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A dynamic *in vitro* method for studying bioprosthetic heart valve calcification

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The most predictable cause of failure of a biological prosthetic heart valve is calcification. The deposition process appears to be related both to the biomaterial composition and to the presence of dynamic stresses in the leaflets. A dynamic *in vitro* test has been developed to investigate the calcification process. The test apparatus consists of a modified Rowan Ash fatigue tester with the test fluid maintained at 37°C. Six valves can be tested simultaneously under similar physical conditions, but with individual incubation media. The valves tested were glutaraldehyde-fixed bovine pericardial valves (the Glasgow Heart Valve). These have been tested in a range of different simple salt solutions containing approximately physiological concentrations of calcium and phosphate. Calcification has been analysed by assay of the incubation media for depletion of calcium and phosphate and direct measurement of uptake of calcium by the valves. There is a wide variability among pericardial valves treated similarly. This may be related to the variability in calcification rate seen in patients with valve implants.

1. Introduction

Calcification of artificial heart valves is reported to be of the dystrophic type and may be intrinisic or extrinsic to the biomaterial involved [1]. The mechanism is obscure and there are several current hypotheses. Dystrophic calcification commonly occurs in tissues which become fibrosed and necrosed, giving rise to hypotheses of nucleation of calcification by tissue damage [2], or crystal initiation by the action of extracellular vesicles formed from cell membrane fragments [1], or mitochondria [3]. Studies of leaflet material implanted subcutaneously in rats and enclosed in Millipore filters showed that calcification still occurred, implying that host cellular factors, at least, are not involved [4]; nor are host immunological factors, as shown in work with athymic mice [5]. *In vitro* experiments have shown that collagen is capable of taking up calcium and phosphate [3, 6]. It has been suggested that phosphate forms covalent bonds connecting inorganic and organic components via the *e*-amino groups of lysine and hydroxylysine in collagen and that proteoglycans and glycoproteins which would normally block these sites are lost during processing of the tissue with glutaraldehyde [7]. It has also been suggested that carbonyl oxygens in the peptide backbone are the binding sites for calcium which then sequesters phosphate to maintain charge neutrality [8].

Several groups postulate a close connection between the mechanical stress applied to a xenograft heart valve and the process of calcification, although it is uncertain whether mechanical stress initiates calcification or vice versa [9–14].

Test methods for the study of valve calcification may be divided into two main groups, *in vivo* and *in vitro*, and each group subdivided into dynamic and static subgroups. Dynamic *in vivo* systems include study of human explants and implantation of intact valves into large animals, e.g. cow or sheep [11, 15–17]. Static *in vivo* tests include subdermal or intramuscular implantation of leaflet material into small animals, e.g. rat or rabbit [18]. Static *in vitro* tests may be carried out in sterile closed sample vials. Dynamic *in vitro* tests are mainly used to test the engineering performance of the valves, e.g. pulse duplicator or fatigue tester [19], but a few have been designed also to study calcification [20, 21]. In general, *in vitro* systems are simpler, cheaper and more easily controlled than *in vivo* systems, although they lack elements present in live biological systems that may influence the calcification process. Static test systems can be used to study small samples of material in large numbers relatively quickly, but cannot give information regarding the response of the material to mechanical stress. *In vivo* studies generally produce rather heavily calcified material, making the study of the early stages of the calcification process difficult. It has also been shown that anti-calcification treatments that show promise in static test systems, e.g. the rat subdermal model, do not always translate their promise to the dynamic *in vivo* model [16].

It seems, therefore, that a need exists for a simple, controllable system, capable of applying mechanical stress to the material, which permits study of early calcification and assists in the initial screening of materials for more extensive investigation.

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A dynamic, *in vitro*, accelerated calcification test has been developed that uses a modified standard fatigue test system. This allows whole valves to be calcified in the laboratory under controlled conditions and to a degree that permits study of the early stages of calcification. The composition of the calcifying medium may be altered or modifications to valves may be tested under conditions of mechanical stress. The fatigue tester is operated at room temperature for straightforward durability testing. In this case, although a few early tests were performed at room temperature, the tester was later modified to allow testing to be carried out at 37 °C so that temperature effects on the biomaterial response would be similar to those *in vivo*.

