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A Histopathologic Study of the Periapex of Monkey Teeth Intentionally Contaminated Prior to Root Canal Filling

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A HISTOPATHOLOGIC STUDY OF THE PERIAPEX OF
MONKEY TEETH INTENTIONALLY CONTAMINATED
PRIOR TO ROOT CANAL FILLING

by

Edward P. Theiss, D.D.S.

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Master of Science

June

1976
DEDICATION

To my wife, Tiki, and children, Tiffany, Christina, and Nathan, whose personal sacrifices have made it possible for me to achieve a late­coming ambition in life.
Edward Peter Theiss was born in Sublette, Illinois, on September 7, 1940 to Gilbert P. and Ruth E. Theiss.

He received his elementary education at St. Mary's Parochial school in Sublette, and his secondary and college education at St. Bede Academy and Junior College in Peru, Illinois.

In September 1960, he entered Marquette University Dental School. He graduated "Cum Laude" and number two in his class of 1964. He was also installed in the honorary dental fraternity, Omicron Kappa Upsilon, and given the Psi Omega scholastic achievement award.


In 1974 he returned to school at Loyola University Dental School to pursue a Masters Degree in Oral Biology in the Department of Endodontics.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.  INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. LITERATURE REVIEW</td>
<td>3</td>
</tr>
<tr>
<td>Development of the Root Canal Culture Technique</td>
<td>3</td>
</tr>
<tr>
<td>Focal Infection Theory</td>
<td>3</td>
</tr>
<tr>
<td>Culturing The Periapical Rarefaction</td>
<td>6</td>
</tr>
<tr>
<td>Improved Prognosis As Related To Negative Culture Results</td>
<td>16</td>
</tr>
<tr>
<td>The Endodontic Culture Challenged</td>
<td>20</td>
</tr>
<tr>
<td>The Importance of Bacteria in Pulp and Periapical Disease</td>
<td>32</td>
</tr>
<tr>
<td>Anachorisis</td>
<td>34</td>
</tr>
<tr>
<td>The Effects of Root Canal Procedures on the Periapical Tissues</td>
<td>37</td>
</tr>
<tr>
<td>Rationale for the Use of <em>Streptococcus faecalis</em></td>
<td>43</td>
</tr>
<tr>
<td>III. MATERIALS AND METHODS</td>
<td>50</td>
</tr>
<tr>
<td>Isolating the Microorganism</td>
<td>50</td>
</tr>
<tr>
<td>Pilot Study</td>
<td>53</td>
</tr>
<tr>
<td>Experiment</td>
<td>55</td>
</tr>
<tr>
<td>Preparation of the Section</td>
<td>59</td>
</tr>
<tr>
<td>IV. RESULTS</td>
<td>62</td>
</tr>
<tr>
<td>Microbiologic Results</td>
<td>62</td>
</tr>
<tr>
<td>Pilot Results</td>
<td>62</td>
</tr>
<tr>
<td>Culture Results</td>
<td>63</td>
</tr>
<tr>
<td>Clinical Results</td>
<td>63</td>
</tr>
<tr>
<td>Radiographic Results</td>
<td>64</td>
</tr>
<tr>
<td>Histologic Results</td>
<td>64</td>
</tr>
<tr>
<td>V. DISCUSSION</td>
<td>71</td>
</tr>
<tr>
<td>VI. SUMMARY AND CONCLUSIONS</td>
<td>79</td>
</tr>
<tr>
<td>VII. BIBLIOGRAPHY</td>
<td>81</td>
</tr>
<tr>
<td>VIII. APPENDIX</td>
<td>94</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Histologic Periapical Tissue Response</td>
<td>68</td>
</tr>
<tr>
<td>II</td>
<td>Histologic Response of Alveolar Bone and Root</td>
<td>69</td>
</tr>
<tr>
<td>III</td>
<td>Microscopic Exam of Root Canal Contents and Filling</td>
<td>70</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION

The art and science of Endodontics has made tremendous strides since the turn of the century, until it has reached today what some refer to as, "The Golden Age of Endodontics". One of the factors that contributed tremendously to this advancement was the development of the culture technique. By verification that a canal could be rendered sterile, the pioneering and perservering dentists were able to refute the focal infection theory of the early 1900's.

These early endodontists had a tremendous battle to wage against the extractionists of that era who considered treatment of pulpless teeth an unjustifiable procedure. One cannot help but admire the fortitude of these men when reading of the vicious attacks made on them. So it is not hard to understand why so much significance was attached to the culture because it aided them through some very difficult and trying times.

