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Response of Pulp Tissue of Rat Molars to Low Temperature Irritation

Stanley Morton Jacobson

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THE RESPONSE OF PULP TISSUE OF RAT MOLARS
TO LOW TEMPERATURE IRRITATION

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B.A., D.D.S.

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1980
THE RESPONSE OF PULP TISSUE OF RAT MOLARS
TO LOW TEMPERATURE IRRITATION

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1980
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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td>Summary of Literature Review</td>
<td>13</td>
</tr>
<tr>
<td>OBJECTIVES</td>
<td>14</td>
</tr>
<tr>
<td>General Objective</td>
<td>14</td>
</tr>
<tr>
<td>Specific Objectives</td>
<td>15</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>16</td>
</tr>
<tr>
<td>RESULTS</td>
<td>19</td>
</tr>
<tr>
<td>Controls</td>
<td>19</td>
</tr>
<tr>
<td>Effects of Experimental Cryosurgical Treatment</td>
<td>20</td>
</tr>
<tr>
<td>Tritiated Proline Deposition in Experimental Animals</td>
<td>29</td>
</tr>
<tr>
<td>Procion H-8BS Experiments</td>
<td>31</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>32</td>
</tr>
<tr>
<td>Cryosurgical Method</td>
<td>32</td>
</tr>
<tr>
<td>Cellular Responses</td>
<td>34</td>
</tr>
<tr>
<td>Irritation Dentin Labelling</td>
<td>37</td>
</tr>
<tr>
<td>FUTURE RELEVANT INVESTIGATIONS</td>
<td>41</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSIONS</td>
<td>43</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>46</td>
</tr>
<tr>
<td>TABLES</td>
<td>51</td>
</tr>
<tr>
<td>FIGURES</td>
<td>55</td>
</tr>
</tbody>
</table>


LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE 1.</th>
<th>Experimental Summary - Number of Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Irritated</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>51</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 2.</th>
<th>Labelled Proline Experiments - Number of Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>52</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 3.</th>
<th>Procion Treatment - Number of Animals Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>53</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 4.</th>
<th>Irritation Dentin Formation Summary - Number of Animals Producing Irritation Dentin/Number of Animals Observed in the Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>54</td>
</tr>
</tbody>
</table>
LIST OF ILLUSTRATIONS

Figure 1. Photograph of the cryosurgical instrument. 56

Figure 2. Schematic diagram of mandibular right first molar and probe placed at experimental site (mesial surface). 56

Figure 3. Photomicrograph of control tooth showing cell layers of normal pulp. 58

Figure 4. Photomicrograph of control tooth demonstrating normal dentin and predentin. 58

Figure 5. Photomicrograph of control tooth showing cuspal attrition and irritation dentin consistently found at these sites. 58

Figure 6. Photomicrograph of experimental tooth of an animal which received one-minute irritation and was sacrificed one day later. 58

Figure 7. Photomicrograph of gingiva of an animal at experimental site which received one-minute irritation and was sacrificed one day later. 60

Figure 8. Photomicrograph of pulp and dentin in an experimental tooth of an animal which received one-minute irritation and was sacrificed one day later. 60
Figure 9. Photomicrograph of central pulpal tissue in mesial horn of experimental tooth of an animal which received one-minute irritation and was sacrificed one day later. 60

Figure 10. Photomicrograph of pulpal tissue in mesial horn of experimental tooth of an animal which received a one-minute irritation and which was sacrificed one day later. 60

Figure 11. Photomicrograph of pulpal tissue in distal pulp horn of an experimental tooth 60

Figure 12. Photomicrograph of pulpal tissue of experimental tooth of an animal which received one-minute irritation and was sacrificed one day later. 60

Figure 13. Photomicrograph of gingival submucosa at experimental site of an animal which received one-minute irritation and was sacrificed one day later. 62

Figure 14. Photomicrograph of pulpal tissue in an experimental tooth of an animal which received one-minute irritation and was sacrificed five days later. 62
Figure 15. Photomicrograph of pulpal tissue of experimental tooth of an animal which received one-minute irritation and was sacrificed five days later. 62

Figure 16. Photomicrograph of distal pulp chamber of an experimental tooth of an animal which received one-minute irritation and was sacrificed five days later. 62

Figure 17. Photomicrograph of an experimental tooth of an animal which received one-minute irritation and was sacrificed five days later. 64

Figure 18. Phase microscopy photomicrograph of an experimental tooth of an animal which received one-minute irritation and was sacrificed five days later. 64

Figure 19. Photomicrograph of mesial wall of experimental tooth of an animal which received one-minute irritation and was sacrificed five days later. 64

Figure 20. Photomicrograph of gingiva at experimental site of an animal which received one-minute irritation and was sacrificed five days later. 64
Figure 21. Photomicrograph of the mesial wall of an experimental tooth of an animal which received one-minute irritation and was sacrificed fifteen days later. 66

Figure 22. Photomicrograph of experimental tooth of an animal which received one-minute irritation and was sacrificed fifteen days later. 66

Figure 23. Photomicrograph of an experimental tooth of an animal which received one-minute irritation and was sacrificed thirty days later. 66

Figure 24. Photomicrograph of an experimental tooth of an animal which received one-minute irritation and was sacrificed thirty days later and examined with phase microscopy. 66

Figure 25. Photomicrograph of the mesial pulp an an experimental tooth of an animal which received one-minute irritation and was sacrificed thirty days later. 68

Figure 26. Photomicrograph of the distal pulp horn of an experimental tooth of an animal which received one-minute irritation and was sacrificed thirty days later. 68
Figure 27. Photomicrograph of external mesial surface of an experimental tooth of an animal irritated for one minute and sacrificed thirty days later. -- -- 68

Figure 28. Photomicrograph of mesial wall of pulp chamber of an experimental tooth of an animal which was irritated for two minutes and was sacrificed one day later. -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- 68

Figure 29. Photomicrograph of an experimental tooth of an animal irritated for two minutes and sacrificed five days later. -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- 68

Figure 30. Photomicrograph of the mesial pulp horn of an experimental tooth of an animal which was irritated for two minutes and sacrificed five days later. -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- 70

Figure 31. Photomicrograph of the mesial wall of the pulp chamber of an experimental tooth of an animal which was irritated for two minutes and sacrificed five days later. -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- 70

Figure 32. Photomicrograph of an experimental tooth of an animal which has received a two minute irritation and was sacrificed fifteen days later. -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- 70
Figure 33. Photomicrograph of an experimental tooth of an animal which has received a two-minute irritation and was sacrificed fifteen days later. 70

Figure 34. Phase microscopy photomicrograph of an experimental tooth of an animal which was irritated for two minutes and sacrificed fifteenth days later. 72

Figure 35. A photomicrograph of an experimental tooth of an animal irritated for two minutes and sacrificed thirty days later. 72

Figure 36. Photomicrograph of the mesial surface of an experimental tooth of an animal irritated for two minutes and sacrificed thirty days later. 72

Figure 37. Photomicrograph of an experimental tooth of an animal irritated for two minutes and sacrificed thirty days later demonstrating brown pigment observed with polarizing light microscopy. 72

Figure 38. Photomicrograph of an experimental tooth of an animal irritated for three minutes and sacrificed one day later. 72

Figure 39. Photomicrograph of an experimental tooth of an animal irritated for three minutes and sacrificed one day later. 74
Figure 40. Photomicrograph of an experimental tooth of an animal irritated for three minutes and sacrificed five days later. 74

Figure 41. Photomicrograph of an experimental tooth of an animal irritated for three minutes and sacrificed five days later. 74

Figure 42. Photomicrograph of an experimental tooth of an animal which received three minutes irritation and was sacrificed fifteen days later. 76

Figure 43. Phase microscopy photomicrograph of an experimental tooth of an animal irritated for three minutes and sacrificed fifteen days later. 76

Figure 44a. Photomicrograph of an experimental tooth of an animal irritated for three minutes and sacrificed thirty days later. 76

Figure 44b. Photomicrograph of an experimental tooth of an animal irritated for three minutes and sacrificed thirty days later. 76

Figure 45. Photomicrograph of the external mesial surface of an experimental tooth of an animal irritated for three minutes and sacrificed thirty days later demonstrating external root resorption. 78
Figure 46. Photomicrograph of a cuspall groove of an experimental tooth of an animal irritated for two minutes, sacrificed thirty days later and stained to demonstrate bacteria. 78

Figure 47. Photomicrograph of the mesial pulp of an experimental tooth of an animal irritated for two minutes, sacrificed thirty days later and stained to demonstrate bacteria. 78

Figure 48. Autoradiograph of an experimental tooth of an animal that received tritiated proline, was irritated for one minute and was sacrificed five days later. 78

Figure 49. Autoradiograph of a control tooth of an animal that received tritiated proline and was sacrificed five days after experimental tooth was irritated. 80

Figure 50. Autoradiograph and dark field microscopy of a control tooth of an animal which was sacrificed five days after irritation. 80

Figure 51. Autoradiograph of an experimental tooth of an animal which received tritiated proline, a two-minute irritation and was sacrificed five days later. 80
Figure 52. Autoradiograph of an experimental tooth of an animal treated with tritiated proline, irritated for one minute and sacrificed fifteen days later. 80

Figure 53. Autoradiograph of a control tooth of an animal in which the experimental tooth received one minute irritation and was sacrificed fifteen days later. 82

Figure 54. Photomicrograph and dark field microscopy of an experimental tooth of an animal which was treated with tritiated proline, irritated for one minute and sacrificed fifteen days later. 82

Figure 55. Photomicrograph of an experimental tooth of an animal treated with tritiated proline, irritated for two minutes and sacrificed thirty days later. 82

Figure 56. Photomicrograph of a distal pulp horn of an experimental tooth of an animal treated with tritiated proline, irritated for two minutes and sacrificed thirty days later. 82

