# *The Role of Glutaraldehyde-Induced Cross-links in Calcification of Bovine Pericardium Used in Cardiac Valve Bioprostheses*

GERSHON GOLOMB, PhD, FREDERICK J. SCHOEN, MD, PhD, MARY SUE SMITH, AB, JUDY LINDEN, AB, MARK DIXON, AB, and ROBERT J. LEVY, MD

*From the Department of Cardiology, Children's Hospital, Department of Pathology, Brigham and Women's Hospital, The Departments of Pediatrics and Pathology, Harvard Medical School, Boston, Massachusetts, and the Division of Pediatric Cardiology, Department of Pediatrics and Communicable Diseases, The University of Michigan Medical School, Ann Arbor, Michigan*

**Calcification is the principal cause of failure of tissue-derived cardiac valve replacements pretreated with glutaraldehyde (GLUT). The objective of this study was to determine the role of GLUT-induced cross-links in bovine pericardial tissue calcification. Various levels of $^3$H-GLUT incorporation were obtained by varying incubation pH, and protein modification was determined by amino acid analysis and resistance to collagenase digestion. Calcification of cross-linked tissue was studied using subdermal implants in rats. Low GLUT uptake (<150 nm/mg) resulted in minimal calcification ($Ca^{2+}$, 12.8 μg/mg) and stability (4% residual weight following digestion) due to a limited crosslinking (lysine + hydroxylysine = 26.1 residues/1000 amino acids [AA]). In contrast, higher GLUT uptake induced more cross-links (Lys + Hyl = 8.2 residues/1000 AA) and consequent higher stability (95% residual wt); such tissues calcified severely ($Ca^{2+}$, 93.5 μg/mg). Incorporation of GLUT two to three times beyond a critical level did not further enhance calcification. It is concluded that the amount of GLUT incorporated controls the extent of cross-links, which in turn directly determines tissue stability and calcification. (Am J Pathol 1987, 127:122–130)**

GLUTARALDEHYDE (GLUT) has been effectively used to stabilize porcine aortic valves or bovine pericardium[1–3] used in the fabrication of clinical heart valve bioprostheses. Although these devices have low thrombogenicity and hemodynamic advantages relative to contemporary mechanical prostheses, they frequently fail because of tears or stiffening resulting from calcification.[2–8] Primary tissue degeneration due to calcification necessitates reoperation or causes death in 20–25% of adult recipients of porcine aortic bioprostheses by 7–10 years after operation[2–5] and in over 50% of children after a comparable period.[4,6] Bovine pericardial bioprostheses also fail frequently because of calcification.[7,8]

Previous studies showed that bovine pericardial tissue and porcine aortic valves pretreated with GLUT, as well as GLUT-fixed Type I collagen, mineralize when implanted subdermally in rats, but fresh (unfixed) implants undergo inflammatory attack and partial digestion without mineralization.[9,10] Thus, it is likely that GLUT incorporation and/or the cross-links introduced upon pretreatment are a prerequisite for mineralization of implanted bioprosthetic tissue. The biochemical reactions of GLUT with tissue proteins are complex and incompletely understood.[11–18] Although it is known that GLUT-protein reactions involve mainly lysinyl residues via Schiff base formation and/or substituted pyridinium salt cross-links,[14–16,19] the relationship between the amount of GLUT incorporated and the resulting cross-links and subsequent calcification is essentially unknown.

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Address reprint requests to Robert J. Levy, MD, C. S. Mott Children's Hospital, University of Michigan Medical Center, Ann Arbor, MI 48109-0204.

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Thus, the objectives of this study were to determine the relationships among GLUT incorporation in treated bioprosthetic tissues, resultant extent of protein cross-linking and tissue stability, and ultimate implant calcification.

## Materials and Methods

In brief, $^3$H-labeled GLUT was incorporated into bovine pericardial tissue (BPT). Protein modification resulting from GLUT pretreatment was determined by amino acid analysis of lysine and hydroxylysine (Lys + HyL) content and resistance to proteolytic (collagenase) activity. Various levels of GLUT incorporation into bovine pericardium were obtained by varying incubation pH, and the availability of BPT functional groups to react with GLUT was studied by examining the diffusion characteristics of the reagent across the tissue. The effect of GLUT incorporation on calcification was determined by chemical and histologic analysis of subdermal implants in rats removed after 21 days.