Consequently, the culture technique almost became a religion. It was relied upon so heavily that teeth were condemned, or a surgical approach was instituted in situations where a negative culture could not be obtained. Some insisted that two consecutive negative cultures were necessary before a root canal should be filled.

With such stress being placed on the need for obtaining a negative culture, many caustic and potentially dangerous nonspecific antibacterial
agents were used to achieve this end. These agents were sometimes used at the expense of sound biological and surgical principles. But, nevertheless, such was the fervor with which some men adhered to these culturing principles.

However, some endodontists began to question the need for obtaining a negative culture prior to filling the root canal and research was undertaken to clarify its impact. It was felt that there are many more important factors involved. Although most studies have demonstrated superior results following a negative microbiologic report, all of the reports supporting the culture seem to indicate that healing might occur in the presence of microbial activity. Hence the development of the two factions in the culture debate.

Since no studies have reported results on treated teeth that were intentionally contaminated, the healing potential of such teeth has remained a question. The purpose of this study was to investigate the healing potential of periapical tissues after endodontic therapy when known viable pathogenic bacteria were present. It was hoped that this research might shed a little more light on the subject.
CHAPTER II

LITERATURE REVIEW

Development of the Root Canal Culture Technique

As early as 1899, Smith\(^1\) outlined six steps which he considered essential to successful root canal treatment. One of the criteria he laid down for filling the root canal was an aseptic condition. However, he had no way of determining when he had achieved this condition. In 1901, Onderdonk\(^2\) recognized this shortcoming in the criteria laid down by Smith, and suggested what he called the "scientific test". This test was a culture of the cotton dressing left in the tooth as an intracanal medicament between appointments. Then with the aid of a bacteriologist, he verified that an aseptic root had been achieved. This was the first mention of an endodontic culture in the literature.

Focal Infection Theory

To try to follow the development of the culture technique further would be impossible without first mentioning the focal infection theory.

The focal infection theory had been suggested, alluded to and discussed in the 1800’s but was not generally accepted. Even Rhein,\(^3\) one of the most renowned endodontists of his era, wrote that he had suggested this theory to his medical colleagues around 1885 to 1900 but was ridiculed by them. Billings\(^4\) and Rosenow\(^5\) were actually the two people most responsible for the theory gaining a foothold. The idea caught on and was given a
big impetus by the address of Hunter\textsuperscript{6} in 1910, at McGill University in Montreal, Canada. He literally lashed out at dentistry, especially as it was practiced in the United States. To quote his words,

Gold fillings, gold caps, gold bridges, gold crowns, fixed dentures, built in, on, and around diseased teeth, form a veritable mausoleum of gold over a mass of sepsis to which there is no parallel in the whole realm of medicine or surgery.

He was trying to draw attention to oral sepsis, which he felt his colleagues were overlooking as the cause of a great group of medical afflictions.

The theory was given more credence by Billings,\textsuperscript{7} Black,\textsuperscript{8} Hartzell,\textsuperscript{9} Rosenow,\textsuperscript{10,11} and Haden.\textsuperscript{12} Rosenow was particularly devoted to demonstrating the affinity of bacteria for certain tissues. He selected patients with systemic disease supposedly caused by a dental infection. He isolated the bacteria from the dental infection, and injected it into an experimental animal, usually the rabbit. The animal then developed the same systemic ailment as the patient. Billings and Haden also reported that they were able to demonstrate this cause and effect type of relationship. Detailing all the work that substantiated the focal infection theory is beyond the scope of this paper.

Coincidental with the onset of the focal infection theory the dental x-ray machine was coming into practical use. For the first time, dental and medical practitioners were able to see the bone structure surrounding the teeth. They developed a sudden awareness of periapical pathosis and began to equate these areas to foci of infection. Consequently an onslaught was made on the pulpless tooth in an effort to purge the ailing
patient of all possible foci.

The fact that the dental and medical profession overreacted to the focal infection theory was recognized early by some. In 1916 Irons\textsuperscript{13} wrote,

\begin{quote}
The startling frequency of alveolar abscess and other extensive dental infection has so impressed many physicians and not a few dentists that they are ready to ascribe all human ailments to disease of the teeth, and to attempt to cure them all by the extraction of teeth. . .
\end{quote}

Howe\textsuperscript{14}, in 1919, refuted the whole theory very well.