2. Materials and methods

The tests were carried out in a standard Rowan Ash (Sheffield) fatigue tester (Wessex Medical Ltd) with the addition of a water-circulator, C-400 (Techne (Cambridge) Ltd), to pump heated water, via 13 mm silicone rubber tubing, around the outside of the valve compartments, enabling the temperature inside the individual valve compartments to be maintained at 37 °C ± 1 °C (Fig. 1). The upper section of the fatigue tester was insulated during the test by using metal foil covered by cotton wool padding. The cycling rate of the fatigue tester was 12 cycles per second. Early tests were carried out at 22 °C (valves 1, 2, 5, 6, 9 and 10). All other valves were tested at 37 °C.

Three different calcifying solutions were used in the tester. Solution composition was specifically chosen to be as simple as possible and still achieve calcification, allowing the parameters involved in the process to be kept to a minimum. This would allow more detailed investigation of the primary binding process resulting in calcification, without the added complications of numerous promoters or inhibitors of the process as are present *in vivo*. Small amounts of trace elements may also be present in solution, in particular, related species such as other divalent cations which may or may not be involved in the calcification process.

Valves 1–4 were tested in solution 1, containing 2.5 mmol l^{-1} $CaCl_2 \cdot 2H_2O$ [22]; valves 5–8 were tested in solution 2, containing 3.0 mmol l^{-1} $CaCl_2 \cdot 2H_2O$, 2.25 mmol l^{-1} KH_2PO_4, 55 mmol l^{-1} KCl [23] and valves 9–12 were tested in solution 3, containing 135 mmol l^{-1} NaCl, 2.0 mmol l^{-1} $CaCl_2 \cdot 2H_2O$, 1.2 mmol l^{-1} KH_2PO_4 in 0.05 mmol l^{-1} MOPS, buffered to pH 7.40 [6]. All solutions were made up by using deionized water, with AnalaR (AnalaR Standards Ltd) grade reagents. Calcium and phosphate concentrations were approximately physiological.

The fatigue tester was cleaned before use with a phosphate-free detergent (Decon 90), rinsed thoroughly with deionized water and sterilized before use with 4% glutaraldehyde in 0.9% NaCl. Solutions were replaced at seven-day intervals both to maintain the concentration of calcium and phosphate available to the valve and to minimize contamination by bacterial