### Glutaraldehyde Tissue Interaction

*Pericardium*

Fresh parietal pericardium was obtained at slaughter from 2–3-week-old calves and immediately placed in iced sterile saline for not more than half an hour. After dissection of superficial fat from the external surfaces, pieces 1 × 1 cm were cut and placed in GLUT.

*Glutaraldehyde Incorporation*

1,5-Tritium-labeled glutaraldehyde was prepared in a custom synthesis by New England Nuclear (Boston, Mass) as previously described.[19] Twenty pieces of BPT were incubated in 20 ml of tritium-labeled glutaraldehyde (16.9 $\mu$Ci/mmol), 0.2% (diluted from 8% glutaraldehyde). Three different pH solutions (each containing 0.2% $^3$H-GLUT) were used: 1) pH 3.0, 0.05 M sodium acetate (acidic), 2) pH 7.4, 0.05 M HEPES (physiologic), and 3) pH 9.5, 0.05 M sodium borate (basic). Solutions were adjusted to 0.15 M with NaCl. BPT was incubated for 1 day at 2 C, rinsed with sterile saline, and incubated again in either fresh, the same, or a different pH solution containing 0.2% $^3$H-GLUT. After incubation for 2 or 7 days, pieces were rinsed free of GLUT with sterile saline, and the GLUT reaction products were reduced by incubation for 24 hours in 0.1 M $NaBH_4$ (in pH 7.4, 0.05 M HEPES). Specimens were subsequently exhaustively washed in saline free of unbound GLUT until background radioactivity levels were obtained on the washings. They were then dried, weighed, solubilized with Protosol (New England Nuclear), and combined with Econofluor (New England Nuclear) for $^3$H radioactivity in an Intertechnique SL32 liquid scintillation counter (IN/US Service Corp., Fairfield, NJ) as previously described.[19] GLUT content was calculated from a series of identically quenched specimens and standard samples containing known amounts of $^3$H-GLUT. The amount incorporated is expressed as the mean; SEM was within 10% of the mean.

### Tissue Cross-links and Stability

Complete amino acid (AA) analyses were done on 6N HCl hydrolysates of freeze-dried tissue,[19] sealed in vacuum, with the use of a Beckman-Spinco 121M automated amino acid (AA) analyzer (Beckman Instruments, Berkeley, Calif). Sensitivity was 0.5 nm, with reproducibility within 7%.

Collagenase digestion of BPT was carried out in HEPES pH 7.4 buffer containing 0.1 M clostridial collagenase (Sigma Chemical Co., St. Louis, Mo) at 37 C for 24 hours. The residual amount was dried, weighed, and expressed as a percentage (wt/wt) of initial weight.

### Glutaraldehyde Diffusion

The diffusion of GLUT across BPT was studied with diffusion chambers constructed as described previously[20,21] having a volume of 1 ml in each side. BPT washed as above was mounted between the two cells after measuring the thickness with a micrometer. The donor cell contained 0.2% $^3$H-GLUT in either acidic, physiologic, or basic buffer described above. The acceptor cell contained the same solution but without GLUT. The diffusion chambers were placed on a shaker at 150 rpm at ambient temperature. At each time point the acceptor cell was evacuated for counting (Atomlight, New England Nuclear), rinsed with double distilled water, and replenished with fresh buffer solution for continuation of the experiment.

### Calcification Studies

*Surgical Procedures*

Two subdermal pouches separated by at least 2 cm were dissected in the abdominal walls of ether-anesthetized rats (CD strain, 3-week-old male weanlings, Charles River Laboratories, Burlington, Mass). A piece of 1 × 1-cm BPT was implanted in each pocket. The wounds were closed with surgical staples. The rats were fed Lab Chow (Ralston Purina Company, St. Louis, Mo). The rats were sacrificed 21 days after implantation by an intraperitoneal overdose injec-

tion of pentobarbital, and the implants were retrieved. Blood sampling was done by cardiac puncture at sacrifice.