However those holding to the theory did not roll over and play dead. Quite the opposite was true. The idea gained great momentum and was a very essential factor in the practice of medicine and dentistry for several decades. That this practice frustrated many of the early dentists that tried to conserve teeth, especially the pulpless tooth, is obvious. They had essentially three tools in their armamentarium on which they could rely upon for evidence to disprove the focal infection theory with regards to the pulpless tooth. These were histologic studies, the radiograph, and the endodontic culture. This was substantiated by Rhein\textsuperscript{15} in 1917, when he lectured to a meeting of the American Medical Association on a way to retain pulpless teeth, without the danger of retaining the tooth as a foci. The thrust of his talk was based around the culture technique he had developed as a way of demonstrating sterility of the root canal and periapical tissues. Then Crane\textsuperscript{16} in 1918 expressed much the same feelings. He felt the medical profession was open minded and was just waiting for substantial proof. He also felt that the radiographic evidence of healing took too long to establish,
consequently the culture method was the only immediate way in which the sterility of the periapical tissues could be demonstrated. Coolidge\(^\text{17}\) also made a very strong case for the culture technique, to be followed in later years by Appleton,\(^\text{18,19}\) Grossman,\(^\text{20}\) Yates and Morse,\(^\text{21}\) Filqueiras,\(^\text{22}\) and others too numerous to mention.

Culturing The Periapical Rarefaction

As mentioned earlier, with the introduction of the Roentgen ray into the dentists' diagnostic armamentarium previously undetected periapical radiolucencies were found. The proponents of the focal infection theory considered these areas to be infected. Thoma\(^\text{23}\) in discussing the Roentgen examination referred to these areas as blind abscesses and considered them all to be infected even if the infection was of a latent character. This was the prevailing theory. However, many skeptical and probing minds found it necessary to study the bacteriologic status of these areas, with interest towards either proving or disproving the focal infection theory.

Several means were employed to obtain a culture of the periapical tissues, by extraction or with the tooth in situ,

1. by going through the root canal.
2. by the use of a dental trocar.
3. by periapical resection.

The possibilities of contamination that exist with all of these methods are obvious.
Coriel\textsuperscript{24} intent upon disproving the diagnostic worth of the dental radiograph, because he felt it had led to the needless slaughter of too many teeth, devised the dental trocar. He sterilized the mucosa and then went through the mucosa and bone with his instrument to culture the periapical tissues. He obtained negative results from teeth which were radiographically positive and positive results from teeth which were radiographically negative.

Appleton\textsuperscript{18} presented the various ways of culturing a tooth upon extraction,

1. from the socket.

2. from the apex of the tooth itself.

3. from the adherent sac.

Rhein,\textsuperscript{15} Prinz,\textsuperscript{25} LaRoche,\textsuperscript{26} Crane,\textsuperscript{27} and Rickert\textsuperscript{28} all put forth ways of culturing the periapical tissues through the root canal. LaRoche's technique was particularly interesting because he actually sealed culture media into the canal for 24 hours before taking his sample.

Head\textsuperscript{29} had a rather barbaric way of insuring that he got positive results from his technique. He made an opening into the periapical tissues through the mucosa by the use of cautery and a drill one day. Two days later he reentered the same area for his culture sample, when there were sure to be bacteria present due to the lowered resistance of the tissue.

Canouse,\textsuperscript{30} Holms,\textsuperscript{31} Garvin,\textsuperscript{32} and Appleton\textsuperscript{18} all described techniques that were essentially root resection techniques, modified to obtain
a culture. Cohen\textsuperscript{33} simply penetrated the tissue with a sterile needle on a hypodermic syringe and went into the periapical tissue to aspirate his sample.

Berwick\textsuperscript{34} disturbed by the lack of uniformity and a reliable bacteriologic technique of previous investigators, carried out what seemed to be a fairly well controlled experiment. Employing a root resection technique after sterilizing the surface mucosa, he studied 71 radiolucent lesions. He found that 9 of these lesions were sterile and commented that his evidence did not corroborate the prevailing feeling as stated by Thoma. He also could not establish any definite relationship between dental infection and systemic disorders except in a very small percentage of the cases.

Gilmer\textsuperscript{35} in a study that was histologic rather than bacteriologic, concluded that pathological zones about the roots of pulpless teeth did not always contain bacteria, and those that did were often so completely walled off by a connective tissue capsule and sclerosed bone, that dissemination of bacteria was difficult.