Figure 57. Photomicrograph of fluorescence in an experimental tooth of an animal injected with Procion H-8BS, irritated for two minutes and sacrificed thirty two days later. 84
Figure 58. Photomicrograph of fluorescence in an experimental tooth of an animal injected with Procion H-8BS, irritated for one minute and sacrificed thirty two days later. 84

Figure 59. Photomicrograph of fluorescence in an experimental tooth of an animal injected with Procion H-8BS, irritated for two minutes, and sacrificed thirty two days later. 84

Figure 60. Photomicrograph of fluorescence in a control tooth of an animal injected with Procion H-8BS and sacrificed thirty two days later. 84
INTRODUCTION

Controversy concerning the cellular mechanisms responsible for the reaction of the dental pulp to external irritation has been present in the dental literature for some time (Baume, 1979). Some investigators have observed that the formation of new aberrant dentin can be the result of external stimuli and is a protective mechanism which permits the organism to respond to external trauma (Lisanti and Zander, 1952; James et al, 1954; Berman and Massler, 1958; Swerdlow and Stanley, 1958; Bradford, 1960; Dubner and Stanley, 1962; Seltzer et al, 1962; Stanley, 1962; Sveen and Hawes, 1968; Luostarinen, 1971). These investigators have argued that undifferentiated cells proliferate and differentiate into new odontoblasts which respond by laying down a protective layer of dentin. Not all investigators accept the notion that the pulpal response is regenerative and therefore protective; rather, some describe it as a consequence of odontoblast injury with subsequent aberrant dentin formation (Manley, 1936; Gurley and van Huysen, 1937; Langeland, 1957, 1959, 1960, 1961). Langeland (1968) concluded that the mesenchymal cells do not differentiate into new odontoblasts to replace those injured or destroyed. Langeland maintains that after irritation the surviving odontoblasts regulate enhanced dentin formation.

A variety of experimental methods have been used to study this controversy. These have included pulp responses to cavity preparation, dental materials, intentional pulp exposure and subsequent pulp capping with various materials in humans (James et al, 1954; Swerdlow and Stanley, 1955; Langeland, 1957; Dubner and Stanley, 1962) and animals (Lisanti and Zander, 1952; Mohammed and Schour, 1955; Weider et al, 1955; Berman and Massler, 1958; Sayegh, 1967; Sveen and Hawes, 1968;
There is no conclusive evidence that pluripotential cells from the pulp differentiate into odontoblasts in mature animals. Most previous studies of pulp repair have been hampered by the experimental disadvantage that in the process of irritating the pulp, structural damage to the enamel and dentin of the tooth is a consequence of the methods used. Exceptions to such studies are those which describe pulpal responses to low temperatures in humans, monkeys, and dogs (Langeland et al., 1969) and in monkeys (Emmings et al., 1970). Langeland et al. (1969) applied extremely low temperatures (−160°C) to dog and human teeth for three minutes. They reported severe destruction of tissue in experimental teeth, both in humans and dogs; however, despite widespread damage, vital tissue remained in the apical third of the roots. Emmings et al. (1970) in a preliminary report used temperatures of −80°C applied for one, two and three minutes in monkeys and found marked pulpal reaction with inflammation present and, after 63 days, irritation dentin had formed. Some remnants of vital pulp tissue were found in all teeth.

This study was undertaken in order to define a range of exposure times to low temperatures (−90°C) which would result in a predictable experimental pulpitis and maintain pulp vitality in rat molars in the absence of gross structural damage. Defining such a procedure will permit the study of dentin formation at the damaged site. In addition, the odontoblasts and the inflammatory response will be examined early during the experimental period. It is expected that an experimental method that produces a range of cellular irritation and damage will be useful in addressing the question of the cellular origin of newly
deposited dentin following experimentally or clinically inflicted pulpal irritation. The results of this study confirm the usefulness of freezing temperatures applied to teeth as a means of inflicting controllable degrees of pulp damage. Using a fixed temperature (-90°C) for one, two, or three minutes consistent pulpal responses were achieved consisting of cell damage, inflammation, and new deposition of irritation dentin.
LITERATURE REVIEW

Histologically aberrant dentin which is deposited adjacent to the pulp and is distinct from its physiological counterpart has been mentioned in the literature as early as the first half of the nineteenth century in the works of Retzius, Owen, and Tomes (Baume, 1979). From that time until the present the debate has continued over the correct terminology to describe this tissue formation. Debate over terminology has been the result of different interpretations concerning the cellular origin and physiological role of this tissue. This tissue has been referred to as secondary dentin (Fish, 1928; Stanley, 1962). The term tertiary dentin has been utilized as well in the literature (Bodecker, 1941; Kuttler, 1959; Fischer, 1970, and Federation Dentaire Internationale according to Baume, 1979). Weider et al (1955); Stanley et al (1966); Sayegh (1967a); Massler (1967); Stewart (1968); Avery (1975) have used the term reparative dentin while Lisanti and Zander (1952) referred to this material as protective dentin. Others view this hard tissue formation as a response to irritation and identify this tissue as irritation dentin (Langeland, 1957, 1961). The term irritation dentin has been adopted to describe the results of the present study.

The functional role of dentin formation resulting from external irritation has been the focus of this discussion. Many investigators feel it is a protective response of the organism against possible further insult (Lisanti and Zander, 1952; James et al, 1954; Swerdlow and Stanley, 1958; Bradford, 1960; Seltzer et al, 1962; Stanley, 1962; Massler, 1967; Zach et al, 1969; Luostarinen, 1971). Some authors would also suggest that the calcific bridging of a pulp exposure is
such a response (Glass and Zander, 1949; Nyborg, 1955; Sveen and Hawes, 1968; Luostarinen, 1971; Schroder, 1972). From this viewpoint new hard tissue formation deposited against existing dentin in response to bacterial, chemical, or mechanical irritants would be characterized as physiologic and, therefore, a protective response. But, this response to external stimuli can be the consequence of irritation in the dental pulp (Manley, 1936; Hurley and van Huysen, 1937; Langeland, 1957, 1959, 1960, 1961, 1976). The observations that tubule architecture is less regular than in pre-existing dentin and that irritation dentin is permeable suggest a non-protective role (Langeland, 1967, 1976; Tronstad and Langeland, 1971). Langeland believes this pulpal reaction to be analogous to scar tissue formation.

Certainly differences of opinion concerning the cellular origin of irritation dentin has prompted much of the debate. The issue of whether or not stem cells in the dental pulp will differentiate into odontoblasts and subsequently will form dentin has been a contentious and, perhaps, the pivotal point. Some investigators are of the opinion that dentin formed after a pulp has been damaged by mechanical, chemical, and bacterial irritants is deposited by odontoblasts which have differentiated from mesenchymal stem cells in the pulp replacing odontoblasts destroyed by irritation and trauma (Zander and Glass, 1949; Lisanti and Zander, 1952; James et al, 1954; Nyborg, 1955; Swerdlov and Stanley, 1958; Berman and Massler, 1958; Seltzer et al, 1962; Stanley, 1962; Stewart, 1968; Sveen and Hawes, 1968; Zach et al, 1969; Feit et al, 1970; Luostarinen, 1971, Schroder, 1973; Searls, 1975; Baume, 1979; Fitzgerald, 1979; Skogedal and Mjör, 1979). Although unsupported, there has been a highly speculative suggestion of a budding-off process of new
odontoblasts from old or injured odontoblasts (Stewart, 1965).

Langeland (1968) has reported that there is no new odontoblast formation after traumatic injury to the pulp. He suggests that what appears to be newly proliferated odontoblasts could be a layer of odontoblasts which survived the trauma intact. He also suggests that the thicker the irritation dentin, the fewer odontoblasts present when compared with other unaltered odontoblast areas of the pulp.

In order to clarify and reconcile the controversy concerning the response of the dental pulp to various stimuli, investigators have altered the pulp environment in several ways. Mohammed and Schour (1955) tested pulpal response in rat incisors to cavity preparation and paraformaldehyde filling material. Weider et al (1955) used rat molars and measured the rate of tertiary (irritation) dentin formation after cavity preparation. Berman and Massler (1958) performed pulpotomies on rat molars and medicated the amputation sites with calcium hydroxide in order to study the healing process. Kakehashi et al (1965) studied pulpal responses in both germ-free and conventional rats. They observed that there was dentinal bridging in the germ-free animals with continued pulp vitality. However, the pulps of the conventional animals did not display bridge formation and developed an advanced state of necrosis by the eighth day of the experimental period. They concluded that the presence of bacteria in the conventional animal was responsible for their negative observations.

Harrop and Mackay (1968) using both light and electron microscopy studied the response of pulps which had been capped with calcium hydroxide. These findings were similar to Berman and Massler (1958) suggesting a fibroblastic origin for the hard tissue of the dentinal
bridge. Lisanti and Zander (1952) used dogs to observe repair after thermal injury. They postulate that the healing or repair which took place would be influenced by the ability of pulp cells to differentiate. Langeland (1960) in a study of reactions to cavity preparation with and without coolant reports a persistant chronic inflammation with a reduced number of odontoblasts in the area subjacent to the cut dentinal tubules. There were no observations of cellular differentiation.

James et al (1954) in assessing human pulpal response to gutta percha and cavity preparation stated that cells which were originally fibroblasts, differentiated into cells (preodontoblasts) which went on to form odontoblasts. James et al (1954) reported that new odontoblast differentiation was responsible for new dentin formation. Langeland (1957) in a study of pulp reactions to cavity preparations observed an inflammatory response but rejected the odontoblast replacement hypothesis. He concluded that surviving odontoblasts were the source of irritation dentin.

Dubner and Stanley (1962) in a study of pulpal reaction to temporary filling materials suggest that a re-organization of the odontoblast layer takes place seven days after irritation and eventually reparative dentin forms from the newly organized cells. Langeland (1957, 1961) in similar studies did not observe a re-organization of the odontoblast layer but noted a reduction in the number of cells in the injured area and increased amounts of irritation dentin.