*Mineral Analysis*

The amount of calcium in the retrieved BPT was determined by atomic absorption spectroscopy of acid hydrolysates as previously described.[9]

*Morphologic Analysis*

The procedure for morphologic analysis of retrieved BPT is detailed elsewhere.[22] In brief, portions of retrieved cusps were immediately fixed in 0.1 M cacodylate-buffered 2.5% glutaraldehyde/2% formaldehyde (pH 7.4). Sections of glycomethacrylate-embedded tissue (3 $\mu$ thick) were examined by light microscopy after staining with hematoxylin and eosin (overall morphology) and the von Kossa reagent (for calcium phosphate).

## Results

### Modification of BPT by GLUT

Incorporation of GLUT into BPT was highly dependent on the pH of incubation (Table 1). In acidic GLUT mean uptake was 65 nm/mg tissue, whereas in physiologic and basic solutions, the amount incorporated was 295 and 418 nm/mg tissue, respectively. Relatively short exposure (1 day) of BPT to physiologic or basic GLUT solutions (after 1 day acidic incubation) resulted in essentially the same amount of incorporation as after 2 days (Table 1). Furthermore, the amount incorporated by the acidic treatment was not increased even after 7 days. However, only physiologic and basic pH treatments rendered the BPT relatively resistant to collagenases, and Hyl and Lys were markedly diminished (Table 1). These groups (physiologic and basic) had a high degree of resistance to collagenase digestion (mean residual weight, 94.7%), compared with the acidic groups, which were modified only slightly (Table 1) and consequently were almost entirely digested by collagenase (mean residual weight, 4%).

GLUT incorporation at pH 7.4 was also dependent on the weight ratio of GLUT in the incubation medium to tissue (Figure 1). As seen in Figure 1, the reagent-to-tissue ratio required for maximal GLUT incorporation was about 0.2 to 0.4 (wt/wt), with higher ratios resulting in comparable GLUT incorporation, and similar tissue modification as determined by Lys + HyL residues and tissue stability. In the low GLUT/tissue ratio groups, GLUT incorporation did not increase following extended incubation (7 days, data not shown), and these tissues were uniformly digested by collagenase.

### Glutaraldehyde Diffusion

GLUT diffused rapidly across fixed and fresh tissue in solutions of various pH (Figure 2). Throughout the entire pH range studied, 40% of the donor chamber GLUT was transported after 150–300 minutes. There was a trend toward faster transport through fresh, rather than fixed, tissue at all pH's studied, with the fastest diffusion rates at pH 7.4.

### Tissue Calcification

Mineralization of implanted tissue was highly dependent on tissue stability and extent of cross-links as induced by a critical level of glutaraldehyde incorporation (Figure 3). The data may be divided into two separate groups, A and B. BPT in the first group (A) had less than 100 nm/mg GLUT uptake, calcified to $12.8 \pm 3.2$ $\mu$g/mg, and were unstable when exposed to collagenase. In contrast, group B had a higher uptake of GLUT (>150 nm/mg), calcified significantly more ($93.5 \pm 12.1$ $\mu$g/mg), and was resistant to collagenase

Table 1—Glutaraldehyde Incorporation in Bovine Pericardium—Tissue Cross-links and Collagenase Stability

| Pretreatment | | Glutaraldehyde incorporation ($10^{-9}$ mol/mg tissue) | Lys + Hyl (residues/1000AA) | Residual undigested tissue (% wt/wt) |
|---|---|---|---|---|
| pH | days | | | |
| 3.0 | 2 | 70 ± 6 | 27.6 | 3 |
| 3.0 | 7 | 60 ± 5 | 24.5 | 5 |
| 7.4 | 2 | 318 ± 7 | 9.8 | 98 |
| 3.0 then 7.4 | 2 | 280 ± 14 | 8.3 | 88 |
| 9.5 | 2 | 420 ± 25 | 7.2 | 98 |
| 3.0 then 9.5 | 2 | 413 ± 10 | 7.8 | 95 |
| Type I collagen* | — | — | 30–35 | 0 |
| Fresh Tissue† | — | — | 41 | 0 |

