

## Glutaraldehyde purity and stability: implications for preparation, storage, and use as a pulpotomy agent

Don M. Ranly, DDS, PhD

### Abstract

*Glutaraldehyde can be prepared and stored for use as a pulpotomy agent in a variety of ways. This study was designed to determine the most effective and practical procedures in the clinical setting. The purity and efficacy of several glutaraldehyde solutions were analyzed before and after six months of storage. Commercial glutaraldehyde (25%) contained considerable organic impurities, but solutions prepared from it proved more effective at fixing protein than the solutions prepared from pure glutaraldehyde. Comparison of refrigerated and room temperature storage of dilute buffered and unbuffered solutions demonstrated that the buffered, unrefrigerated preparations developed organic impurities, some of which were different chromatographically from those seen in stock solution. In addition, fixation of pulp protein by the aged buffered preparations was diminished, as determined by measurements of residual enzyme activity. The results suggest that while some of the impurities in stock glutaraldehyde solutions are polymers which enhance fixation, some that appear in buffered solutions stored at room temperature are converted species which have lost their cross-linking properties.*

The search for a substitute for formocresol (FC) had been prompted by a series of negative reports questioning both its local tissue effects<sup>1-4</sup> and its extrapulpal toxicity.<sup>5,6</sup> Recently, glutaraldehyde (GA), a standard fixative for electron microscopy,<sup>7</sup> has been recommended<sup>8,9</sup> and tested<sup>10,11</sup> as an alternative pulpotomy medicament for deciduous teeth. Its efficacy as a protein cross-linking agent has been demonstrated with bovine pulp,<sup>12-14</sup> and several clinical studies are ongoing<sup>15,16</sup> or completed.<sup>11</sup> Therefore, it is likely that the use of GA as a pulpotomy agent will become more widespread. Since users of GA for histologic fixation have noted that purified solutions of GA are unstable, resulting in a variable composition of stock solutions,<sup>17,18</sup> this study was initiated to determine if these concerns might have clinical implications. Specifically, this report will deal with the effects of preparation and storage on the accumulation of chemical

impurities in GA solutions and their impact on the fixation of bovine pulp.

### Methods and Materials

#### Preparation and Storage of Solutions

An aliquot of a 25% stock solution of commercial glutaraldehyde (pH 2.9)<sup>a</sup> was vacuum distilled at 80°C to obtain the pure dialdehyde. The distillate was diluted immediately with either distilled water to make 2 and 5% unbuffered solutions or with 0.1 M phosphate buffer (pH 7.2) to make 2 and 5% buffered solutions. The initial pH of the freshly made unbuffered preparations was 5.8. An aliquot of each was refrigerated at 4°C; another aliquot of each was stored at room temperature (25°C).

#### Analysis of Impurities

Immediately after preparation and monthly for six months the aliquots were analyzed spectrophotometrically for the presence of impurities, determined by an increase in the 235/280 nm absorbance ratio (impurity index). This index was chosen because it has been reported that the dialdehyde is responsible for absorption at 280 nm and extraneous material for the absorption at 235 nm.<sup>19,20</sup> Thus, in this study an increase of the ratio 235/280 nm denotes a conversion of glutaraldehyde into contaminating molecules or polymeric forms of GA.

#### Cross-Linking Assay

The ability of various preparations of GA to fix pulps was determined by comparing the degree of cross linking following exposure of pulp tissue to each of the solutions. The procedure for the cross linking followed the protocol of Hassel and Hand.<sup>21</sup> After thawing and mincing frozen samples of calf molar pulp, weighed aliquots (~0.5 g) were placed in vials containing 10 ml of 0.1 M phosphate buffer or one of two preparations of 2% buffered GA, prepared either from purified distillate or a stock solution with demonstrated impurities. The samples were agitated on a

<sup>a</sup> Aldrich Chemical Co., Milwaukee, WI.