In order to study the permeability of dentin, labels such as silver nitrate (Langeland, 1966) and tritiated prednesilone (Langeland, 1977) have been utilized. Tritiated thymidine has been used to study repair
after pulp exposure in rat molars (Sveen and Hawes, 1968; Luostarinen, 1971) and rat incisors (Feit et al., 1970; Luostarinen, 1971). In addition, tritiated thymidine was used to study repair following trauma induced by a high speed handpiece (Sveen and Hawes, 1968; Cotton, 1968; Zach et al., 1969). Zach reported a slight increase in the index of tritiated thymidine labelling in the pulp. Furthermore, there was an increase in labelled cells in the odontoblast layer subjacent to the area of cavity preparation. These results were interpreted as evidence of cell proliferation followed by odontoblast differentiation.

In a similar experiment Cotton (1968) also reported a slight increase in the labelling index of tritiated thymidine but he was not able to identify labelled cells in the odontoblast layer subjacent to the area of trauma. Sveen and Hawes (1968) found labelled cells in the odontoblast layer three days after injection of the label and twenty-four to forty-eight hours after tooth grinding. They concluded from this experiment that fibroblasts differentiated into new odontoblasts following cell division. Feit et al. (1970) using tritiated thymidine as a label and the rat incisor as a model reached similar conclusions. Luostarinen (1971) who studied both incisors and molars in young and old rats which had been subjected to trauma and tritiated thymidine concluded that pulp cells differentiated into odontoblasts which produce the material for the dentinal budge. Similarly, Fitzgerald (1979) studying pulp exposure in monkeys reaches the same conclusion.

Researchers who believe new odontoblasts differentiate as replacements for those destroyed by the irritation point out that
mitosis takes place followed by differentiation (Stanley, 1962). This position cannot be reconciled with the findings of Langeland (1968) who stated that he had never seen mitosis in the odontoblast layer but had observed mitotic figures in the periphery of an inflamed area in the pulp. Cotton's report supports these observations. Cameron (1971) has categorized odontoblasts with neurons and cardiac muscle as non-renewing populations. The idea of a mitotic requirement before differentiation is still very much an open question (Holtzer et al, 1972) and there are investigators who believe mitosis may not be a requirement for differentiation and a quiescent cell population may respond immediately without mitosis, to an irritant (Ruch et al, 1976).

While the cellular responses remain obscure and controversial, the synthesis and organization of dentin matrix following irritation are more easily documented. Obersztyn (1966) used tritiated glycine to study collagen formation in healing rat incisors after they had been intraosseously exposed and capped with various agents. He reported a reconstituted odontoblast layer subjacent to newly formed dentin in the area of injury.

Searls (1967, 1975) used both the electron microscope and tritiated proline to evaluate pulpal damage produced by high-speed cavity preparation in rat incisors. He noted that although collagen synthesis is markedly reduced ten minutes after cavity preparation, the histological appearance of odontoblasts does not change until four days after trauma.

In addition to tritiated proline, Sayegh (1967b) injected tetracycline as well into rats to measure irritation dentin formation in rat molars after grinding with a high speed water cooled handpiece. He
concluded that the original odontoblasts were responsible for dentin formation of the bridge over exposure sites and were also responsible for tertiary (irritation) dentin formation.

Stallard (1966) used a tritiated proline and tritiated thymidine double-label technique to study repair in rat maxillary molars after occlusal reduction. He determined that odontoblasts were not directly involved in the initial formation of a dentinal bridge, and suggested that fibroblasts of the pulp produced collagen which eventually calcified to form a dentinal bridge.

Atkinson (1976) transplanted maxillary molar tooth buds of ten-day old mice to homogeneic and heterogeneic adult mice in order to study reparative phenomena in tooth transplants. Mice were injected with tritiated proline twenty four hours before sacrifice over a period of twenty to fifty days after transplantation. Isografts formed dentin-like projections across the cervical pulp cavity after twelve days and an odontoblast layer was present. In contrast, allografts developed a narrow tubular floor without repair of the odontoblast layer. Atkinson proposed from his study that the first tertiary (irritation) dentin formed is characterized by bizarre and irregular architecture and is the product of non-polar, i.e. unorganized and random, secretion by atrophying odontoblasts which then became entrapped by their product. Later, as the tissue architecture became similar to regular dentin he proposed that this was a result of newly differentiated odontoblasts from the pulp stem cell pool.

Fischer et al (1970) used Procion H8BS as a marker of tertiary (irritation) dentin formation in monkey teeth after cavity preparation. Procion H8BS is a monochlorotriazinyl derivative of cyanuric chloride.
This dye is believed to link covalently with free hydroxyl or amino substituted substrates under alkaline conditions (Golland and Brand, 1968). When viewed with ultra-violet light the area fluoresces where the dye has been attached. By timing their dosages Fischer et al were able to determine the earliest appearance of tertiary (irritation) dentin and the rate of formation. They found that tertiary (irritation) dentin formation was minimal in the first fifteen days of the experimental period. Maximum formation occurred from the fifteenth to the sixtieth day and decreased thereafter. They concluded that the average daily rate of formation was 4.0 μm.

There have been studies in which pulpitis has been induced experimentally as a model for pulpal repair (Langeland, 1957, Mjör and Tronstad, 1972; Bergenholtz and Lindhe, 1975). The results of these studies do not provide unequivocal evidence for the production of reproducible injury. These studies introduce experimental variables which appear to be overcome by a cryosurgical technique for inducing odontoblast injury (Langeland et al, 1969; Emmings et al, 1970). For example, the cryosurgical technique can produce an injury without hard tissue destruction and pulp vitality can be maintained during the experimental period.

A study of pulp damage after osseous cryosurgery in dogs (Pollan et al, 1974) has been reported but did not provide a reproducibly controlled application of the cryoirritant to crowns of experimental teeth. Shepherd (1976) studied the effect of cryoprobe stimulation to the crowns of rat molars. Unfortunately he applied the probe to the occlusal surface of the tooth where because of attrition there is continual irritation dentin formation in the age group of his experimental animals.

The exact mechanism of cryosurgically induced injury is not fully understood. Whittaker (1974) in an excellent review discussed six
probable mechanisms of cell death as a result of lower temperatures: cell compression due to ice crystal formation; concentration of electrolytes; changes in the osmotic pressure gradient that alter membrane permeability; holes formed in the semi-permeable plasma membrane by either intracellular or extracellular ice crystals; disruption of intra-cellular enzyme activity; and, a rapid loss of water after freezing which ruptures the semi-permeable membrane. He postulated that tissue injury is most likely to be due to the concentration effect of cell constituents as opposed to direct cell membrane rupture. He believed that there was no evidence from the electron microscope studies of cell membrane rupture.
SUMMARY

Deposits of irritation dentin at the sites of injury is well documented. The dental literature contains many discussions of the nature of this response of the dental pulp to external stimulation. Certain investigators believe that the deposition of hard tissue after injury is a protective mechanism while others believe that there is no protective quality in the cellular response, and rather, it is thought to be a consequence of cellular injury.

Pivotal to solving the controversy is the identification of the cell type that is responsible for hard tissue deposition. It has not yet been demonstrated unequivocally whether or not new odontoblasts differentiate from pluripotential cells in the pulp although this notion persists in the literature. Equally plausible is the notion that the remaining odontoblasts at the site of irritation which have survived the injury respond and form irritation dentin.

Many investigative approaches to studying this problem have been attempted; yet each methodology has certain inherent variables which preclude a definitive explanation of the phenomenon. Recently, a novel approach has used freezing temperatures delivered by a cryosurgical instrument to inflict pulpal irritation. This method permits production of pulpal injury without anatomical disturbances of the hard tissue of the crown. The ability to induce pulpal injury without exposing the pulp chamber provides a potentially versatile approach in the quest for greater insight into questions of pulpal differentiation.
OBJECTIVES

General Objective

The general objective of this investigation was to study the response of the dental pulp to low temperature irritation. To accomplish this goal, the mandibular right first molar of a rat was used to test the hypothesis that the pulp tissue of the experimental teeth can repair itself and maintain vitality after irritation with a cryosurgical instrument (-90°C).

This experimental animal provides an excellent subject for a preliminary study of the proposed method. The rat molars are able to respond to various forms of external irritation thus lending themselves to histopathologic study. Once it is established that the experimental tooth does form irritation dentin and maintains vitality, a series of experiments can be undertaken to identify the sources of irritation dentin and to describe this response as compared with dentinal production in other areas of the pulp.

This study was also undertaken to provide more information concerning the histological differences between irritation dentin and healthy dentin during the early stages of matrix deposition. It is of interest to observe at several sacrifice times the cellular relationships of the pulp relative to both types of dentin. This may clarify the role played by the cells during irritation dentin production. An experimental model of this nature will be a versatile tool in the quest for greater insight into reparative potential of the dental pulp of rats.
Specific Objectives

The specific objectives of the study were the following:

1. To irritate the rat molar pulp cryosurgically and, thus, to destroy a localized area of odontoblasts while maintaining the vitality of the pulp.

2. To examine the fate of the odontoblasts present at the time of insult.

3. To determine the extent of new dentin formation at the damaged site.

4. To examine the morphology of the cells actually secreting the dentin matrix.

5. To establish the cellular response to damage and then of repair at early and later sacrifice times after cryosurgical insult.