*Value from Miller and Gay.[44]
†Value from Schoen et al.[19]

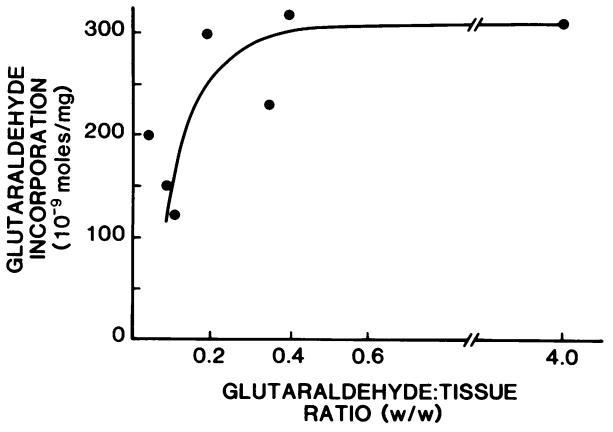


**Figure 1**—The amount of glutaraldehyde incorporated in bovine pericardial tissue as a function of the ratio between the amount of glutaraldehyde in the treatment solution and tissue weight.

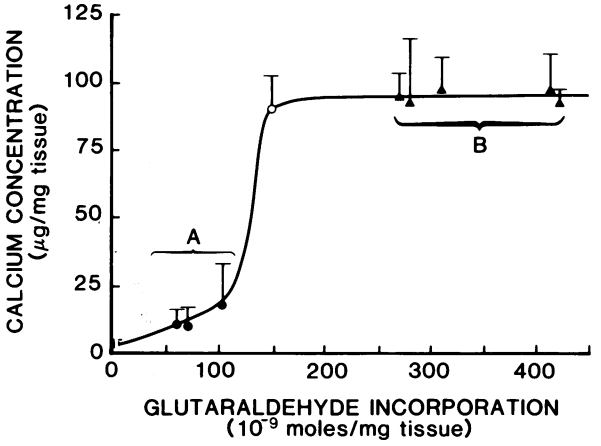


**Figure 3**—Calcification of glutaraldehyde-treated bovine pericardial tissue after 21 days of a subdermal implant in rats as a function of glutaraldehyde incorporation. Treatment solutions' pH: ●, 3.0; ▲, 7.4 and 9.5 incubated for 2 or 7 days (see Table 1); ○, 7.4 (24-hour incubation, modified in part from Schoen et al.[19]) The ● symbol with no glutaraldehyde uptake was fresh tissue (not treated with glutaraldehyde). Each point represents the mean of $Ca^{2+}$ concentration (n = 10) and standard error of the mean (*bars*). Specimens in Group A exhibited both a low degree of cross-linking and stability, but specimens in Group B exhibited both a high degree of cross-linking and tissue stability (see Table 1).

digestion. Groups A and B each had various levels of GLUT incorporation. Nevertheless, calcification levels were insignificantly different within each group (Table 1). The BPT calcification was most strongly associated with the extent of cross-links induced (see Table 1). Group A tissues (with a mean of 65 nm/mg GLUT uptaken) were only slightly modified (Lys + Hyl = 26.1 residues) and calcified less, in comparison with group B tissues (with >150 nm/mg GLUT), which were markedly more modified (Lys + Hyl = 8.2 residues) and yielded significantly greater calcification. Calcification levels of explanted BPT pretreated with sodium borohydride ($Ca^{2+}$, 118.7 ± 10.4 μg/mg) were not significantly different from control (not reduced) tissue levels ($Ca^{2+}$, 93.5 ± 12.1 μg/mg).

Histologic observations on BPT explants demonstrated a wide spectrum of extent of mineralization and confirmed the chemical analyses (Figure 4). Specimens with low GLUT incorporation developed no mineralization after implantation, but had peripheral inflammation with early organization and central degeneration with homogenization of tissue structure, consistent with autolysis (Figure 4B and C). In contrast, specimens pretreated to obtain a high degree of GLUT incorporation yielded diffuse calcification with markedly diminished host cell reaction (Figure 4D and E).