Haden\textsuperscript{12} substantiated Rosenow's selective localization in experimental animals in selected cases, but by using the extraction technique to collect his sample he came up with the same results as Coriell. Therefore he concluded that the pulpless tooth was not necessarily infected.

Hardnt\textsuperscript{36} used extracted human teeth with attached periapical lesions in his study and concluded that all solid granulomae with no tears in the
fibrous capsule were sterile. He found no bacteria within the granulomae and observed them only on the surface of the lesions as contaminants. He always found bacteria in granulomae and cysts that were acutely inflamed and had caused clinical symptoms of pain.

Bulleid\textsuperscript{37,38} took a stand somewhere in the middle in the focal infection theory debate. He felt it was impossible to sterilize a "dead tooth", and therefore it would always be a potential source of infection. However, the medical condition of the patient tempered his decision to extract. He studied the bacteriological status of 400 periapical granulomae by the extraction method and concluded that the granuloma was always infected. But, he was not able to show the selective localization described by Rosenow when the isolates were injected into experimental animals.

Hatton\textsuperscript{39} also reported bacteria in granulomae at the apices of pulpally involved teeth but that not all pulpless teeth were infected.

Lucas\textsuperscript{40} reported that 90\% of all previously infected periapical tissues remain infected even though they might have been temporarily sterilized. He labeled as radical those dentists that wanted to save the pulpless tooth in light of all the evidence that implicated such teeth as the etiologic factor in a myriad of systemic diseases.

In 1931 Freeman\textsuperscript{41} concluded, from histopathological studies on dental granulomae, that from the focal infection standpoint there was undoubtedly no question that bacteria or their toxins were not limited by the fibrous capsule. He felt there was a direct communication between the
inner portion and the circulation via a capillary network in the capsule.

Grossman 42 modified Coriell's dental trocar for use with a straight handpiece in still another attempt to provide a sterile avenue of approach to the periapical tissues. His concern was to determine the status of a tooth suspected of being a focus of infection, and also whether sterility of the apical tissues, if once obtained, could be maintained over an extended period of time. In that same year, Cramer and Reith 43 reported that granulomae are invariably infected. They used the extraction method to collect their sample.

In 1934, Boyle 44 reported on another histologic study involving 63 cases where he used Giemsa, Gram, and eosin methylene blue stains. He actually demonstrated the presence of intracellular gram positive bacilli within the phagocytic cells of a solid dental granuloma. Because they were intracellular, he was confident that they were not contaminants. He expressed a skepticism of the studies that supported the focal infection theory and felt that most of their positive results were due to bacterial contamination. Also, in that year, Mela 45 cultured 500 intact extracted periradicular granulomae. He found nine sterile lesions. All the rest yielded streptococci.

Stein 46 used extraction and apicotomy to collect his samples which showed that 27% of 300 pulpless teeth were sterile. Of those that had complete root canal fillings, 50% were sterile. He felt that good root canal treatment was in order and that routine extraction of all pulpless teeth
was an indefensible procedure.

Ellingham operated in the belief that restored vital teeth could be chronically infected even though they were asymptomatic and radiographically negative. He studied 42 such teeth by the extraction method and found that 57% were infected. He believed that these teeth presented a much more serious problem than the frankly dead tooth in relation to systemic disease. He felt the reason some systemic diseases did not respond to the extraction of pulpless teeth was that the real culprit was the chronically infected vital teeth which had been overlooked. Taylor and Bulleid also were able to grow streptococci from apparently normal pulps, despite rigorous precautions to prevent post-extraction contamination.

However in 1936, Fish and Maclean were able to show that the histologic picture of the supposedly infected pulp tissue was incompatible with the theory that they contained bacteria. They showed bacteria in the vessels and lymph spaces of teeth extracted without cauterization of the periodontal tissues and postulated they were pumped there by the forces of extraction. This work was really a monumental step towards the salvation of the pulpless tooth. In addition to the above, they made several other conclusions.

1. In the case of an infected pulpless tooth, the infection is confined to the root canals or the pus of an associated chronic abscess.

2. The surrounding bone and soft tissue were sterile, though irritated by diffusible toxic products.
3. The bacteria must have a necrotic nidus in which to grow in the tissues, otherwise it provokes an acute reaction or is immediately destroyed.

Kronfeld\textsuperscript{51} in a histobacteriologic study of pulpless teeth, always found bacteria in the canal, but the granulation tissue at the apicies was often free of microorganisms. He concluded that cysts and granulomae are sterile in a large percentage of the cases. He felt that the granulation tissue destroyed bacteria because it was a healing tissue.