reciprocal shaker for four hours at room temperature, after which any extracted protein was separated by filtration. Protein in the filtrate (extractable fraction) was precipitated with 10% trichloroacetic acid (TCA) and washed several times with 5% TCA. The final precipitates were solubilized in 1 NaOH at 60°C overnight and assayed for total protein. The filtered residue from the original treated samples was rinsed and homogenized in distilled water using a hand homogenizer and then centrifuged at  $750 \times g$  at 4°C for 20 minutes. The supernatant (soluble fraction) and the remaining pellet (insoluble fraction) were treated as described above. The amount of protein in the fractions was determined by the Bradford assay<sup>22</sup> using bovine serum albumin (BSA) as a standard, and the mean  $\pm$  S.D. was calculated for five samples at each condition.

### Enzyme Assay

Lactic dehydrogenase activity was determined as a measure of tissue viability following fixation. The procedure for the assay of lactate dehydrogenase activity was modified from a previous study.<sup>12</sup> In initial experiments comparing enzyme inactivation by stock and pure preparations, pulp samples were incubated for one hour in 0.2% buffered GA solutions diluted from either stock solution or fresh distillate. Phosphate buffer (0.1 M, pH 7.2) served as the control solution. The residual fixative property of stored GA was determined by treating pulp samples with the 2% preparations that had been stored for six months. In this latter experiment, buffer, 2% buffered and unbuffered glutaraldehyde (stock), and 2% buffered and unbuffered glutaraldehyde (distillate) served as control solutions. After treatment, each sample was washed and homogenized<sup>b</sup> for 1 minute in 10 ml of 0.2M Tris HCl, pH 7.3. Following centrifugation<sup>c</sup> for 10 minutes at 300 rpm the supernatant was decanted. Fifty microliters of the supernate was added to a cuvette containing 2.8 ml of Tris HCl, 1.0 ml of 6.6 mM NaOH, and 0.1 ml of 30 mM sodium pyruvate, then vortexed for 10 seconds. Change in absorbance per minute was measured with a spectrophotometer at 345 nm at room temperature. The activities were calculated as units/mg of wet weight, and a mean  $\pm$  S.D. was calculated for five samples in each group.

### Chromatographic Analysis

The presence of chemical impurities was determined by comparison of thin layer chromatograms of each of the test solutions with freshly distilled GA. Thin layer chromatograms were prepared by application of distilled GA, stock GA, or stored GA solu-

tions to chromatography plates.<sup>d</sup> The mobile phase consisted of benzene: methanol 4:1, v/v. Spots were visualized following oxidation with 50% sulphuric acid and heat.

### Results

The effects of preparation and storage are shown in Figure 1 where the ratios of the absorbance readings at 235 and 280 nm are plotted. The presence of impurities appears to be segregated into three distinct groups. All of the unbuffered preparations, regardless of GA concentration or storage temperature, demonstrated little or no change in their absorbance ratios. The pH of these solutions ranged from 6.3 to 6.6 at the end of six months. In contrast, buffering the solutions promoted an increase in the 235 nm absorbing molecules, in some cases dramatically. It also is apparent that refrigeration slowed the process. The buffered 2 and 5% preparations stored at 4°C exhibited an increase in their impurity index to 2.1 and 2.9, respectively. When left at room temperature the buffered 2% GA reached a ratio of 28.2 and the buffered 5%, 12.7. In both instances the peak values began to fall and their respective ratios at six months were 17 and 5.2. The final pH of these solutions was 7.9 for the 2% preparation and 7.7 for the 5%.

To ascertain whether the accumulation of polymeric forms in GA can enhance its ability to react

<sup>d</sup> EM Science, Cincinnati, OH.