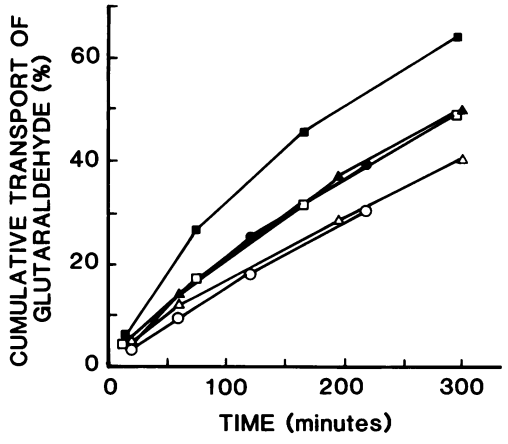


**Figure 2**—Glutaraldehyde diffusion across fixed (pH 7.4) and fresh bovine pericardial tissue in solutions of various pH. Full symbols, fresh tissue; empty symbols, fixed tissue. pH: ○, ●, 9.5; □, ■, 7.4; △, ▲, 3.0.

## Discussion

The primary result of this study is that GLUT potentiates severe calcification of BPT implants, but only when a sufficient level of cross-linking/material stabilization has occurred. A critical level of glutaraldehyde incorporation was required to induce maximal cross-links and to confer material stability, and only when this level was reached did severe implant mineralization occur. Nevertheless, incorporation of GLUT two to three times beyond this critical level did not further enhance calcification. These results suggest that glutaraldehyde modification of BPT structural components is central to the mechanisms of BPT collagen calcification and may contribute to the stabilization of devitalized cellular structures, leading to their calcification as well. The glutaraldehyde molecule itself and its stoichiometric incorporation may only be significant as determinants of a critical level of material cross-linkages and stability.