In 1937, Grossman\textsuperscript{52} felt that the peak of the focal infection wave had passed. He presented evidence that root canal therapy was a safe procedure compatible with bodily health, if done properly. He also predicted that the future success of root canal operations would depend to a great extent upon a bacteriologic examination of the tooth to determine its sterility before filling the canal.

Fish and Maclean\textsuperscript{50} plus the work of Tunnicliff and Hammond,\textsuperscript{53} and Kanner\textsuperscript{54} virtually discredited all previous bacteriologic studies of the teeth when done via the extraction method.

Burket\textsuperscript{55} studied periapical lesions at autopsy with a modified root resection technique to obtain his culture sample. He found that 49\% of the granulomae were infected.

The classical work of Fish\textsuperscript{56} although not directly involving a pulpless tooth and periapical tissues, vividly depicted the fate of microorganisms in relation to viable tissue. By drilling a hole in the bone and
placing into it a bacterially contaminated cotton pledget, he demonstrated the development of four distinct zones.

1. A zone of infection: where the polymorphonuclear leukocytes rapidly controlled the organisms.

2. A zone of contamination: free from infection but poisoned by the soluble toxic products of the infection.

3. A zone of irritation: characterized by active phagocytosis.

4. A zone of stimulation: where a fibrous tissue capsule was being developed.

He felt that this confirmed that infection was confined to the necrotic nidus and was not present among the living cells. Sommer and Crowley\(^5\) also published data contrary to the general belief that all granulomae were infected. Figg, et. al.\(^5\) also substantiated the work and conclusions of Fish.

Considerable light was shed on the idea of residual periapical infection after root canal therapy by Hedman\(^5\) in 1951. By using only anterior teeth with periapical rarefactions that had both infected periapical tissues and root canals, he was able to show that when the root canal had been sterilized by endodontic procedures, the periapical tissues were also sterile. His method of sampling the periapical tissues was done by inserting a sterile cannula through the root canal to the apex of the tooth. Then sterile wires were passed through the cannula to the periapical tissues thereby avoiding contamination from the root canal contents. He also
verified his results by tissue gram stains of the periapical tissues after
curettage.

Grossman\textsuperscript{60} did another study of the bacteriologic status of periapical
lesions using the root resection technique. He operated 150 cases but had
reliable data in only 109 of these cases. Of these 109 cases, 85.3\% yielded
negative cultures. In 49 of these cases, he had also cultured the canal
and got only 18.2\% negative cultures. He speculated as to why he would
get a negative culture from the canal and still have a periapical lesion.
He felt that toxic products from tissue breakdown could also cause bone
destruction, and possibly that bacteria present initially had used up
their nutrients and were no longer viable.

Shindell\textsuperscript{61} was not convinced by the work of Hedman. He felt that
Hedman had contaminated the periapical tissues by his canal debriding
procedures before taking his periapical culture. Therefore the experi-
ment was repeated using essentially the same technique but with greater
care in the debridement procedures. Shindell was able to get only 3 posi-
tive cultures from the periapical tissues out of 63 cases. All but one of
these cases showed a positive root canal culture. Melville and Birch\textsuperscript{62}
using the root resection technique came up with essentially the same
results as Shindell. They found that the periapical area may be sterile
even when bacteria were present in the pulp canal.

In 1972, Winkler, et. al.\textsuperscript{63} still felt that the issue of what really
caused a periapical radiolucent lesion had not been satisfactorily resolved.
Was it the bacterial toxins and autolytic pulpal products within the canal, or rather an actual bacterial invasion of the periapical tissues? They used a modified Gram tissue stain on histologic sections of 15 intact periapical lesions collected by extraction. They checked the integrity of the fibrous capsule both clinically and histologically. All fifteen lesions were diagnosed histologically as granulomae and one of these showed cyst formation. They found bacteria were dispersed uniformly throughout the lesions in slight to moderate concentrations in all but two instances. The single cyst also had bacteria present within its cavity. They also noted necrotic debris within the root canal which was heavily infiltrated with bacteria. In each specimen, the heavy infiltrate of bacteria within the canal stopped at the apical foramen. Frequently, bacterial contaminants were detected on root surfaces and on the outer surfaces of the capsules. This study has a lot of credence because of the rigid criteria they established for bacterial identification, the most impressive of which was, the inflammatory cell response of the tissues had to be present in the same plane as the bacteria, and adjacent to or engulfing the microorganisms. This study would be in agreement with Hedman because he was able to culture bacteria from the periapical tissues. However, it was not in total agreement with the work of Fish and Maclean.