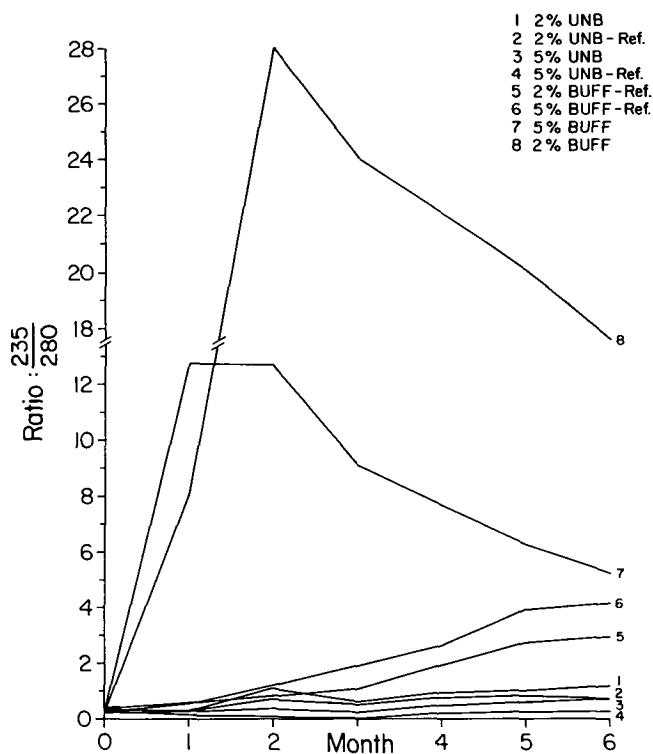


FIGURE 1. Plot of the ratios of the absorbance at 235 and 290 nm, recorded from eight glutaraldehyde preparations at monthly intervals for six months.

<sup>b</sup> Tekmar Tissumizer, Tekmar Co., Cincinnati, OH.

<sup>c</sup> IEC clinical centrifuge, International Equipment Co., Needham, MA.

with protein, bovine pulp was treated with either stock or pure GA preparations and then analyzed for the status of the cross linking and enzyme suppression. The cross-linking capacity of stock and purified GA is compared in Figure 2. During the four-hour incubation, 68% of the protein was extracted from the control pulp. The remaining protein was divided almost evenly between the soluble and insoluble fractions. The GA preparations significantly modified the distribution of these fractions. Distilled GA increased the insoluble protein to almost half (49%) of the total while substantially reducing the extractable fraction (26%). The solution prepared from a stock source had an even greater influence on the tissue. Not only was the percentage of the insoluble fraction increased (55%), but the extractable fraction was reduced by more than half when compared to the group treated by distilled GA.

The inhibition of lactic dehydrogenase by GA solutions prepared from stock or the distillate is depicted in Figure 3. When compared to the control tissue, the 0.2% solution of distilled GA reduced the enzyme activity 44%. In contrast, the solution prepared from the stock bottle inactivated the enzyme almost totally, leaving less than 2% of the former activity.

In order to determine if stored dilute GA develops polymeric forms or undergoes a degenerative process, the 2% preparations were analyzed for their impact

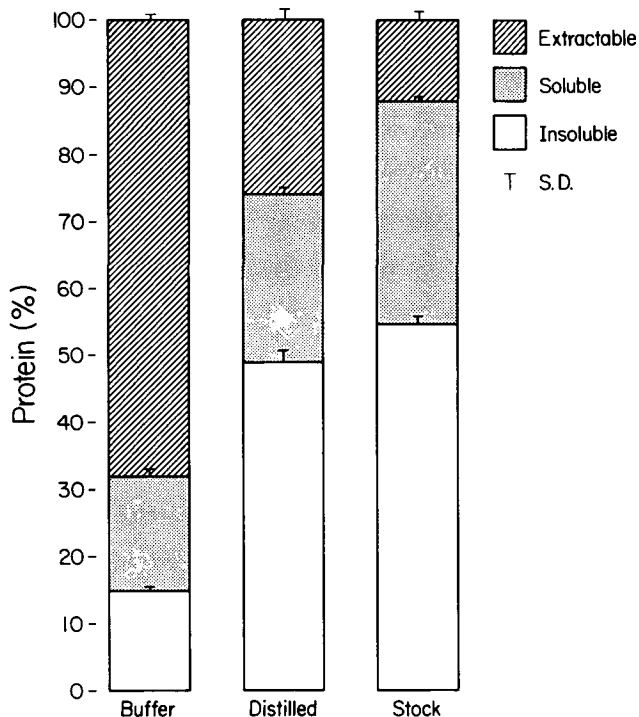


FIGURE 2. Comparison of the distribution of three protein fractions of bovine pulp following treatment with buffer or two glutaraldehyde preparations.