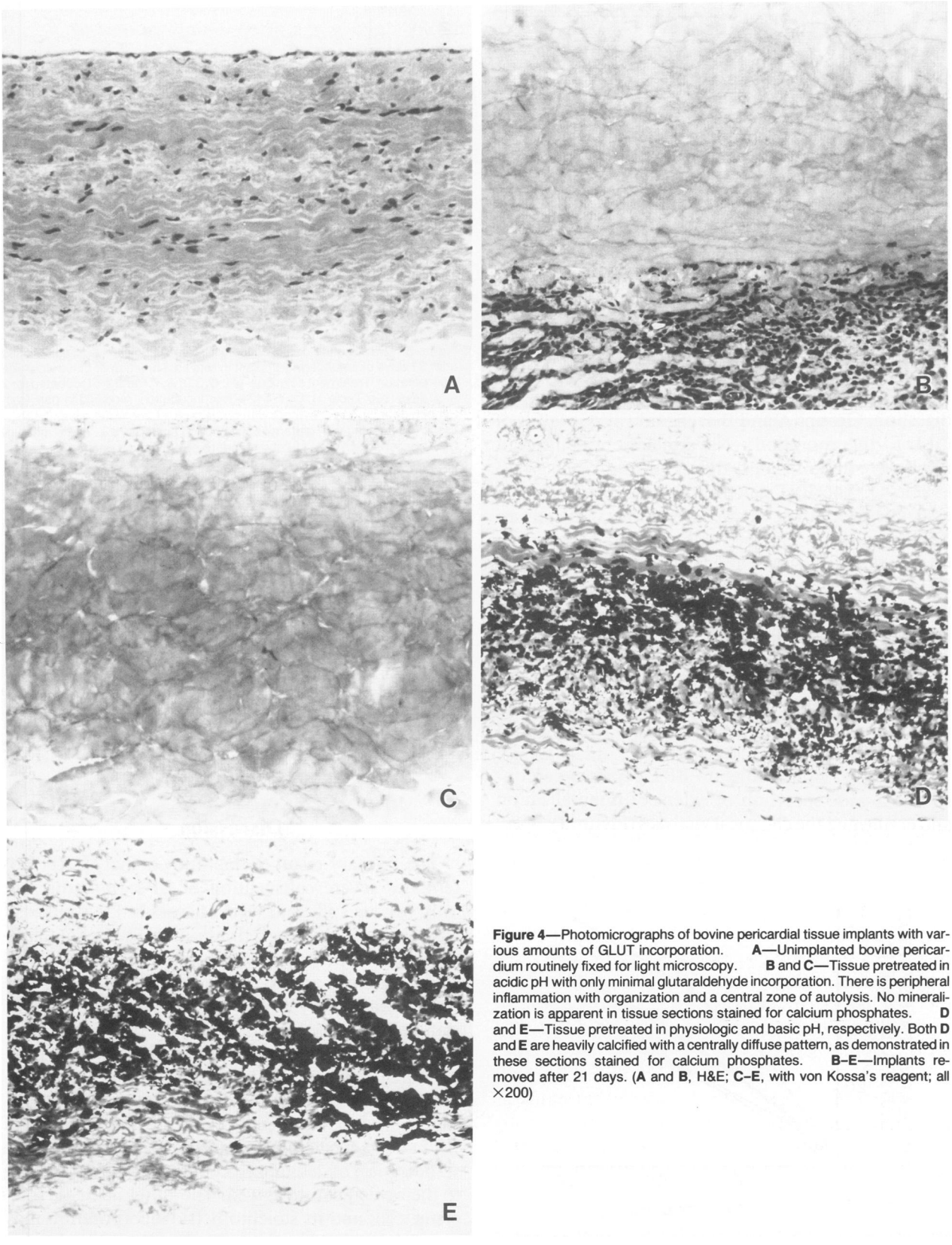


**Figure 4**—Photomicrographs of bovine pericardial tissue implants with various amounts of GLUT incorporation. **A**—Unimplanted bovine pericardium routinely fixed for light microscopy. **B** and **C**—Tissue pretreated in acidic pH with only minimal glutaraldehyde incorporation. There is peripheral inflammation with organization and a central zone of autolysis. No mineralization is apparent in tissue sections stained for calcium phosphates. **D** and **E**—Tissue pretreated in physiologic and basic pH, respectively. Both **D** and **E** are heavily calcified with a centrally diffuse pattern, as demonstrated in these sections stained for calcium phosphates. **B–E**—Implants removed after 21 days. (**A** and **B**, H&E; **C–E**, with von Kossa's reagent; all ×200)

### Glutaraldehyde Incorporation and Tissue Cross-links

The amount of GLUT incorporated into BPT was dependent on the pretreatment conditions (Table 1). In acidic solutions the amount of GLUT incorporated was approximately one-fifth of the levels found in the physiologic or the basic incubations. This strong pH dependency of incorporation agreed with the results of Bowes and Cater,[23] who showed that GLUT tanned kangaroo tail tendons at a faster rate at high pH levels. This may be explainable by the reaction rate, which is determined by the available concentration of R-$NH_2$ with an unshared pair of electrons.[13] If the unshared pair of electrons on the nitrogen of R-$NH_2$ is protonated (in acidic pH), it cannot then attack the carbon of the aldehyde carboxyl group. On the other hand, protonation of the carboxyl group should enhance its reactivity toward nucleophilic agents. The favorable combination of reactants is the protonated carboxyl and lone pair electrons on the aminic nitrogen obtained in neutral to basic pH range. In addition, when GLUT reacts with a solution of amino acids or proteins the solution slowly becomes more acidic.[24] The reaction of GLUT with free amino groups causes the following equilibrium to be shifted to the right:

$$\text{Protein} - NH_3 + \rightleftharpoons \text{protein} - NH_2 + H+$$

Hence, neutral to basic pH will enhance this reaction.[25,26]