The experimental objectives were executed in three parts. First, experimental animals were irritated cryosurgically with one, two or three minutes of probe application and sacrificed at one, five, fifteen and thirty days after irritation. Second, experimental animals received a pulse of tritiated proline three days before cryosurgical irritation of one and two minutes, they then received another pulse of tritiated proline at three days after cryosurgical irritation. Third, experimental animals received one, two, and three minutes of cryosurgical irritation and were immediately injected with Procion H-8BS. They were injected again on the second, fourth and thirtieth day after cryosurgical irritation and sacrificed on the thirty-second day.
MATERIALS AND METHODS

Male Sprague-Dawley rats 50-65 days old (200 gms) were used in this study. At this time the first molars are in occlusion as evidenced by the presence of irritation dentin under cusp tips due to attrition which begins at approximately 35-40 days (Hoffman and Schour, 1940). The rats were housed at 28°C and fed laboratory chow and water ad libitum. The lighting was on a timed on-off schedule of twelve hours. The animals were anesthetized with an intraperitoneal injection of Vetalar (Parke-Davis) 100 mg per ml, containing azopromazine 0.5 cc/ml.

Freezing temperatures were produced by a cryosurgical instrument (CT-73, Frigitronics, Shelton, Ct.) which delivered a probe-tip temperature of -90°C (Fig. 1). The probe tip has a 2 mm diameter. The mesial surface of the mandibular right first molar was selected as the experimental subject (Fig. 2).

The number of animals, duration of irritation, and sacrifice schedule used to determine the effect of freezing temperatures are shown in Table 1. The irritation durations were selected because pilot studies established that one minute was the minimal time necessary to produce a detectable response. One, two, and three minute stimulations at -90°C caused marked pulpal responses in proportion to the duration of irritation. The contralateral (left) mandibular first molars from two animals from each experimental group were used as controls in addition to molars from two untreated animals.

At designated intervals, animals were sacrificed by Vetalar overdose. Mandibular segments containing all three molars were dissected and the root tips were removed with a water-cooled diamond wheel. The mandibular segments were fixed in 10% neutral formalin for one week and
decalcified for two weeks in 44% formic acid buffered with sodium citrate. The specimens were washed in running tap water for 24 hours and then processed for histological examination.

The specimens were infiltrated with paraffin in a vacuum oven at 58°C for one hour and then embedded. Serial buccal-lingual sections were cut at 6-7 μm with a sledge microtome and stained with hematoxylin and eosin. The Brown and Brenn (1931) stain for bacteria was also used on some sections.

Twelve male Sprague-Dawley rats weighing approximately 200 grams were given tritiated proline to identify sites of collagen deposition. Four μc/gm of body weight of tritiated proline (specific activity 2 curies/m mole) was administered three days before cryosurgical irritation. Three days after irritation the same dose of tritiated proline was administered. The animals were treated according to the schedule summarized in Table 2.

The sacrifice periods were selected because previous studies by Sayegh (1967a), Sveen (1968), and Luostarinen (1971) reported that dentin formation in adult rats appeared approximately five days after stimulation. The duration of irritation was selected on the basis of the first series of experiments (Table 1).

After sacrifice the animals were processed as described above, but before staining, serial sections on subbed slides were dipped in NTB-3 liquid emulsion according to the method described by Messier and LeBlond (1957). The specimens were dried and placed in air-tight plastic boxes containing vials of silica to reduce condensation. Included with the labelled slides were slides of untreated controls to monitor background radiation. The boxes were wrapped twice in aluminum foil and placed in
a larger container which was also double-wrapped in aluminum foil. The specimens were exposed in the cold for four weeks and then developed in D-19 (Kodak), fixed and stained with hematoxylin and eosin.

Eight male Sprague-Dawley rats weighing approximately 220 grams after irritation with the cryosurgical probe were given Procion Red H-8BS (50 mg/kg) in 0.85% NaCl (Maltha, 1977). The animals were divided randomly into experimental groups (Table 3). The Procion Red was injected immediately after irritation and two, four, and thirty days after irritation. The animals were all sacrificed on the thirty-second day after irritation. The decalcified sections were examined with transmitted ultraviolet light microscopy using a Zeiss photomicroscope.

Six areas of observation will be described:

(a) **Irritation dentin** will be described as present or absent and in the tritiated proline and Procion experiments the presence of labelled collagen will be described.

(b) **Inflammation** will be described as present or absent. The cytoology of the inflammatory infiltrate will be described.

(c) **Vascular damage** will be observed using birefringence of disintegrating blood pigments (Langeland, 1957).

(d) **Cellular damage** in the odontoblast layer will be described with reference to inflammatory cells, nuclei in dental tubules, and cavity formation.

(e) **Fibroblast density** in the mesial portion of the pulp will be compared with control teeth and the distal portion of the pulp in treated molars.

(f) **External resorption of the crown or root** will be described as either present or absent.
RESULTS

Controls

The lower left first molar (contralateral, untreated), the lower right second molar and the distal pulp chamber of experimental right first molars were used as controls. In these specimens the cellular and hard tissue characteristics were not altered.

Morphologically, a healthy dental pulp consists of an odontoblast layer three or four cells thick with cell processes penetrating the predentin and continuing into the dentin (Fig. 3). Empty spaces or cavity formations are observed within the extracellular spaces of the odontoblast layer of the controls and the cell layer subjacent to the dentin in the experimental areas. These will not be described further in the results but will be referred to in the Discussion section.

Calcified dentin contains characteristic dentinal tubules coursing through it in a symmetrically regular pattern (Fig. 4). Basal to the cell bodies of the odontoblasts there is a thin, cell-rich layer. Centrally, the pulp contains fibroblasts, fibrocytes, and mesenchymal cells with an occasional inflammatory cell or resting macrophage. Blood vessels are found most commonly in the central portion of the pulp. In the tips of the pulp horns, irritation dentin formation is present (Fig. 5). The dentinal tubules in this irritation dentin could be traced to areas of attrition on the cusp tips of the tooth (Fig. 5). This irritation dentin is usually found beneath the enamel-free areas of rodents and represents reaction to continual attrition occurring at these sites.
Effects of Experimental Cryosurgical Treatment

The data are derived from three experimental groups (Table 1) following one-, two-, and three-minute durations of irritation. The sacrifice periods are one, five, fifteen and thirty days after each treatment.

One-minute irritation, one-day sacrifice period

One day following the application of the cryosurgical instrument to the mesial aspect of the mandibular right first molar for one minute duration with a temperature of -90°C at the probe tip, an obvious alteration of odontoblasts along the mesial wall of the most mesial pulp horn was seen (Fig. 6) including cell damage and disruption of the odontoblast layer. The site at which the probe was placed could be determined by reference to gingival damage (which will be discussed below) and is visible in the low power view of sections of mandibular segments (Fig. 7).

Inflammatory cells were readily observable in the odontoblast layer (Fig. 8) one day after treatment. The inflammatory cells were predominantly polymorphonuclear leucocytes and were seen adjacent to the predentin layer. Additional accumulations of polymorphonuclear leucocytes were also found in the central portion of the pulp horn (Fig. 9).

Figure 10 illustrates an obvious increase in the number of fibroblasts in the mesial pulp chamber in contrast to control teeth (Fig. 5) and also when compared with the undamaged distal half of the experimental tooth (Fig. 11). Another characteristic feature typical of this experimental group is the dilatation of blood vessels in the
The gingiva on the mesial aspect of the first molar displayed sloughing of the dead gingival epithelium where the probe had been applied (Fig. 7). Damage was confined to this area in all experimental groups. There was evidence of necrotic cells in the epithelial layer and an inflammatory infiltrate in the damaged submucosa (Fig. 13).

**One-minute irritation, five-day sacrifice period**

By the fifth day after freezing, the number of inflammatory cells was reduced (Fig. 14) in contrast to the numbers seen one day following treatment. Dilated blood vessels persisted in the cell layer subjacent to the injured area (Fig. 14). The increased cellularity of the more central portion of the pulp was still evident (Fig. 15) compared with Figure 16.

Of particular significance is the observation that small amounts of dentin matrix were deposited at the site of injury (Fig. 17). This area of new dentin matrix was very irregular and a discrete predentin layer could not be identified on the basis of staining characteristics (Fig. 17). The newly deposited irritation dentin matrix contained fewer dentinal tubules in contrast to control areas (Figs. 3 and 4) and typically contained alterations of the usually regular dentinal tubule pattern (Fig. 18). Phase microscopy confirmed that cellular processes were inserted into the irritation dentin from the subjacent cell layer (Fig. 18). The cells lining this area were fibroblast-like and dilated blood vessels are present (Fig. 19).

At this sacrifice time, a regenerated gingiva had repaired its normal architecture and disruption of epithelial integrity was no longer evident (Fig. 20). Despite the initial loss of crevicular and attachment
epithelia seen in the previous experimental period, the gingival epithelia has approximated the enamel surface by five days after experimental treatment.

One minute irritation, fifteen-day sacrifice period

Fifteen days after experimental damage, an inflammatory reaction was virtually absent, only an occasional leucocyte could be detected in this group. Although there was increased fibroblast density in the mesial pulp it was reduced in contrast to previous groups and the cells subjacent to the irritation dentin took on a more organized and layered appearance (Fig. 21).

Dilated blood vessels were still evident in the cell layer subjacent to the irritation dentin. However, this vascular response had decreased in contrast to previous groups.

The amount of irritation dentin, however, was obviously increased from the previous group examined five days after freezing. A predentin layer was now evident between the irritation dentin and the cells of the pulp. The tubules in the irritation dentin were fewer in number and were less regular in contrast to pre-existing dentin (Fig. 22). As additional evidence of aberrant tissue architecture, cellular inclusions were evident in the irritation dentin bordering the normal dentin (Fig. 22).

One minute irritation, thirty-day sacrifice period

Although the appearance of this group is similar to teeth examined fifteen days after one minute of irritation, there are certain significant differences. Increased amounts of irritation dentin had formed and only rarely were there dilated capillaries in the cell layer subjacent to the irritation dentin.
cent to the irritation dentin. The cells lining the irritation dentin had organized into a definitive layer of cells which appeared to be odontoblast-like (Fig. 23). These cells had processes which extended into the irritation dentin (Fig. 24).