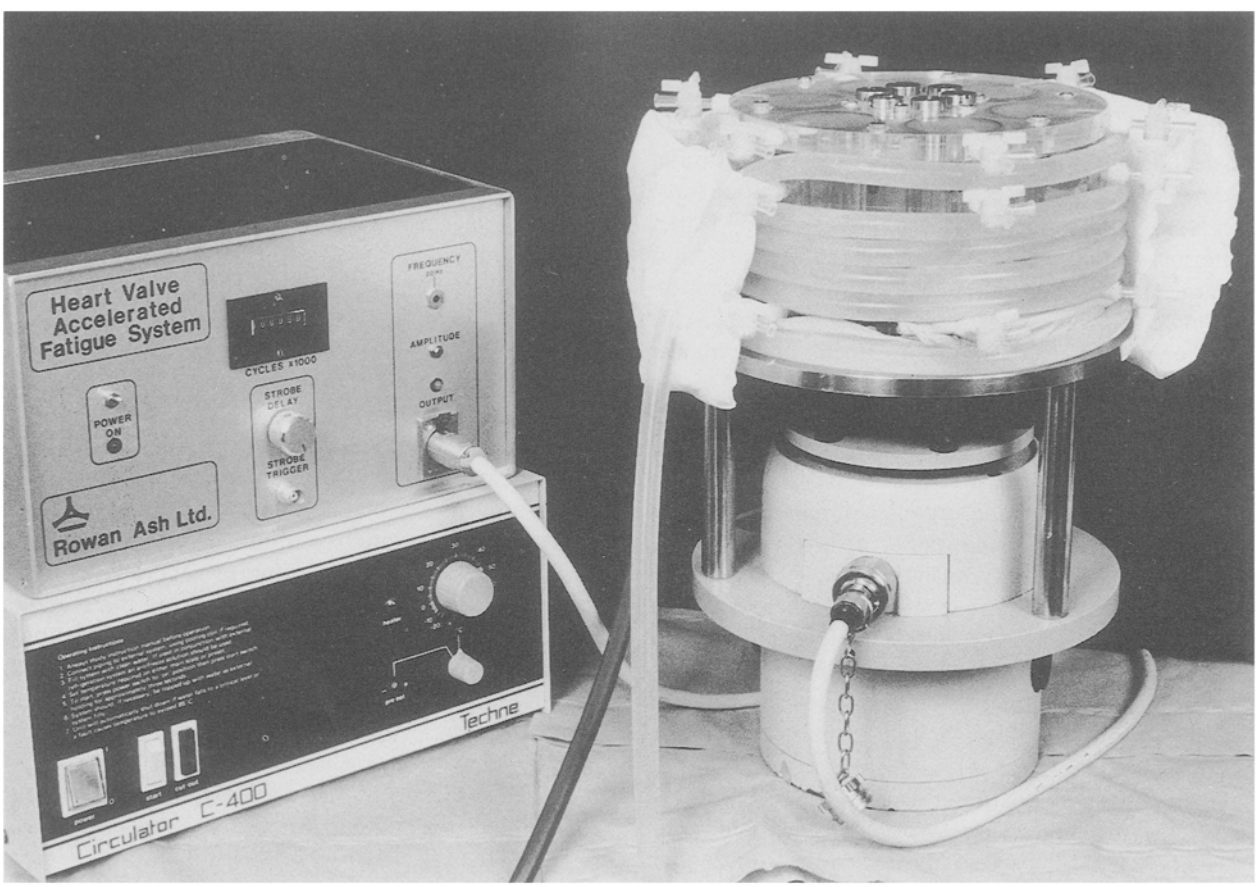

Figure 1 The dynamic *in vitro* test system.

or fungal growth. It was decided against the addition of anti-bacterial or anti-fungal agents as these might have an unpredictable effect on the calcification process.

The tester was operated, before valve testing, with compartments containing calcifying solution but no valve, for 3.2×10^6 cycles. Similar control compartments were also included in the test runs (two controls for each test run).

The system has been tested by using the Glasgow Heart Valve, an improved bovine pericardial valve, fixed with 0.25% (v/v) glutaraldehyde in 0.1 mmol l^{-1} phosphate buffered to pH 7.40 and stored in buffered 4% formaldehyde [19].

The progress of calcification was monitored by both direct and indirect methods. The test media were assayed when fresh and on replacement for depletion of calcium and phosphate, by standard photometric techniques: calcium by the cresolphthalein complexone method of Baginski *et al.* and phosphate by the modified malachite green method of Hohenwaller and Wimmer [24]. The rate of calcium depletion from solution was used as an indicator of calcium uptake by the valves. At the end of the test, valves were examined radiographically for shadowing caused by calcification. Samples of the leaflets were dried to constant weight and ashed, in boiling tubes, by using 2 ml ARISTAR (BDH Ltd) grade HNO_3 at 120 °C, in a heating block. The dissolved samples were made up to 10 ml volume with deionized water, diluted 1:33 with 0.1% v/v SpectrosoL (BDH Ltd) $LaCl_3$ to reduce interference from phosphate, then introduced into the flame (lean, oxidizing, air/acetylene) of a Perkin Elmer 3030 Atomic Absorption Spectrophotometer, wavelength 422.7 nm. Tests were compared with a series of standards in the range 0–600 mg l^{-1} calcium, made up from SpectrosoL calcium standard solution (100 p.p.m.) in 20% ARISTAR HNO_3. All glassware was washed before use in 20% AnalaR HNO_3 and rinsed three times with deionized water. Results were expressed as milligrams calcium per gram dry mass of tissue.

Control samples of leaflet material, having no contact with calcifying media, were also analysed for tissue calcium content.

3. Results

After an initial uptake of calcium (mean ± standard deviation = 1.41 ± 0.34 mmol l^{-1}, $n = 6$), and phosphate (mean ± standard deviation for solution 1 = − 2.22 ± 0.09 mmol $^{-1}$, $n = 2$, and for solutions 2 and 3 = − 0.97 ± 0.07 mmol l^{-1}, $n = 4$) in the first five days of contact with the calcifying solution, the control compartments showed an average uptake of − 0.12 ± 0.05 mmol l^{-1} calcium and − 0.06 ± 0.07 mmol l^{-1} phosphate ($n = 12$) on subsequent contact. This implies that long-term uptake of calcium and phosphate on to the tester itself was not significantly greater than zero.

