number of artificial membranes (curve 1 in Fig. 6). A second set of experiments was carried out with membranes of denaturated collagen (▽) called "Wursthaut" (sausage hide) in laboratory jargon.

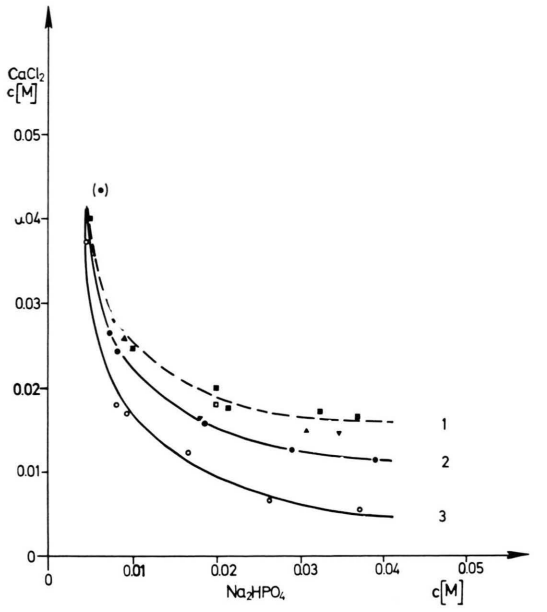

Fig. 6. Dependence of calcification on bulk concentration for different membranes. Curve 1: artificial membranes; (■) cellulose nitrate, pore size between 0.45 and 0.8 μm, Satorius SM 13006; (□) cellulose acetat, pore size 0.45 μm, Satorius MFA SM 11106; (▲) PVC membrane, pore size 0.8 μm, Satorius 12801; (△) silver, pore size 5 μm, Flotronics Membrane FM 47. Curce 2: denaturated collagen (▽); Naturin and collagen buffered with citric acid (●). Curve 3: native collagen (○).

These showed nearly the same behavior as the membranes made of native collagen and buffered with citric acid (●) (curve 2 in Fig. 6). The inhibition of calcium phosphate formation by citrate was previously found by Termine and Posner [8]. Curve 3 in Fig. 6 shows the behavior of membranes made of native collagen buffered with Tris/HCl. At low Na_2HPO_4 concentrations, it behaves roughly like the other membranes, whereas collagen needs a significantly lower concentration product to calcify with increasing phosphate concentration.

To verify the specific precipitation effect of collagen by phosphate alone, experiments were repeated with sodium bicarbonate instead of phosphate. In this case we found no difference between collagen and the other membranes.

Discussion

The exact mechanism involved in the process of deposition of calcium phosphate in biological tissues is still not well understood. A large number of factors such as geometrical parameters [9], matrix macromolecules [10] and influence of pyrophosphate [11, 12] are involved in this process. A new method is proposed to investigate one of these parameters: the calcium and phosphate concentration. By varying these concentrations independently over a wide range on each side of the membrane we found that with increasing phosphate concentration the collagen calcifies at lower calcium contentration than in membranes built from other macromolecules. This effect seems to be specific for collagen and does not occur with bicarbonate. (See also the Ca/P and P/Ca ratio in Fig. 7.) The calcification of a membrane made from native collagen shows a distinctly stronger dependence on the phosphate concentration as it does on the calcium concentration.

The biological significance of these different degrees of concentration dependence for *in vivo* calcification can not be determined without knowing additional parameters. On the other hand, it is well known that the phosphate concentration and not the calcium concentration is correlated with the velocity of growth. One, too, has observed a change of phosphate concentration of the extracellular fluid in some diseases like rickets or renal insufficiency, where the process of calcification is disturbed.

The method shown in this study could also be helpful to investigate the time behavior of calcification and the effects of the addition of other substances. The convenient and independent variation of external parameters makes this method ideal in verifying some of the results found in experiments with metastable solutions.

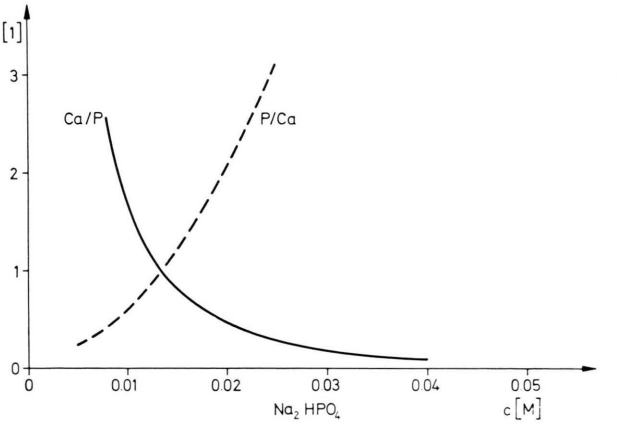

Fig. 7. Ca/P and P/Ca ratio of calcification versus sodium-phosphate concentration of one bulk phase. The values are taken from curve 3 in Fig. 6.

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