The fibroblast density, although decreased from earlier sacrifice periods, was still increased (Fig. 25) when compared with controls (Fig. 26). Root resorption was present subgingivally (Fig. 27).

Two-minute irritation, one-day sacrifice

This experimental group was similar to specimens obtained one day after a one-minute exposure to freezing. The normal relationship of odontoblast processes within predentin was severely disrupted (Fig. 28), and there was clear evidence that the odontoblast layer was disrupted (Fig. 28). The inflammatory response was greater than seen one day after a one-minute period of irritation. Inflammatory cells were very numerous in areas next to the predentin and in the central portion of the mesial pulp (Fig. 28). As in the previous experimental group employing a shorter duration of freezing described above, there were many dilated blood vessels within the cell layer subjacent to the predentin in contrast to control sections.

Gingival damage was similar to that seen after one-minute of irritation and examined one-day after freezing.

Two-minute irritation, five-day sacrifice period

As was observed five days after one-minute irritation, the inflammatory response was reduced at this time. A re-organization of the cell layer subjacent to the treated mesial wall of the pulp chamber was observed here as well (Fig. 29). Phase microscopy confirmed cell
processes extending from the cells into the irritation dentin. Dilated blood vessels were present in this cell layer. A marked increase in fibroblasts was still evident in the mesial chamber in contrast to control sections (Fig. 30).

As noted earlier, irritation dentin deposition was observed at this time (Fig. 31) and the newly formed matrix was columnar in appearance with an irregular deposition front interdigitated between the cells (Fig. 31).

Despite the long duration of freezing temperature in this experimental group, gingival structure had returned to normal by this sacrifice period as it had in the previous experiment.

Two-minute irritation, fifteen-day sacrifice period

An inflammatory response was not present fifteen days after two minutes of irritation. The fibroblastic cellularity in the mesial pulp had decreased when compared with the previous group, and only an occasional blood vessel was observed in the cell layer subjacent to the irritation dentin. Brown pigment indicating erythrocyte breakdown could be seen (Fig. 32) for the first time in these experimental specimens within the odontoblast layer and in the pulp tissue. The cell layer had become organized as a definitive cell layer with cell processes extending into the irritation dentin (Fig. 33). There were fewer dentinal tubules in the irritation dentin and these ran irregularly with a definite change in direction from tubules in the healthy dentin (Fig. 34). Occasionally cellular inclusions were observed deep within the irritation dentin near the border of the normal dentin (Fig. 34).
Two-minute irritation, thirty-day sacrifice period

There was virtually no observable inflammatory response and only an occasional dilated capillary subjacent to the irritation dentin thirty days after a two-minute probe application. This group also presented a marked decrease in fibroblastic cellularity (Fig. 35) when compared with one-, five-, and fifteen-day sacrifice periods. The cells subjacent to the irritation dentin on the mesial wall of the chamber appeared well organized as a definitive cell layer quite separate from the central portion of the pulp and the underlying cell-rich zone (Fig. 35). The cell processes of the odontoblasts subjacent to the irritation extended into the predentin and into the irritation dentin.

External resorption of tooth structure subgingivally was also present in this experimental group as well (Fig. 36). Again, there was evidence of brown pigment formation (Fig. 37).

Three-minute irritation, one-day sacrifice period

At this duration of freezing, the extent of cellular damage in the mesial pulp horn was such that an odontoblast layer was not observable (Fig. 38). A marked infiltration of polymorphonuclear leucocytes was seen in the central portion of the mesial pulp as well as adjacent to the predentin (Fig. 39) and in the middle portion of the pulp as well (Fig. 39). Extravascated red blood cells were evident subjacent to the mesial dentinal wall of the chamber extending around the perimeter of the mesial pulp horn (Fig. 39). Dilated blood vessels were not present in what was previously an odontoblast layer of the mesial chamber and sparse deposits of brown pigment formation were seen. The
degree of fibroblast accumulation evident in sections of the one-day sacrifice period after one- and two-minute irritation was not present in this experimental group.

Gingival destruction was similar to that seen after one- and two-minutes of irritation. These disruptions included severe damage to the gingival epithelium with an intense inflammatory infiltration.

**Three-minute irritation, five-day sacrifice period**

The inflammatory cell response (polymorphonuclear leucocytes with some lymphocytes) was still present at five days after treatment; yet, it was not as great as the previous group. As before, capillaries were observed in the cell layer subjacent to the damaged region and sparse brown pigment formation was present as well. Some of these blood vessels were partially surrounded by the early stages of matrix deposition (Fig. 40). The cell layer next to the newly deposited irritation dentin had cell processes extending into the irritation dentin (Fig. 41). There was increased fibroblastic density in the mesial chamber. As observed previously in the early stages of irritation dentin formation, this tissue had a columnar, irregular appearance.

Gingival repair which had occurred at this time in the two previous experimental groups was evident in these specimens as well.

**Three-minute irritation, fifteen-day sacrifice period**

At this experimental period the inflammatory response had subsided. An occasional dilated blood vessel was seen in the cell layer subjacent to the irritation dentin but the vascular response was not as profound as that of the three-minute stimulation specimens sacrificed on the fifth day after irritation. Small amounts of brown
pigment was present subjacent to the irritation dentin and in the central pulp in two specimens. The fibroblast population was reduced when compared with the previous groups (Fig. 42). The cells subjacent to the irritation dentin were organized into a definitive cell layer with processes extending into the irritation dentin (Fig. 43). The amount of irritation dentin was greater in this three-minute experimental group than that seen at previous sacrifice times. In the irritation dentin near the border of normal dentin, there were cellular inclusions and obvious dentinal tubule irregularity (Fig. 43). As the pulp is approached the tubules become more regular and lead into and through a predentin layer (Fig. 43). External root resorption was observed in two of the six animals.

Three-minute irritation, thirty-day sacrifice period

The appearance of this group was very similar to the previous group sacrificed fifteen days after irritation. An inflammatory response was absent. There was no increase in fibroblast density and dilated blood vessels were rarely present in a well-organized cell layer subjacent to the irritation dentin (Figs 44a, 44b). Blood pigments were present in the mesial pulp horn. The well organized odontoblast layer under the definitive predentin of the irritation dentin had processes extending through the predentin into the irritation dentin (Fig. 44b). As the field of observation moved away from the pulpal side of the irritation dentin toward the healthy dentin the tubular architecture and direction became increasingly more irregular finally reaching an area of cell inclusions. Immediately mesial to this there was pre-existing dentin.
External root resorption below the epithelial attachment was extensive in the 3-minute irritation, thirty-day sacrifice group (Fig. 45). It was slightly evident as well in the two-minute irritation, thirty-day sacrifice period but to a reduced extent. When resorption was more advanced it extended coronal to the epithelial attachment. Howship's lacunae-like patterns were observed which contained osteoclasts.

Selected sections of two- and three-minute irritations in a thirty day sacrifice period were stained by the Brown and Brenn method (1931) in order to detect the presence of bacteria. Bacteria was observed in the cuspal grooves of the experimental teeth (Fig. 46) but at no time were bacteria evident in the pulp chamber (Fig. 47).
Tritiated Proline Deposition in Experimental Animals

Controls

Silver grain deposition as two discrete bands resulting from our dual injection schedule was observed in control sections (Fig. 48). These bands were evident in the roof and floor of the chamber as well as in the proximal walls of the chamber (Figs. 48 and 49). As the bands were followed into the pulp horns they joined together and entered the pulp horns as a very faint, barely visible, single and more diffuse light band (Fig. 49). Under dark field illumination which optically enhanced grain detection, silver grain deposition was observed around the whole perimeter of the pulp (Fig. 50). There was active isotope incorporation throughout the pulp tissue as well (Fig. 50).

One- and two-minute irritation, five-day sacrifice period

Two bands demonstrating the uptake of tritiated proline in dentin were observed in the floor, roof and proximal walls of the chamber (Fig. 51). The double lines united before entering the pulp horns both mesially (Fig. 51) and in the distal unresponsive regions. With dark field illumination a single band was observed encompassing the perimeter of the pulp tissue in the pulp horns. Increased silver grain deposition was noted both mesially and distally to a degree greater than background deposition in the mesial horn.

One- and two-minute irritation, fifteen-day sacrifice period

Silver grains were deposited in two bands in the proximal walls, floor and roof of the chamber (Fig. 53). As the bands were followed into the mesial pulp horn they joined together to become a single band. The single bands in the mesial pulp were observed to be darker than the
single band in the distal pulp horn when viewed under light microscope (Fig. 52 and 53). A single band was observed in the perimeter of all pulp horns with the silver grain deposition greater than background evident in the pulp tissue (Fig. 54).

Two-minute irritation, thirty-day sacrifice period

Silver grains were deposited as two bands on the proximal wall, floor and roof of the pulp chamber (Fig. 55). As the band of silver grains entered the pulp horns they joined to form one band (Fig. 55). The band in the mesial pulp (Fig. 55) was obviously darker than the single band in the distal pulp horn (Fig. 56). This band outlined the area of irritation dentin formation very precisely (Fig. 55). Silver grain density was greater in the pulp tissue than background activity.
Procion H-88S Experiments

The animals in this experiment received cryosurgical irritation of one, two, and three minutes. Immediately after irritation they were injected with Procion H-88S and subsequently at two, four, and thirty days after irritation. They were sacrificed on the thirty-second day after irritation. Every specimen of each group contained two fluorescent lines evident on the mesial and distal side of the irritation dentin (Figs. 57 and 58) when viewed with ultra-violet light. Irritation dentin formation was outlined at its apical origin extending around the entire perimeter of the pulp horn (Fig. 57). There was evidence of Procion incorporation on the pulpal perimeter of the distal wall of the pulp chamber of the experimental teeth (Fig. 59). However, at no time was there evidence of incorporation of Procion in the dentinal tissues of the control areas in either treated or untreated teeth (Fig. 60).
DISCUSSION

Cryosurgical Method

The results of this study demonstrate that a freezing temperature of \(-90^\circ C\) inflicts severe localized pulpal injury. It is possible to deliver a temperature sufficiently low to provoke an inflammatory reaction followed by the deposition of irritation dentin. Despite the freezing temperature, the vitality of the pulp tissue was maintained in this experimental model. Langeland et al (1969) reported severe injury in the teeth of dogs and humans at much lower temperatures but observed vital tissue in the apical third of human teeth. Emmings et al (1970) in similar experiments reported irritation dentin circumscribing the pulp. An inflammatory reaction was noted as well as the persistence of vital pulp tissue. Both of those studies describe a more generalized and severe pulpal response to freezing temperatures than was observed in this study even at the longest irritation times.