As can be seen from all these conflicting reports, there still is no agreement on the issue. The extremes go from 100% infected to 100% not infected.
Improved Prognosis As Related To Negative Culture Results

Many studies have been published over the years to point out the efficacy of obtaining a negative culture prior to filling the root canal. The first of these articles was written by Appleton\textsuperscript{64} in 1932, but it was actually an analysis of the data published by Rhein, Krasnow, and Gies\textsuperscript{3} in 1926. Of the 340 cases that were filled after obtaining a negative culture, 94\% were judged successful clinically and radiographically. No cases were considered successfully treated unless all radiolucencies had disappeared and there had been a regeneration of osseous tissue. No specific post-operative time interval was used in this study. In 152 cases the canals were filled while the cultures were still positive and 84\% were successful. Their differential was 10\%. Rhein, Krasnow, and Gies used an aerobically incubated blood agar medium for their cultures. Appleton had always advocated the desirability of applying bacteriologic methods to check the treatment of periapical infection just on general principles, but this, he felt, gave him substantial evidence for his case.

Buchbinder\textsuperscript{65} compared the success of cases that were filled after obtaining a negative culture to the success of cases that were filled without the benefit of a culture. These were all done by students and the failure rate was 8\% of the 151 cultured cases and 18\% of the 94 non-cultured cases. These studies were not done simultaneously and ran over a period of eight years. Here again, there is a 10\% differential, but there was no way of
telling whether the uncultured cases would have yielded positive or negative results. An aerobic broth medium was used to culture these teeth.

In 1961, Abramson made a report of 135 cases done over a three year period. He required two consecutive negative cultures to fill and had 96.6% success rate as opposed to 84.2% success when the teeth were filled after obtaining two positive cultures. He used clinical and roentgenographic criteria one year postoperatively to determine success or failure.

Oliet used only the culture results he obtained immediately prior to filling the root canals of 98 teeth. Of those 98 cultures were positive and 31 negative. His figures showed a 94% success with negative cultures as opposed to 79% success with positive cultures. He used a trypticase soybean broth with 0.1% agar as his culture media and incubated them for 72 hours at 38°C. He filled the anterior teeth with gutta percha, either single cone or laterally condensed, and the posterior teeth were filled with silver cones, as were small tortuous canals. No other factors such as quality of fill, etc., were used in analyzing the results.

In 1963, Zeldow and Ingle published the results of a two year follow up study of 89 single canal teeth treated by the endodontic staff at the University of Washington. The teeth were cultured immediately after the access cavity was made, and then they were cleansed and shaped using sterile water as an irrigant. A sterile paper point and cotton pellet were sealed into the canal and the patient reappointed. At the second appointment,
48 hours later, the paper point which had been sealed in the canal was cultured plus an additional culture was taken. If the tooth was comfortable, it was filled with laterally condensed gutta percha at that time. The culture media used was brain heart infusion broth incubated aerobically for 48 hours. At the time of filling, 67% of the teeth yielded positive cultures. They were able to follow 67.7% of these for two years, and 83.3% were judged successful on the basis of negative posttreatment history, clinical, and roentgenographic evidence. Of the 27 teeth that yielded a negative culture they were able to follow only 51.8% for two years. All except one of these were judged successful. Since their sample size was too small to allow for statistical analysis, they chose to compare their results of the positive culture cases to the results of a couple of surveys that showed a success rate of 94.5% and 95.9% when the conventional approach to endodontics was employed.

In still another study published a year later, Engstrom, et. al., used a more conventional approach. They used only teeth which had yielded a negative culture on a previous visit, but had turned to positive on the fill appointment. Dextrose serum broth and thioglycollate broth were used as the culture media in all cases. The cases were followed for four to five years and they tried to correlate their results to other factors which contribute to failure such as overfill, non-negotiable canals, and large radiolucencies. They followed 137 teeth that yielded positive cultures and judged 24.1% as unsuccessful, 6.6% as uncertain, and 69.3% as
successful. Of the 169 teeth that yielded negative cultures, 10.7% were considered unsuccessful, 6.5% as uncertain, and 82.8% as successful. They found that this difference was statistically significant at the .01 level. Oliet and Sorin published a statistical analysis of 360 cases which they had done. In this study they also analyzed such factors as diagnosis of tooth, tooth distribution, age of patient, and root canal filling materials to see what effect they had on success or failure. They found that any such differences that did occur were due to chance and therefore the differences in healing or retrogression that they sustained were dependent upon culture results. Their figures showed a 94% success rate when a negative culture was obtained and a 78.9% success rate with a positive culture, a differential of 15.1%. They concluded that the elimination of cultures, without substitution of a better technique to evaluate the root canals bacteriologically, is indefensible.