Four valves were tested in solution 1, which contained no phosphate ions. The loss of calcium from the test medium was variable, valve 1 producing the greatest loss (Fig. 2a). Phosphate concentrations in the medium rose during the test (Fig. 2b). Valves 2–4 calcified to a minimal extent (Table I). Valves 1 and 2 were tested at 22 °C and valves 3 and 4 at 37 °C.

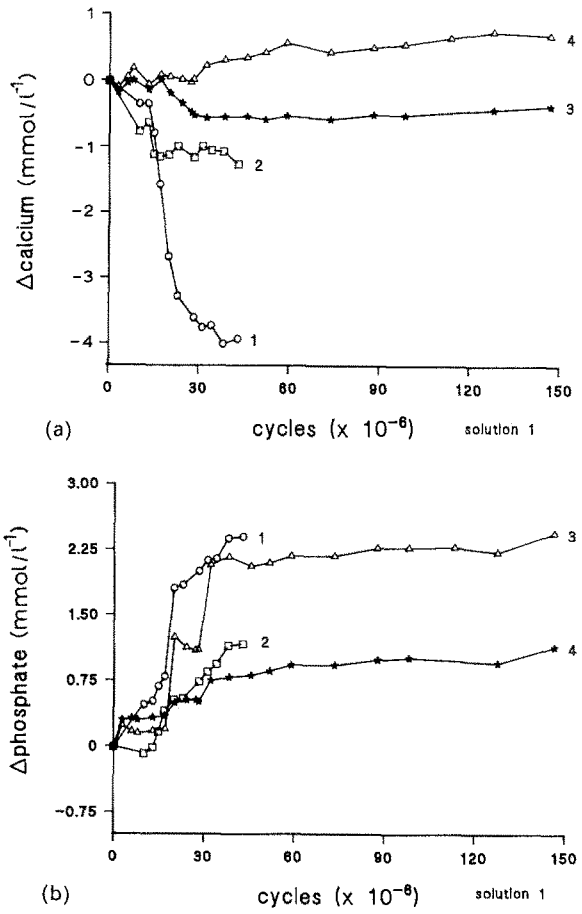

Figure 2 (a) Calcium depletion from solution for valves tested in solution 1. (b) Phosphate uptake by solution for valves tested in solution 1.

TABLE I Calcium, phosphate and buffering in calcifying solutions

Solution	Calcium (mmol l^{-1})	Phosphate (mmol l^{-1})	Buffer
1	2.5	none	none
2	3.0	2.25	none
3	2.0	1.2	MOPS (pH 7.40)

Solution 2 had higher concentrations of both calcium and phosphate than the others; however, valves tested in this medium calcified minimally (Table I), the medium lost comparatively little calcium (Fig. 3a) and, in all cases, the medium gained phosphate during the test (Fig. 3b). Valves 5 and 6 were tested at 22 °C and valves 7 and 8 at 37 °C.

Four valves were tested in solution 3. Valves 9 and 10 were tested at 22 °C and all others at 37 °C. Valves 10–12 lost significant amounts of calcium from the test medium (t-test, significant at the 5% level), although the rates of depletion were highly variable (Fig. 4a). The depletion of phosphate did not correlate well with calcium depletion, except for valve 10, which calcified early (Fig. 4b).

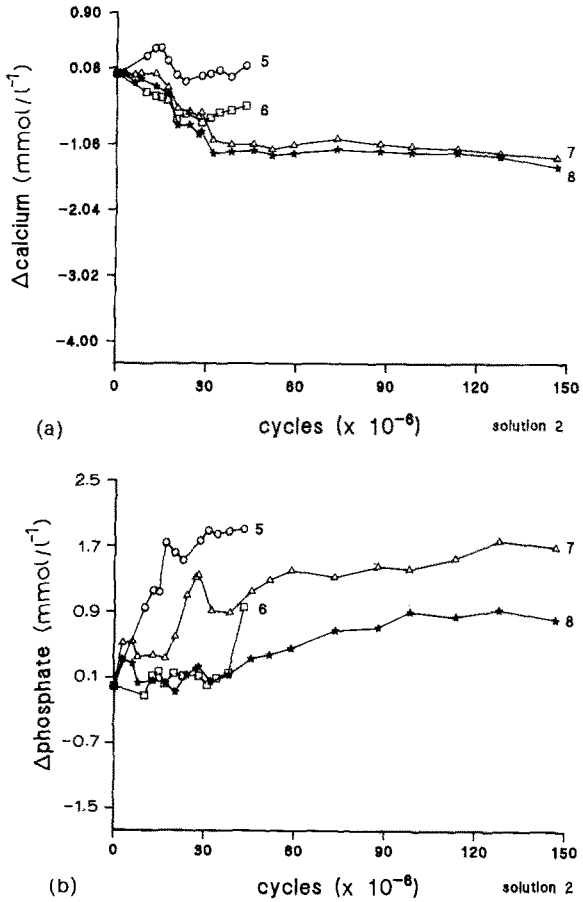

Figure 3 (a) Calcium depletion from solution for valves tested in solution 2. (b) Phosphate uptake by solution for valves tested in solution 2.