Emmings et al (1970) did not describe the size of the probe that was used but Langeland reported that his probe was 10 mm in diameter. The probe diameter in the present study was 2 mm and this could be the reason for the more localized action of the probe tip. In addition the probe employed by Langeland delivered a temperature of \(-160^\circ C\) at the probe tip while the instrument in this study was able to deliver only \(-90^\circ C\) at the probe tip.

The mesial surface of the mandibular molar in a Sprague-Dawley rat is small relative to the probe tip used and it must be applied in a confined area. It can be seen from Table 4 that occasionally an animal did not respond to irritation. There is good probability that the
probe tip slipped from the tooth in these cases. However, the cryo-
surgical instrument can be placed more easily than a high speed hand-
piece which requires a water spray and a dissecting microscope. The
depth of cavity preparation is always an experimental variable. A
diamond wheel in a handpiece does not offer any alternative advantage
since it is very difficult to determine when cusp tips have been
reduced sufficiently and pulpal exposure has not been achieved in a
rat molar. This criticism is supported by the studies of Sayegh
(1967).

One of the unique features of the cryosurgical method is the
ability to produce a pulpal response without inflicting structural
damage to the tooth. In all studies reviewed previously except that
of Langeland et al (1969) and Emmings et al (1970) a high speed hand-
piece has been used in conjunction with various filling materials,
surgical diathermy or carious dentin. The method used here precludes
these factors as variables, thus the anatomical integrity of the
tooth was never compromised at any time in the experiment. In addi-
tion, the pulpal irritation in the present experiments was introduced
at one time only. On the other hand, in previous experiments because
of the toxic effect of filling materials or faulty margins of restora-
tions (which would permit the ingress of oral fluids and, possibly,
bacteria) would result in a continual pulpal irritation creating in-
accuracies in defining the strength of irritation, length of irritation,
or both.

Because it was presumed that the cryosurgical method eliminated
possible ingress of bacteria, the Brown and Brenn stain was used on
sections of specimens thirty days after irritation. There was organic
material in the cuspal grooves which stained positively for bacteria.
The pulpal tissue did not stain positively and the absence of bacteria confirm the structural integrity of the teeth and, thus, bacteria were not involved in the pulpal response. At no time at the thirty-day sacrifice time after two or three minutes of cryosurgical irritation was there positive staining for Brown and Brenn within the pulp tissue of the experimental teeth.

An obvious disadvantage of the cryosurgical instrument is the lack of flexibility in varying temperatures at the probe tip. The fixed temperature necessitated a variation of treatment time rather than testing pulpal response to a range of probe tip temperatures. Although the temperature within the pulp no doubt varies proportionately with the time the probe is in place a combination of temperature variations at the probe tip coupled with the ability to vary experimental durations would increase the efficacy of the experiments resulting in injury that is more discriminating within the odontoblast layer.

**Cellular Responses**

The histopathological response observed early in the experimental period confirms the observations of Langeland (1957) in his study of pulpal response to various forms of external irritation. The presence of inflammatory cells and cellular destruction unequivocally demonstrate the degree of odontoblast injury. Spaces were found between the odontoblasts at the experimental site but these are observed frequently within the odontoblast layer of the control specimens. These spaces have been shown by Langeland (1957) to be an artefact of histological processing. Nonetheless, there is no doubt that the inflammatory cells present in the early part of the experiment contribute to the observation of extra-cellular empty space; but, it is impossible to quantitate their role with the methods used.

The degree of the inflammatory response noted in this study is pro-
portional to the length of time the irritation had been applied to the experimental site. The intensity of the irritation also affected the appearance of the odontoblast layer in a predictable fashion. A damaged odontoblast layer was present one day after a one-minute irritation. However, extravasated blood cells and neutrophilic leucocytes were observed subjacent to the experimental site one day after the probe was applied for one minute.

In all experimental groups regardless of duration of irritation new dentin was first observed five days after the probe had been applied to the experimental site. The degree of response was related to the duration of the irritation. Evidence of synthesis at this time is confirmed by the work of Sveen and Hawes (1968) and Luostarinen (1971). In the early stages new dentin was observed around cells and capillaries. As deposition continued the tubules became more recognizable but, certainly, they were not as regular or as numerous as in pre-existing dentin. Langeland (1957) has suggested that irritation dentin has fewer tubules because some odontoblasts have been destroyed and those remaining have re-organized to form irritation dentin. This reorganization could result in fewer tubules per area of dentin. This may be the case at one minute of irritation with the cryosurgical probe. However, when specimens were observed after three minutes of irritation, the odontoblast layer was destroyed and there was no evidence of cell processes extending into the predentin or dentin. It was observed in the experiments that after three minutes of irritation and a five-day recovery period, cell processes did extend into the irritation dentin which was forming at that time. When the animals which received three-minute cryosurgical irritation were observed at thirty days after the irritation cell processes were observed entering the
predentin of the irritation dentin in a more typical fashion than at five days. They were fewer in number than in control dentin. This decrease in tubule number suggests a possible restructuring of the cell layer subjacent to the irritation dentin. However, the origin of these cells could not be determined due to the limitations of the present study.

One day after irritation, an increase in fibroblast density was observed in specimens sacrificed after one and two minutes of irritation. However, an increased fibroblast density was not observed to the same extent in the specimens which had been irritated for three minutes and observed one day after the cryosurgical probe application. This is undoubtedly related to the severity of the irritation. The samples subjected to one- and two-minute irritations were not injured to the same degree as the three-minute groups, consequently the three-minute specimens required more time during recovery to respond with an equivalent degree of fibroblast density. The increased density of fibroblasts could be the result of either migration or proliferation but cell division was not observed and the limited observation periods did not permit a resolution of this question of the origin of the cells found in the repairing pulp.

The presence of blood pigments was observed in two- and three-minute experimental groups. Langeland (1957) has shown that blood pigment presence is a measure of vascular damage. The blood pigment presence in this study was only observed in small depositions although vascular damage was present, particularly in the three-minute irritation group. This may be due to the time between observation periods. Thus, it may be necessary to shorten the interval between the observa-
Dilated capillaries were observed within the cell layer subjacent to the experimental site in animals sacrificed at one and five days after a one- and two-minute probe application. The dilated capillaries were also noted in this cell layer at the five-day sacrifice period after three minutes of probe application. At day fifteen of all experimental groups, the dilated capillaries were still present but were less prevalent than in earlier sacrifice periods. Langeland (1957) also observed the presence of dilated capillaries in the odonto-blast layer early in the experimental period such that it would not have been possible for them to have been formed within the time the tooth had received the irritation and the time of observation.

External root resorption including multinucleated clast cells was a consistent finding present in the group sacrificed at thirty days after a three-minute irritation. Langeland et al (1969) supports the observation of the presence of external resorption in teeth subjected to low temperatures. This phenomenon should be investigated more carefully and suggests an experimental model for the study of external resorption.

Irritation Dentin Labelling

The use of tritiated proline was successful in establishing the areas of irritation dentin formation. However, there are some observations which affect the interpretation of the data. The viability of cells and rate of synthesis at the beginning of the experiments will affect incorporation of the tritiated proline greatly such that much of the tag can be lost if availability in the system is not timed.
The observation that isotope was not readily taken up in control pulp horns or the distal pulp horns of experimental teeth was an important finding. Further, the observation of two dark lines (representing the two pulses of tritiated proline) blending into one faint line entering all pulp horns with the consistent finding that the line in the experimental horn was always denser than control pulp horns is related to the increase in label uptake by the cells which synthesize the collagen which later calcifies to form irritation dentin. This increase in label uptake in the experimental pulp horns is related to the application of the cryosurgical probe which provides the signal to transform the collagen producing cells from a quiescent state to a more active role of collagen synthesis.

Synthesis continued at a seemingly constant rate after irritation in the roof and floor of the pulp chamber as well as the distal wall of the chamber of experimental and control teeth. The two label lines which formed in these areas were darker than the single lines in the control and experimental pulp horns suggesting that at the time of label uptake the cells in areas of dark label lines were synthesizing at consistently higher rates than the cells further up in the pulp horns.

Tritiated proline used as a labelling method defined the boundaries of the irritation dentin present at the sacrifice times in this study. It also provided information about levels of cellular activity in control in contrast to experimental sites. This finding provides the basis of an interesting future study on cell activity in various areas of the rat molar pulp.

In order to confirm the findings of the tritiated proline experiments, Procion H-88S was used in nine animals. The material is not
costly, requires no special precautions and is well tolerated by the animals. Its advantage over tritiated proline is the fact that it will label any available collagen. There are no special laboratory procedures other than caution in staining procedures such that materials that interfere with fluorescence such as eosin counter-stain should not be used (Maltha et al, 1977).