Despite the better success rate shown by these authors by obtaining a negative culture before filling the canal, a large percentage of the teeth filled with a positive culture still gave successful results. This would indicate that healing can take place, even in the presence of bacteria, and other factors must be involved in deciding the ultimate fate. Torn I summarized this concept very well. In reviewing the role that microorganisms play in endodontic disease, he concluded that there are several etiological factors which can delay or prevent healing of the periapical tissues, and the presence of microorganisms is one of these factors.
However, their effect seems to be secondary and cumulative and they must coexist with some factor that would prevent repair. He felt that not all microorganisms would have an influence and even those that did would have to be present in critical numbers.

Many studies have been done that show the efficacy of the culture although they have not been directly correlated with percentages of success or failure. Grossman\textsuperscript{72} showed that without taking a culture but using criteria such as appearance and odor of the dressing, a clinician was able to guess the correct microbiologic status of the root canal only 58\% of the time. He concluded that with only a slightly better than even chance for correctly guessing the status of the root canal, a great need for bacteriologic control seemed apparent.

Dixon and Rickert\textsuperscript{73} did a histologic study on the results of root canal therapy on dogs' teeth. They purposely infected these teeth with streptococci from a clinical case and waited for lesions to develop. After lesions were apparent radiographically they instituted endodontic therapy and filled the teeth after obtaining two consecutive negative cultures. They then observed the periapical tissues histologically one and a half years later, and noted regeneration of cementum in areas of resorption with a normal periodontal membrane over the secondary cementum.

The Endodontic Culture Challenged

The fact that healing can take place in the presence of bacteria was demonstrated by Kitamura\textsuperscript{74} in 1956. He attempted to sterilize previously
infected root canals in dogs with the usual endodontic medicaments, and then filled these canals with a calcium hydroxide paste. These procedures were evaluated by a histopathological and a histobacteriological study of the periapical tissues. The report stated it was almost impossible to exterminate bacteria in the deep part of the dentine wall or from the cementum lacunae, and the bacteria propagated towards the surface as the medicament weakened. However, periapical repair was the same whether or not microorganisms were present in the root canal, and the microorganisms tended to die out in time. These findings were supported by the work of Kukidome and Matsumoto.

In 1963, Seltzer, et al. published the results of a study on the factors affecting successful repair after root canal therapy. Here again a certain amount of doubt was cast on the importance of the negative culture. They found that in teeth without areas of rarefaction, there was no statistically significant difference between success of repair in teeth yielding positive cultures at the time of filling and those yielding negative cultures. However, in teeth with lesions the underfilled canals or those filled flush with the apex had a more favorable prognosis when negative cultures were obtained. Other interesting observations resulted from this study. One was the extent to which the degree of filling affected success. Those overfilled were significantly less successful, 70.6%, than either the underfilled or flush filled case, 87%. They also showed a statistically significant difference in the success rate of the tooth without preoperative area of
rarefaction, 92%, as opposed to the tooth with an area, 76%. This brought to light factors other than the culture results which might affect repair.

In a histologic study of the periapical tissues of dogs Seltzer, et. al. 78 compared the healing after filling with a negative culture to the healing after filling with a positive culture. They concluded that there were no discernable differences in the reaction of the two groups. They found that the most severe reaction occurred as a result of overfilling the root canal.

The real bombshell was dropped on the endodontic community that same year by Bender, et. al. 79 It was this article that touched off the ensuing culture debate. They concluded from a study designed to answer the question of the value of cultures in endodontic therapy, that success is dependent on many factors, but culture results is not one of them. The presence or absence of microorganisms within the root canal prior to filling appeared to have surprisingly little effect on the eventual outcome. The end results appeared to depend more on method and completeness of canal filling than on sterility. These results agreed very favorably with Kitamura.