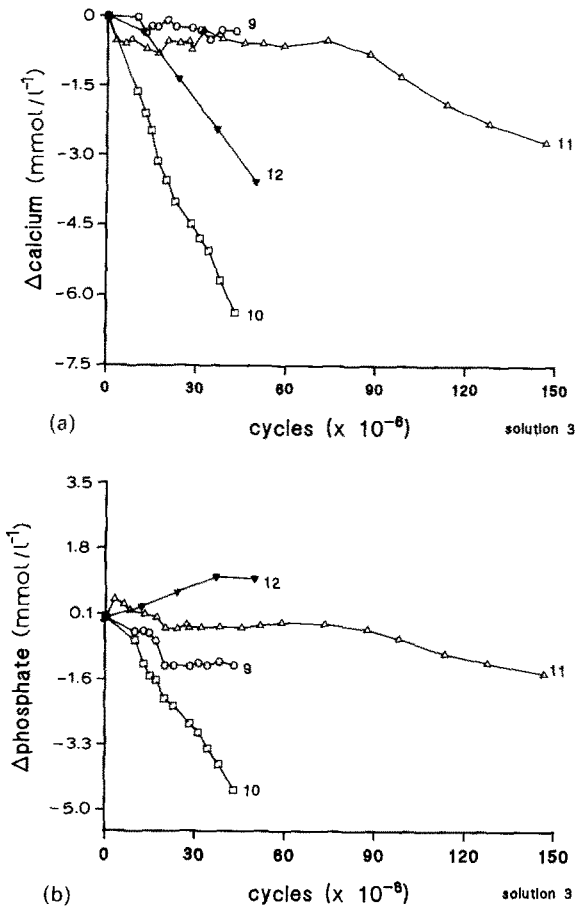

Figure 4 (a) Calcium depletion from solution for valves tested in solution 3. (b) Phosphate depletion from solution for valves tested in solution 3.

In general, tissue calcium content was approximately proportional to depletion of calcium from the test medium (Table II). Rank correlation of tissue calcium with depletion of solution calcium was − 0.86. Overall, depletion of phosphate from solution did not correlate with calcium depletion.

All valves tested in solutions 1 and 3 had significantly greater tissue calcium concentrations than the control group (0.10 ± 0.13 mg calcium per g tissue, $n = 11$). Valves 5 and 6, tested in solution 2 were not significantly different from the control group.

Direct tissue calcium assay was the most sensitive method for detection of calcification. In most valves, the tissue concentration of calcium was not sensitive to diagnostic X-ray, nor visible to the naked eye. In a few valves, however, the calcification appeared localized in heavy deposits, both visible and apparent on X-ray, e.g. valve 11 (Fig. 5a, b). This valve has a small tear at the free edge of the leaflet, near the calcified area.

There was no significant relationship between calcification and the size of the valve (Kruskal–Wallis test, comparing 21 mm ($n = 3$), 25–27 mm ($n = 4$) and 29 mm ($n = 5$) valves). Further testing of larger numbers of valves is required to confirm this finding.

TABLE II Calcium uptake by valves under dynamic test

Valve	Size (mm)	Solution	ΔCalcium (soln) (mmol l^{-1})	Calcium (mg g^{-1}) tissue)
1	21	1	− 3.92	2.35
2	29	1	− 0.88	1.13
3	27	1	0.60	0.74
4	29	1	− 0.20	1.03
5	21	2	− 0.13	ND
6	29	2	− 0.47	ND
7	27	2	− 1.39	0.66
8	29	2	− 3.56	1.54
9	21	3	− 0.31	0.83
10	29	3	− 6.36	46.77
11	27	3	− 2.69	4.98
12	25	3	− 3.56	4.28

4. Discussion

The dynamic *in vitro* accelerated calcification system demonstrates calcification of "bioprosthetic" heart valves under simple, defined conditions, with physiological concentrations of calcium and phosphate. This permits economical and fast calcification of relatively large numbers of valves allowing study of the effects of mechanical stress at physiological temperatures on biomaterial calcification; however, it is not implied that the mechanical loading of the valves in this system relates directly to that found *in vivo*. The system also allows study of the early stages of calcification and may thus aid in defining the mechanism of calcification.