GLUT pretreatment of connective tissue biomaterials (porcine aortic valves and bovine pericardium) as well as purified Type I collagen affects primarily the $\epsilon$-amino groups of lysinyl residues.[12,18,19,27] This was confirmed in the present study, because relatively few cross-links were formed in the acidic incubations, but in the physiologic and basic pH GLUT incubations only the amount of Lys + Hyl residues was markedly diminished. The level of Lys + Hyl modification was essentially the same in all the nonacidic groups (mean, 8.2 $\pm$ 0.5 residues/1000 AA), indicating maximum cross-linkages. This is in agreement with results of other studies.[12,23,28] In addition, the resistance of the treated BPT to collagenase activity was associated with the extent of cross-links. Cross-linked tissues exhibited a high degree of resistance to digestion (mean residual weight, 94.7%), but partially cross-linked tissue (Lys + Hyl, 26.1 residues/1000 AA) was digested almost entirely (mean residual weight, 4%). These data suggest that relatively few cross-links were formed at low incorporation of GLUT and thus the tissue was susceptible to collagenase digestion; however, higher levels of incorporation of GLUT (280–420 nm/mg) introduced uniformly high levels of cross-links and resistance to proteolytic action.

The permeability of the BPT to GLUT was evaluated with diffusion studies. Although GLUT diffused rapidly across both fixed and fresh tissue at each pH studied, there was a tendency for faster transport through fresh, rather than fixed, tissue. This tendency of slower penetration through fixed tissue may be explainable by the observation of firmer and fuller texture of tanned tissue[25,29] due to inter- and intramolecular cross-links. In addition, the reaction of GLUT with the unfixed tissue could serve as a driving force for enhanced GLUT diffusion. Although GLUT autopolymerizes rapidly at basic pH, yielding polyunsaturated aldehyde oligomers,[12,23,27,30,31] no retarded transport occurred in pH 9.5 in comparison with pH 3 (Figure 1). These results suggest that although cross-linked BPT was less permeable than fresh tissue, it is porous enough to allow free permeation of even higher molecular weight species of GLUT. These data indicate that the interstitium of the tissue is readily accessible for cross-linking by GLUT. Cheung et al[18] proposed that interstitial cross-links may be affected by steric hindrance caused by initial cross-linking or nucleation sites to which further GLUT molecules may attach. However, our diffusion results, as well as the observed maximal degree of cross-links obtained after only 2 days in neutral and basic pH do not support their hypothesis. Nevertheless, it is conceivable that the existence of lysinyl residues with different reactivity may affect the degree of modification, because about 20–30% of the lysinyl residues did not react even when a high ratio of GLUT to tissue was used.[17,28]

In a previous study an intermediate level of GLUT incorporation (150 nm/mg, Schoen et al,[19] Figure 2) was obtained by using a different ratio of GLUT to tissue weight in the tanning solution. The present study demonstrated a direct relationship between the amount of unreacted GLUT in solution and the amount that has combined with the protein; this result was in agreement with other studies.[11,12,17,30,32] Above a ratio of about 0.4 wt/wt (GLUT/tissue) the amount of GLUT incorporated reached a plateau of approximately 300 nm/mg at physiologic pH, with a maximal and constant degree of Lys and HyL modification. Furthermore, the amount of GLUT incorporated and the extent of cross-links introduced in the tissue remained constant even after incubations of 7 days at acidic or physiologic pH. This observation is in agreement with results of other studies.[19,28] Our quantitative finding is of importance in the preparation of bioprostheses for clinical use, because incom-

plete cross-linking can lead to poor mechanical properties and biodegradation.

Although high incorporation of GLUT was obtained with the high pH treatment (>400 nm/mg, Table 1), the same extent of cross-links (8.2 ± 0.5 Lys + Hyl residues/1000 AA) was introduced at pH 7.4, although with less GLUT incorporation (mean, 295 nm/mg). This excess amount of GLUT incorporated in the basic incubation was probably in the form of polymeric GLUT, which underwent rapid polymerization at high pH levels.[12,23,27,30,31] We observed a brownish discoloration of the tissues treated with basic GLUT solution. This color was darker than the yellowish-amber and whitish color of BPT treated in physiologic and acidic GLUT solutions, respectively. Upon reduction with sodium borohydride, all BPT turned white. A similar observation was reported by Hardy et al,[14] Hopwood et al,[13] and Korn et al,[27] who examined the interaction between amino groups and GLUT. Indeed, Hardy et al[14] proposed that GLUT reaction with primary amino groups of proteins is followed by condensation of additional free GLUT and leads to the formation of a 1,3,4,5-substituted pyridinium salt, which could be subject to hydride reduction. Our results suggest that these unsaturated chromophoric products did not affect either tissue stability or calcification (see below), because borohydride-reduced and nonreduced BPT had similar properties.