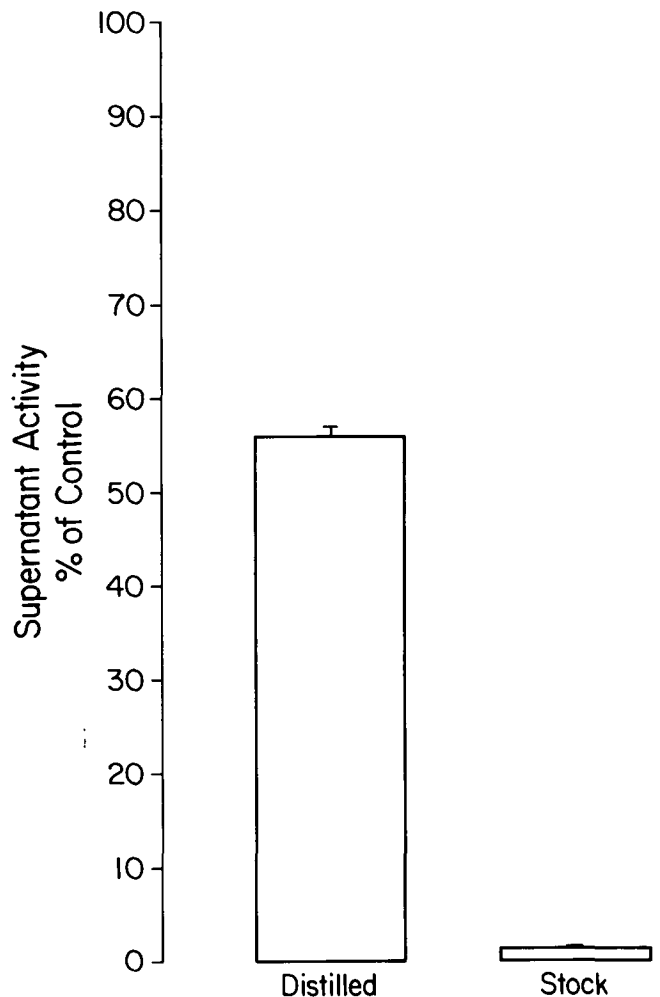


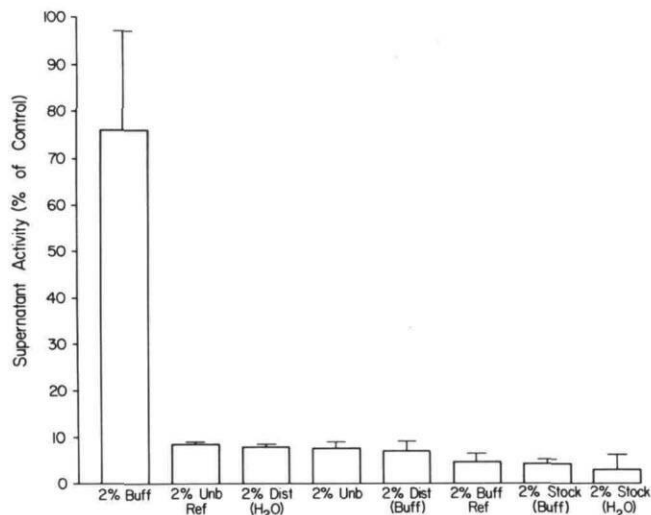
FIGURE 3. Comparison of residual lactate dehydrogenase activity of bovine pulp following treatment with either distilled or stock glutaraldehyde.

on lactic dehydrogenase activity. The results of the enzyme study comparing the activity of fresh and aged dilute solutions are presented in Figure 4. Only one of the preparations, 2% buffered and unrefrigerated GA, proved ineffective in inhibiting the activity of lactic dehydrogenase. Although there was some reduction in this group the assays were variable and not statistically different from the control. All of the other preparations significantly reduced the activity of the enzyme.