It is our observation that clinical valves explanted early for reasons such as endocarditis, unrelated to the

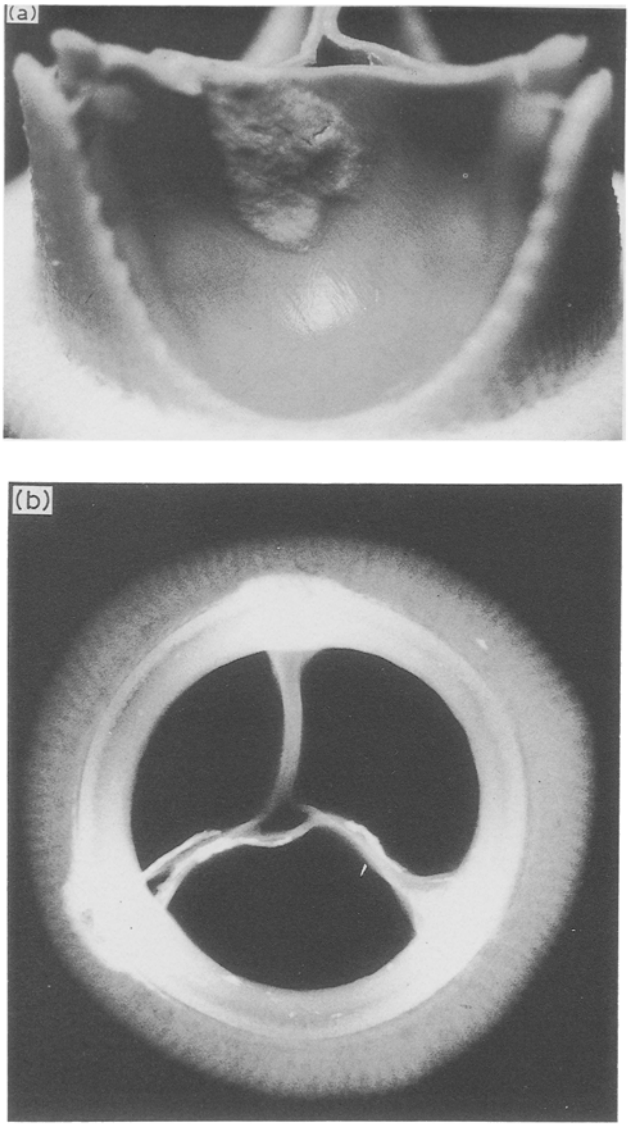

Figure 5 (a) Valve 11, showing localized heavy calcification adjacent to small leaflet tear. (b) Valve 11, diagnostic X-ray showing electron-dense areas corresponding to localized patches of calcification.

development of calcium and where calcification, if visibly present, is at a very early stage, present a similar gross morphological appearance to the calcification on the valves tested *in vitro* where calcification was clearly evident.

It is apparent that calcification occurs in the absence of proteins present in the fluid medium or other host-related factors. The results obtained from testing in solution 1 indicate that calcification can be supported by the phosphate present in the valve material (in this case probably supplied by the fixation buffer). This suggests that a fixation buffer other than phosphate might be beneficial. Calcification occurred most reliably in valves tested in solution 3. It is likely that solutions 1 and 2 are poorer for this purpose partly because of the lack of buffering in solution: they have pH values in the region 5.0–6.0, thus making it more likely for calcium and phosphate to remain in solution than would be the case at higher pH. Solution 3 has, however, been selected for future testing in view of both its ability to calcify and its simplicity of composition.

The variability in calcium uptake by different valves is considerable (Table II), in spite of the fact that all the valves had similar treatment before testing. The valve variability encountered here would be adequate in itself to explain the variability experienced in valves implanted into patients [25–27]. Further study is in progress in our laboratory to investigate the relationship of calcification to valve trauma, stress distribution and collagen orientation, utilizing this *in vitro* test system, and to relate the processes occurring *in vitro* to those *in vivo*.

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