### Effect of GLUT-Induced Cross-links on Calcification

After subdermal implantation in rats for 21 days, BPT with less than 100 nm/mg of GLUT incorporated calcified significantly less than those with more than 295 nm/mg. Our previous study showed extensive calcification with 150 nm/mg GLUT uptake.[19] Calcification might be due to either the incorporated GLUT and/or to the modification of the tissue by the introduction of cross-links. In this study, fresh (nontreated) BPT did not calcify (Figure 3). Furthermore, our previous studies have demonstrated that both nontreated porcine aortic valves and Type I collagen sponges do not calcify.[9,10] Nevertheless, despite the wide range of GLUT incorporation that was present in the highly cross-linked tissues (280 to 413 nm/mg), calcification levels within this group were not significantly different. Thus, the degree of cross-links induced by GLUT, rather than the specific amount of GLUT incorporated, determines both the extent of calcification as well as tissue stability.

The molecular nature of the products formed upon the reaction of GLUT with protein has not been completely determined. It is likely that the chromophores or residual aldehydes formed in the tissue from the reaction with GLUT are not playing a role in calcification, because BPT calcification occurred at the same levels despite hydride reduction.

### Mechanism of Calcification and Implications for Prevention

The mechanism by which collagen cross-linking with GLUT facilitates implant calcification is not known. Recent studies demonstrate that initial mineral deposits *in situ* are localized to residual porcine aortic valvular and bovine pericardial connective tissue cells.[19,33] In these degenerative cells modified by GLUT reaction, calcium entry probably occurs passively and unimpeded, but the mechanisms for calcium removal are no longer active.[19] This phenomenon of subcellular and cellular degradation products serving as a nidus of calcification[34–36] is somewhat analogous to mitochondrial calcification, which occurs when cardiac myocytes irreversibly damaged by severe ischemia are reperfused[37] and might explain the calcification of tissue valves fabricated from fascia lata[38] or dura mater,[39] which contain cellular degradation products. However, mineral deposits in bioprosthetic heart valve implants also involve collagen fibrils.[19,33,40,41] Furthermore, acellular Type I collagen (the most abundant protein in both BPT and porcine aortic valve cusps[19] pretreated with either GLUT or formaldehyde undergoes calcification.[10] Similar mechanisms are also thought to contribute to physiologic and other forms of pathologic mineralization.[35,42] Thus, it is likely that GLUT not only affects BPT cells but also exposes other affinity sites in the tissue for hydroxyapatite nucleation.

This study suggests that strategies to prevent BPT calcification by reducing GLUT incorporation will not be fruitful because BPT with low levels (<150 nm/mg) of GLUT had poor stability, as evidenced by their susceptibility to collagenase digestion. Moreover, calcification levels in the highly cross-linked tissue were not significantly different despite a wide range of GLUT incorporation.

One possible approach to prevention of bioprosthetic heart valve calcification might be avoiding the use of GLUT, in view of the present results, and substituting another reagent. However, the important advantages of GLUT, its water-solubility, its reacting at physiologic pH, and its stability of its reaction products, would strongly favor its continued use. Another possible prevention method could be the binding of anticalcification agents to the tissue and/or their release from a controlled-release drug delivery system directly to the cusps.[20,43]

It is concluded that a critical level of glutaraldehyde

incorporation into BPT tissue confers a potential for severe implant calcification. The mechanism of this potentiation of calcification is most closely associated with the cross-linkages and tissue stability resulting from the glutaraldehyde–tissue chemical reactions, rather than the quantitative amount of glutaraldehyde actually taken up. The amount of GLUT incorporated determines the extent of cross-links with constant extent of modification above 150 nm GLUT/mg tissue. The degree of cross-links induced by GLUT treatment determines both the tissue stability to collagenase digestion and the extent of calcification.

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