Chromatograms demonstrated the distillate to be a pure preparation (Figure 5). While the patterns of the stock solution and six-month buffered and unrefrigerated solutions were identical in many respects, the latter preparation exhibited new molecular species. The unbuffered, unrefrigerated solutions and all the refrigerated preparations underwent little or no change as demonstrated by chromatography.

## Discussion

The purity, efficacy, and stability of GA was evaluated by spectrophotometric and biochemical methods in order to develop principles concerning the

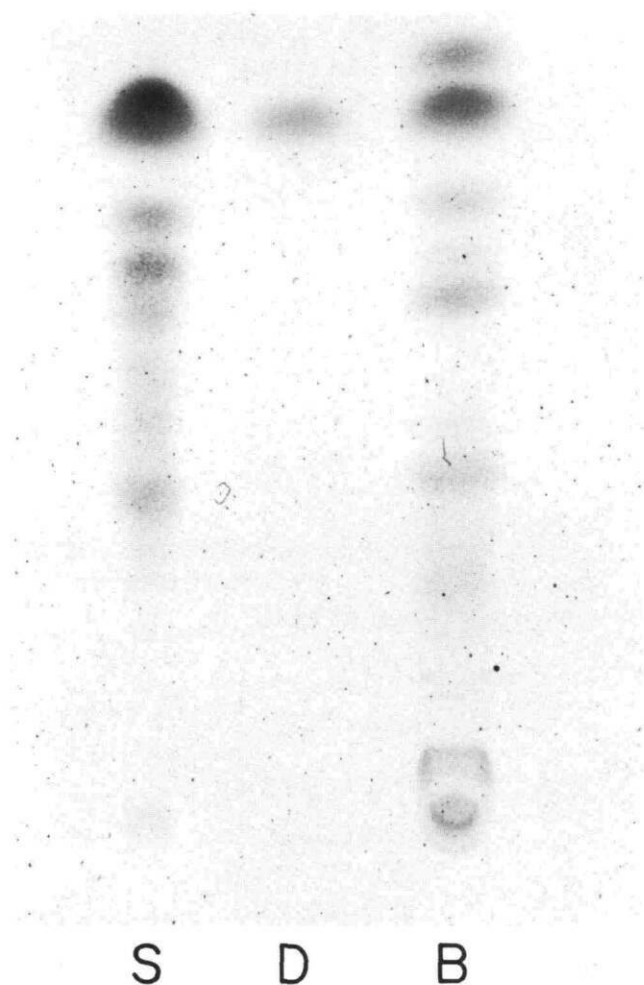


**FIGURE 4.** Comparison of residual lactate dehydrogenase activity of bovine pulp following treatment with four stored preparations (2% Buffered, 2% Unbuffered-Refrigerated, 2% Unbuffered, and 2% Buffered-Refrigerated) or with four fresh glutaraldehyde solutions.

preparation and storage of dilute solutions. Two and 5% dilutions were selected since both have been proposed as possible concentrations for clinical practice.<sup>9,10</sup> Because certain treatment settings might find the cold storage of a pulp medicament to be impractical, refrigerated and nonrefrigerated preparations were compared for the development of impurities and possible deterioration over a six-month period.

There is good reason to believe that the polymeric forms are responsible for the majority of the cross linking by GA.<sup>23</sup> These results substantiate the theory that the accumulation of polymeric forms in GA enhances its ability to cross link protein. Not only was the insoluble component of the treated bovine pulp increased following treatment with the stock preparation, but the extractable fraction was reduced substantially, indicating a rapid fixation. By the other criteria, enzyme inactivation, the stock preparation was again more effective. The activity of lactate dehydrogenase was destroyed almost totally by the treatment, whereas the tissue treated with the distillate demonstrated significant residual activity. The results of the enzyme assay agree with the study of Anderson,<sup>19</sup> in which the remaining activity of several enzymes of skeletal muscle were compared following treatment with glutaraldehyde of various impurities.