Our injection schedule of Procion Red H-8BS successfully labelled the borders of the irritation dentin. Binding of the stain in the early stages of irritation dentin formation effectively outlines the pattern of irritation dentin formation. Note especially the diffuse pattern of label easily related to this early stage of new dentin secretion at the damaged sites. This label also confirms that the hard tissue response was one of increased formation in contrast to control areas. Since Procion labels uncalcified collagen the absence of a line within the dentin of the control areas suggests that any collagen labelled by the Procion in control areas never calcified during the experimental period. However, at the experimental sites a labelled line was evident within the dentin and another labelled line was observed at the border between the pulp and the irritation dentin. This fluorescent line within the dentin could only be separated from the pulpal line if tissue deposition and calcification had taken place within the experimental period.

Procion labelling provides an effective means of defining the hard tissue response elicited by a rat molar pulp. It describes the boundaries of such formation as well as providing information on the pattern of tissue deposition. This method in conjunction with tritiated proline will allow future investigations to make more
definitive conclusions concerning the rate, pattern, and location of cellular response of pulpal tissue to irritation.
FUTURE RELEVANT INVESTIGATIONS

The results of this study lead to several areas of research which may prove useful in gaining more knowledge of developmental problems of dental pulp. Irritation dentin was present by the fifth day after irritation. Experiments between the first day and the fifth day after irritation should be carried out to determine the origin of cells which participate in the early stages of irritation dentin formation. The location and morphology of these cells will be studied to provide data on whether cells differentiate into odontoblasts in the early stages of pulpal response.

Investigations should be carried out in an effort to improve the capacity of the cryosurgical instrument to provide a more localized injury. A more localized odontoblast injury in larger animals such as dogs or monkeys might provide data on the patterns of irritation dentin in relation to the cells subjacent to it at the time of inception of irritation dentin formation.

A large number of animals should be used to repeat the tritiated proline study. By counting grain deposition in experimental and control pulp horns a rate of collagen synthesis in the experimental pulp horns might be determined which would be a measure of the ability of an external irritation to increase the rate of collagen synthesis. This will produce data which will provide insight into which areas of the dental pulp have the greatest capacity for repair.

A study should be carried out using dental materials in teeth which have had their pulps compromised by cryosurgical irritation. This study would provide information on dental materials in a more realistic fashion since most materials are placed in teeth that have
been previously compromised by caries or previous restorative procedures in combination with dental materials.

A study could be carried out to investigate the procedures and materials which could arrest external resorption. As observed in the results, external resorption could be induced consistently with a three minute irritation and when the tooth was observed thirty days later external resorption had progressed to quite an extent. The cryosurgical method provides a convenient means of inducing this type of response.

A study is presently in progress to study the anachoretic effect in pulp tissues compromised by a low temperature irritation. This study would be useful in providing information regarding the response of compromised pulps in patients who have acute and chronic infections. The data provided by such a study may permit a more definitive clinical protocol to be developed for acute and chronically ill patients.
SUMMARY AND CONCLUSIONS

The experimental methodology effectively fulfilled the specific objectives of the experiment. The rat molar pulp was shown to maintain its vitality after a localized damage by a low temperature to an area of the odontoblast layer. This was a consistent finding in all experimental groups. At no time was the pulp of an experimental tooth at any sacrifice time observed to be necrotic. Quite the opposite occurred; instead the experimental teeth observed at thirty days had healthy and uninvolved pulps except for the presence of irritation dentin at the experimental site.

While the presence of irritation dentin was readily observable the behavior of the odontoblast layer, its disruption and the organization of a cell layer subjacent to the irritation dentin was of great interest. It was consistently found that a cell layer was present subjacent to the irritation dentin in all groups sacrificed at thirty days. This finding is suggestive of a re-organization of the odontoblast layer particularly in animals which had received a three minute probe application and the odontoblast layer was observed to be totally destroyed at one day observation. It is not possible to definitively state the origin of these cells within the limits of the present methodology and further experiments will be carried out to observe the response between one-and five day sacrifice times after irritation.

The experimental animals responded to cryosurgical irritation by forming irritation dentin. Animals responded with irritation dentin production by the fifth day after probe application. The animals which received a three minute probe application were observed to be responding more aggressively than the one-and two-minute irritation
groups. This was expressed by the production of more irritation dentin at five days which had progressed between and around the cells subjacent to it to a greater extent than the other groups. The irritation dentin had tubular structures but they were less numerous and regular than in healthy dentin. When the irritation dentin is first formed it is very irregular but becomes more regular as deposition increases during the experimental period.

It was possible to define the parameters of irritation dentin formation with the use of tritiated proline. This label determined not only the deposition of irritation dentin formation but also the degree of cellular synthesis in the experimental pulp horn compared to control pulp horn. It was concluded that the cryosurgical instrument provoked the collagen producing cells of the experimental pulp horn to become more active than controls as evidenced by the density of label in the experimental pulp horn compared to the control pulp horns.

It was also evident that Procion H-8BS could be used to confirm the findings of tritiated proline by defining the parameters of irritation dentin formation. Since Procion labelled available free collagen it was effectively used to determine the level of increased collagen production at the experimental site.

The cellular response was consistent in all experimental groups. The pulps of the experimental teeth all responded with an acute inflammatory infiltrate by the first day after probe application. As the length of time of the probe application increases, the inflammatory response becomes proportionately more severe. However, by the thirtieth day an observable inflammatory response was not present in any of the experimental groups.
All experimental teeth responded with an increase in fibroblast density. This was evident on the first day after the probe had been applied for one and two minutes to the experimental site. The teeth of the animals which had the probe applied for three minutes displayed an increase in fibroblast density by the fifth day after use of the cryosurgical instrument. It is concluded that, because of the severity of the injury, the pulps that had been irritated for three minutes required more time to respond with an increase of fibroblast density.

The cell layers subjacent to the experimental site in all teeth all had an increase in dilated capillaries within this cell layer. It is concluded that this is a reproducible finding in the odontoblast layer of any tooth subjected to external irritation.

The cells which organize subjacent to the irritation dentin appear to be odontoblasts. They have cell processes which extend into the irritation dentin. Although it is not possible to state definitively that they are odontoblasts because of the limits of the methodology used in this experiment they appear to be very similar to odontoblasts.

It was concluded that hard tissue damage did not take place during the experiment as a result of low temperature probe application. This was confirmed by the negative results in the pulp tissue after histological staining by the Brown and Brenn method.

It can be concluded that the pulp tissue of rat molars can be irritated with a low temperature of -90°C for one, two, and three minutes and when examined at one, five, fifteen, and thirty days after the irritation the pulp tissue remains vital.
BIBLIOGRAPHY


TABLE 1

EXPERIMENTAL SUMMARY

Number of Animals

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TABLE 2

LABELLED PROLINE EXPERIMENTS

Number of Animals

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SACRIFICE SCHEDULE (Days)
### TABLE 3

**PROCION TREATMENT**

**Number of Animals**

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TABLE 4

IRRITATION DENTIN FORMATION SUMMARY

Number of Animals Producing Irritation Dentin/
Number of Animals Observed in the Group

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Plate I

Figure 1. Photograph of cryosurgical instrument - probe tip diameter is 2 mm.

Figure 2. Schematic diagram of mandibular right first molar and probe placed at experimental site - note size of probe tip in relationship to experimental site (cp, cryosurgical probe).
Figure 3. Photomicrograph of control tooth showing all layers of normal pulp - note odontoblast layer (OD), cell rich layer (CR). X220.

Figure 4. Photomicrograph of control tooth demonstrating normal dentin (ND) and predentin (PD). X560.

Figure 5. Photomicrograph of control tooth showing cuspal attrition (A) and irritation dentin (ID) consistently found at these sites. X140.

Figure 6. Photomicrograph of experimental tooth of an animal which received one-minute irritation and was sacrificed one day later. White arrows point to disrupted odontoblast layer with polymorphonuclear leucocytes present. X560.
Plate III

Figure 7. Photomicrograph of experimental tooth of an animal which received one-minute irritation and was sacrificed one day later. Note damage to gingival epithelium with ulceration and sloughing. X140.

Figure 8. Photomicrograph of pulp and dentin in an experimental tooth of an animal which received one-minute irritation and was sacrificed one day later. Black arrows indicate the presence of polymorphonuclear leukocytes. Note disruption of odontoblast layer. X1400.

Figure 9. Photomicrograph of pulpal tissue in mesial horn of experimental tooth of an animal which received one-minute irritation and was sacrificed one day later. Black arrow indicates a polymorphonuclear leucocyte. X1400.

Figure 10. Photomicrograph of pulpal tissue in mesial horn of experimental tooth of an animal which received a one-minute irritation which was sacrificed one day later. Note increased fibroblast density. X140.

Figure 11. Photomicrograph of control pulpal tissue in distal pulp horn of experimental tooth. Compare fibroblast density to that in Fig. 10. X140.

Figure 12. Photomicrograph of pulpal tissue of experimental tooth of an animal which received one-minute irritation and was sacrificed one day later. Black arrow indicates dilated capillary. Note odontoblast layer disruption. X560.
Plate IV

Figure 13. Photomicrograph of gingival submucosa at experimental site of an animal which received one-minute irritation and was sacrificed one day later. Black arrows indicate the presence of polymorphonuclear leucocytes. Note the presence of extravasated red blood cells. X1400.

Figure 14. Photomicrograph of pulpal tissue in an experimental tooth of an animal which received one-minute irritation and was sacrificed five days later. White arrow indicates dilated capillary. X560.

Figure 15. Photomicrograph of pulpal tissue of experimental tooth of an animal which received one-minute irritation and was sacrificed five days later. Note increased fibroblast density. X140.

Figure 16. Photomicrograph of distal pulp chamber of an experimental tooth of an animal which received one-minute irritation and was sacrificed five days later. Note fewer fibroblasts compared to Figure 15. X140.
Plate V

Figure 17. Photomicrograph of mesial pulp chamber of an experimental tooth of an animal which received one-minute irritation and was sacrificed five days later. Note presence of irritation dentin (ID) deposition with less numerous and more irregular dentinal tubules. X560.