This study also demonstrated that the buffering of GA augments the accumulation of substances that absorb at 235 nm, particularly when the solution is stored at room temperature. The impurity that absorbs at 235 nm in a stock solution is likely a dimer or other polymer of GA.<sup>17,22</sup> However, the chromatographic evidence suggests the presence of converted molecular species besides polymerized GA.



**FIGURE 5.** Thin layer chromatogram of stock(S), distilled(D), and buffered (B) glutaraldehyde, the latter being stored at room temperature for six months.

Thus, dilute buffered GA stored at room temperature appears to manifest a variety of molecular forms which differ from those in the 25% stock solution.

Other proposed impurities in stock GA such as acrolein, glutaric acid, or glutaraldoxime have not been demonstrated. One possible impurity, glutaric acid semialdehyde, has not been examined. In this study the authors did not attempt to define the new molecular forms found in the preparations. Although no evidence exists, the initial rise and subsequent fall of the impurity index of the two buffered and unrefrigerated preparations might have represented an early polymerization followed by deterioration.

Despite the failure of several investigators to identify an extraneous chemical, concentrated stock GA obviously undergoes an oxidative process to form organic acids since the pH of these solutions is invariably acidic. Studies on the pK of GA suggest that a few molecules rapidly oxidize to the hemiacid of GA, and the acidic pH catalyzes polymerization.<sup>17</sup> The unbuffered preparations in this study did not acidify,

which might account for the reduction in polymer formation. Apparently, the dilution of pure GA minimizes the effects of oxidation.

Assays of residual enzyme activity in pulp treated with the preparations of stored dilute GA substantiated the chromatographic evidence that buffered GA at room temperature undergoes conversion into non-cross-linking molecular species. Whereas the buffered, unrefrigerated solution developed the highest impurity ratio, it also demonstrated the least enzyme suppression. These findings suggest that polymeric forms are not responsible for the increased absorbance in this preparation. Although new molecular species were demonstrated by chromatography, they were not effective in cross linking the enzyme. This observation is of some interest since an earlier study<sup>14</sup> showed that buffered GA was twice as effective in cross linking bovine pulp as a similar concentration prepared with distilled water. Although the clinical use of nonbuffered GA would fail to optimize its capacity for fixation, these findings suggest that unless buffered preparations can be stored cold, they will deteriorate in time.

## Summary and Conclusions

This study demonstrated that a concentrated stock solution of GA that typically might be used in the preparation of a pulpotomy agent contained a variety of molecules other than the GA monomer. Distillation purified the dialdehyde, but cross linking and enzyme assays revealed that preparations of the monomer were less effective than the solutions prepared from the commercial source. This study also demonstrated that buffered GA solutions prepared from distillate and stored at room temperature developed new molecular species. Unbuffered dilute preparations remained stable regardless of the temperature, but buffered dilute solutions benefited from cold storage. Unlike stock solutions containing polymers of GA which are effective in fixation, some of the impurities in the buffered, unrefrigerated aliquot probably were not cross-linking molecules. Previous studies have demonstrated that preparations using the pure dialdehyde are more effective when buffered, but these current findings suggest that buffered preparations must be stored in the cold to slow deterioration. If refrigeration is not practical, the use of unbuffered preparations at a slightly stronger concentration is recommended.

Dr. Ranly is a professor, Department of Pediatric Dentistry, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78284. Reprint requests should be sent to him.

The author would like to acknowledge the technical assistance of Ms. Diane Horn.

This study was supported by Biomedical Grant #RR-05654 from the National Institutes of Health, Bethesda, Maryland.