Figure 18. Phase microscopy photomicrograph of an experimental tooth of an animal which received one-minute irritation and was sacrificed five days later. Note cell process (black arrow) located in irritation dentin with irregular tubule pattern. X1400.

Figure 19. Photomicrograph of mesial wall of experimental tooth of an animal which received one-minute irritation and was sacrificed five days later. Note fibroblast-like appearance of cells subjacent to irritation dentin. Black arrows indicate dilated blood vessels. X1400.

Figure 20. Photomicrograph of gingiva at experimental site of an animal which received one-minute irritation and was sacrificed five days later. Note gingiva has re-epithelialized. X140.
Plate VI

Figure 21. Photomicrograph of the mesial wall of an experimental tooth of an animal which received one-minute irritation and was sacrificed fifteen days later. Note organized appearance of cell layer subjacent to irritation dentin. X140.

Figure 22. Detail photomicrograph of experimental tooth shown in Figure 21 of an animal which received one-minute irritation and was sacrificed fifteen days later. Black arrow indicates cell inclusion within irritation dentin. Note that tubules of irritation dentin are highly irregular and less numerous compared to pre-existing dentin. X1400.

Figure 23. Photomicrograph of an experimental tooth of an animal which received one-minute irritation and was sacrificed thirty days later. Note cells subjacent to irritation dentin are observed to form a definitive layer. Note also irregularity of dentinal tubules. X140.

Figure 24. Photomicrograph of an experimental tooth of an animal which received one-minute irritation and was sacrificed thirty days later and examined with phase microscopy. Black arrows indicate cell processes located within irritation dentin. X1400.
Plate VII

Figure 25. Photomicrograph of the mesial pulp of an experimental tooth of an animal which received one-minute irritation and was sacrificed thirty days later. Note increase in fibroblast density. X140.

Figure 26. Photomicrograph of the distal pulp horn of an experimental tooth of an animal which received one-minute irritation and was sacrificed thirty days later. Note control fibroblast density. X140.

Figure 27. Photomicrograph of external mesial surface of an experimental tooth of an animal irritated for one minute and sacrificed thirty days later. Note root resorption at experimental site. X140.

Figure 28. Photomicrograph of mesial wall of pulp chamber of an experimental tooth of an animal which was irritated for two minutes and was sacrificed one day later. Black arrows indicate polymorphonuclear leucocytes. Note disruption of odontoblast layer. X560.

Figure 29. Photomicrograph of an experimental tooth of an animal irritated for two minutes and sacrificed five days later. Black arrow points to a re-organized cell layer subjacent to the mesial wall of the pulp chamber. X560.
Plate VIII

Figure 30. Photomicrograph of the mesial pulp horn of an experimental tooth of an animal which was irritated for two minutes and sacrificed five days later. Note increase in fibroblast density. X140.

Figure 31. Photomicrograph of the mesial wall of the pulp chamber of an experimental tooth of an animal which was irritated for two minutes and sacrificed five days later. Small and large black arrows indicate irregular tubule direction within irritation dentin. X560.

Figure 32. Photomicrograph of an experimental tooth of an animal which has received a two minute irritation and was sacrificed fifteen days later. White arrows indicate brown pigment formation. X560.

Figure 33. Photomicrograph of an experimental tooth of an animal which has received a two-minute irritation and was sacrificed fifteen days later. Black arrows indicate tubules within irritation dentin which contain cell processes. Note branching of tubules. X1400.
Plate IX

Figure 34. Phase microscopy photomicrograph of an experimental tooth of an animal which was irritated for two minutes and sacrificed fifteen days later. Note change in tubule direction of irritation dentin compared to healthy dentin. Black arrow points to cell inclusion in irritation dentin. X350.

Figure 35. A photomicrograph of an experimental tooth of an animal irritated for two minutes and sacrificed thirty days later. Note fibroblast density compared to controls (Figs. 5 and 26). Also a definitive cell layer is present subjacent to the irritation dentin. X140.

Figure 36. Photomicrograph of the mesial surface of an experimental tooth of an animal irritated for two minutes and sacrificed thirty days later. Note external resorption. X140.

Figure 37. Photomicrograph of an experimental tooth of an animal irritated for two minutes and sacrificed thirty days later demonstrating brown pigment observed with polarizing light microscopy. Black arrows indicate brown pigment formation viewed under polarized light. X1400.

Figure 38. Photomicrograph of an experimental tooth of an animal irritated for three minutes and sacrificed one day later. Black arrows indicate polymorphonuclear leucocytes. Note absence of odontoblast layer. X560.
Plate X

Figure 39. Photomicrograph of an experimental tooth of an animal irritated for three minutes and sacrificed one day later. Note presence of many polymorphonuclear leucocytes, extravasated blood cells, and absence of odontoblast layer. X\text{560}.

Figure 40. Photomicrograph of an experimental tooth of an animal irritated for three minutes and sacrificed five days later. Black arrow indicates a dilated capillary. Note presence of irritation dentin surrounding cells subjacent to it. X\text{1400}.

Figure 41. Photomicrograph of an experimental tooth of an animal irritated for three minutes and sacrificed five days later. Black arrows indicate a cell process located in irritation dentin. Note columnar appearance of forming irritation dentin. X\text{560}.
Figure 42. Photomicrograph of an experimental tooth of an animal which received three minutes of irritation and was sacrificed fifteen days later. X140. Compare with Fig. 15.

Figure 43. Phase microscopy photomicrograph of an experimental tooth of an animal irritated for three minutes and sacrificed fifteen days later. Black arrows indicate cell inclusions within irritation dentin. Note cell processes within irritation dentin. Note also the increase in number and regularity of dentinal tubules as pulp is approached. X350.

Figure 44a. Photomicrograph of an experimental tooth of an animal irritated for three minutes and sacrificed thirty days later. Note a definitive cell layer subjacent to irritation dentin. X560.

Figure 44b. Photomicrograph of an experimental tooth of an animal irritated for three minutes and sacrificed thirty days later. Note definitive cell layer subjacent to irritation dentin. Fibroblast density here is the same as seen in control pulps (Fig. 26). X220.
Plate XII

Figure 45. Photomicrograph of the external mesial surface of an experimental tooth of an animal irritated for three minutes and sacrificed thirty days later demonstrating external root resorption. Note external resorption with Howship lacunae-like patterns. X350.

Figure 46. Photomicrograph of a cuspal groove of an experimental tooth of an animal irritated for two minutes, sacrificed thirty days later and stained to demonstrate bacteria. Black arrows indicate presence of bacterial colonies. X350.

Figure 47. Photomicrograph of the mesial pulp of an experimental tooth of an animal irritated for two minutes, sacrificed thirty days later and stained to demonstrate bacteria. No bacterial colonies are present. X140.

Figure 48. Autoradiograph of an experimental tooth of an animal that received tritiated proline, was irritated for one minute and was sacrificed five days later. Black arrows indicate two discrete bands of grain deposition on floor and roof of pulp chamber. X140.
Plate XIII

Figure 49. Autoradiograph of a control tooth of an animal that received tritiated proline and was sacrificed five days after experimental tooth was irritated. Black arrows indicate the two bands of grain deposition uniting to form one band. X140.

Figure 50. Autoradiograph and dark field microscopy of a control tooth of an animal which was sacrificed five days after irritation. Note silver grain deposition around the entire perimeter of pulp with grain incorporation within the pulp tissue. X140.

Figure 51. Autoradiograph of an experimental tooth of an animal which received tritiated proline, a two-minute irritation and was sacrificed five days later. Black arrows indicate two distinct bands of grain deposition on floor, roof and proximal wall of chamber uniting into one band as band enters pulp horn. X140.

Figure 52. Autoradiograph of an experimental tooth of an animal treated with tritiated proline, irritated for one minute and sacrificed fifteen days later. Note that the bands in the mesial pulp horn are darker than the bands in the distal pulp horn in Figure 53. X140.
Plate XIV

Figure 53. Autoradiograph of a control tooth of an animal in which the experimental tooth received one minute irritation and was sacrificed fifteen days later. Note bands entering pulp horn are lighter and less evident than bands in mesial horn in Figure 52. X140.

Figure 54. Photomicrograph and dark field microscopy of an experiment tooth of an animal which was treated with tritiated proline, irritated for one minute and sacrificed fifteen days later. X140.

Figure 55. Photomicrograph of an experimental tooth of an animal treated with tritiated proline, irritated for two minutes and sacrificed thirty days later. Note grain deposition in two bands in roof, floor and proximal wall form one band as they enter mesial pulp horn. Note as well delineation of irritation dentin by tritiated proline. X140.

Figure 56. Photomicrograph of a distal pulp horn of an experimental tooth of an animal treated with tritiated proline, irritated for two minutes and sacrificed thirty days later. Note that single band is lighter in distal horn than mesial horn of Figure 55. X140.
Figure 57. Photomicrograph of fluorescence in an experimental tooth of an animal injected with Procion-H8BS, irritated for two minutes and sacrificed thirty two days later. Note irritation dentin (ID) outlined by the fluorescent lines. X220.

Figure 58. Photomicrograph of fluorescence in an experimental tooth of an animal injected with Procion H-8BS, irritated for one minute and sacrificed thirty two days later. Irritation dentin (ID) is outlined by two fluorescent lines. X350.

Figure 59. Photomicrograph of fluorescence in a experimental tooth of an animal injected with Procion H-8BS, irritated for two minutes, and sacrificed thirty two days later. Note only one fluorescent line in walls of distal pulp dentin of experimental tooth. X350.

Figure 60. Photomicrograph of fluorescence in a control tooth of an animal injected with Procion H-8BS and sacrificed thirty two days later. Note one line in mesial wall of the control tooth. X350.