1. Rolling, I., Hasselgren, E., Tronstad, L. Morphologic and enzyme histochemical observations on the pulp of human primary molars three to five years after formocresol treatment. *Oral Surg* 42:518-28, 1976.
2. Magnusson, B.O. Therapeutic pulpotomies in primary molars with the formocresol technique: a clinical and histological followup. *Acta Odontol Scand* 36:157-65, 1978.
3. Block, R.M., Lewis, R.D., Sheaths, J.B., Fawley, J. Cell-mediated immune response to dog pulp tissue altered by formocresol within the root canal. *J Endod* 3:424-30, 1977.
4. Block, R.M., Lewis, R.D., Sheaths, J.B., Burke, S.G. Antibody formation to dog pulp tissue altered by formocresol within the root canal. *Oral Surg* 45:282-92, 1978.
5. Pruhs, R.J., Olen, G.A., Sharma, P.S. Relationship between formocresol pulpotomies on primary teeth and enamel defects on their permanent successors. *JADA* 94:698-700, 1977.
6. Myers, D.R., Pashley, D.H., Whitford, G.M., McKinney, R.V. Tissue changes induced by the absorption of formocresol from pulpotomy sites in dogs. *Pediatr Dent* 5:6-8, 1983.
7. Dawes, C.L. *Biological Techniques for Transmission and Scanning Electron Microscopy*. Burlington, VT; Ladd Research Industries, Inc., 1979, p 52.
8. s'Gravenmade, E.J. Some biochemical considerations of fixation in endodontics. *J Endod* 1:233-37, 1975.
9. Davis, M.J., Myers, R., Swetkes, M.D. Glutaraldehyde: an alternative to formocresol for vital pulp therapy. *J Dent Child* 49:176-80, 1982.
10. Kopel, H.M., Bernick, S., Zachrisson, E., DeRomero, S.A. The effects of glutaraldehyde on primary pulp tissue following coronal amputation: in vivo histologic study. *J Dent Child* 47:425-30, 1980.
11. Garcia-Godoy, F. Clinical evaluation of glutaraldehyde pulpotomies in primary teeth. *Acta Odontol Pediatr* 4:41-44, 1983.
12. Cunningham, D.W., Lazzari, E.P., Ranly, D.M. The effect of formocresol and glutaraldehyde on certain enzymes in bovine dental pulp. *Oral Surg* 54:100-103, 1982.
13. Ranly, D.M., Lazzari, E.P. A biochemical study of two bifunctional reagents as alternatives to formocresol. *J Dent Res* 62:1054-57, 1983.
14. Ranly, D.M. The effect of pH and concentration on the glutaraldehyde fixation of bovine pulp: an in vitro study. *Acta Odontol Pediatr* 4:45-47, 1983.
15. Noble, S. (personal communication).
16. Ranly, D.M., Bugg, J.L., Brandt, S. (unpublished data).
17. Robertson, E.A., Schultz, R.L. The impurities in commercial glutaraldehyde and their effect on the fixation of brain. *J Ultrastruct Res* 30:275-87, 1970.
18. Fahimi, H.D., Drochmans, P. Purification of glutaraldehyde: its significance for preservation of acid phosphatase activity. *J Histochem Cytochem* 16:199-204, 1968.
19. Anderson, P.J. Purification and quantitation of glutaraldehyde and its effect on several enzyme activities in skeletal muscle. *J Histochem Cytochem* 15:652-61, 1967.
20. Gillet, R., Gull, K. Glutaraldehyde—its purity and stability. *Histochemie* 30:162-67, 1972.
21. Hassel, J., Hand, A.R. Tissue fixation with diimidoesters as an alternative to aldehydes. I. Comparison of cross linking and ultrastructure obtained with dimethylsuberimidate and glutaraldehyde. *J Histochem Cytochem* 22:223-39, 1974.
22. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-54, 1976.
23. Richard, F.M., Knowles, J.R. Glutaraldehyde as a protein cross-linkage reagent. *J Mol Biol* 37:231-33, 1968.