In 1970, Morse 80 published a non-scientific article in which he critically evaluated the culture technique. He discussed many of the ramifications of the culture, and the fallacies or possible alternatives. He established his criteria for filling a root canal as a tooth which is asymptomatic and in which the canals are clean, shaped and dry. He did
emphasize that, although it is not necessary to culture, it is essential to reduce the microbial population to as low a level as possible.

Although not directly challenging the culture technique there have been many publications that have cast doubt on its veracity. Some of these articles revolved around the false negative cultures obtained when using antibiotics as intracanal medicaments.

Buchbinder and Bartels \(^{81}\) agreed with Seltzer and Bender \(^{82}\) that a mixture of streptomycin and chloramphenicol was a promising combination of antibiotics for endodontic therapy, but they concluded that the bacterial culture method of evaluating the sterility of the canal after their use was unreliable, and should not be used until an agent which would neutralize their effect in a medium could be found. Therefore, any appraisal of the efficacy of these antibiotics had to be largely clinical in nature.

Bender and Seltzer \(^{83}\) showed that where antibiotic combinations were employed a 13% margin of error could be expected when culture results were used as a criterion of the bacteriologic status of the root canal. This was true provided that the cultures were incubated for one week. If incubated only 48 hours, the percentage of false negatives would be 31%. Champhorated monochlorophenol and sodium caprylate were sufficiently diluted by 10 ml. of broth so as not to give false negative cultures.

Even among the people who use it, there is considerable disagreement as to how the culture should be taken. Bender and Seltzer \(^{83}\) suggested
that if a combination of penicillin, bacitracin, streptomycin, and sodium caprylate (PBSC) as used at the University of Pennsylvania, was used as a dressing, the first three absorbent points should be discarded to lower the incidence of false negative cultures.

Garber using single-canal teeth not previously treated, showed that wiping out root canals with paper points before taking a culture considerably reduced the accuracy of the culture result. He concluded that the first and second points used for obtaining cultures were the most reliable. He speculated that this effect might be more pronounced in treated teeth because the quantity of organisms would have been reduced by mechanical cleansing and drugs. Yet Ingle proposed that when intracanal medicaments are present, the third or fourth paper point, moistened at its terminus for a distance of approximately 1 to 2 mm, be used to minimize the chance of medicament transfer to the culture tube.

Serene and McDonald did a statistical study on culturing root canals treated with nonspecific medicaments and concluded that wiping out the canal with paper points before taking a culture apparently reduces the inoculum, and thereby significantly reduces the accuracy of the culture results. When only one paper point was used, the first point gave the most reliable results. They also showed that a combination of points number 1, 2, and 3 in the same culture tube may be the most desirable combination.
Another phenomenon that has confused the culturists and questioned their creditability is that of the culture reversal. Cognizant of this possibility Yates and Morse, Dixon and Rickert, Buchbinder, Appleton, Moller, Ingle and Zeldow, and Sommer, Ostrander and Crowley all recommend that two consecutive negative cultures be obtained before a root canal is filled. However Van Amerogen showed that the probability of the next test being positive is the same whether one or two negative cultures had been obtained earlier. This was corroborated by Seltzer who found a 16.6% culture reversal rate after the previous culture had been negative, and by Engstrom and Lundberg who showed a 16.2% reversal rate after two consecutive negative cultures, and a 21.2% reversal rate after one negative culture. The differences in the latter study were not significant. They did find three factors that had an influence on the reversal phenomenon:

1. The clinical and radiographic diagnosis.
2. The condition of the crown.
3. The time elapsed between the negative tests and the root filling.

Nevertheless it becomes obvious that 16 to 20% of the culture results are going to be positive when root canals are filled.

Still another aspect of the culture technique which will produce equivical results is the type of culture media used and the conditions of incubation. Hobson summarized the problem very well. She felt that the present-day culture procedures were inadequate to reveal the complete
bacterial flora of the canal, because it is a well-known microbiologic fact that all organisms will not grow in the same medium. Although the media used in endodontics will support the growth of a large portion of the bacterial flora, an element of uncertainty exists in regards to fastidious organisms present in the canal or periapical region.

The early endodontists were concerned with only one species of bacteria and that was the streptococci. This concept was still subscribed to as late as 1950 by Appleton. Consequently, they needed only a medium that would grow streptococci. However even when they obtained negative cultures they still experienced some failures. Convinced that these failures were due to microorganisms in one way or another more sophisticated culturing techniques were developed.

Grossman described both a smear and a culture technique as bacteriologic examinations for the pulpless tooth. At that time he felt hormone broth, Rosenow's glucose brain broth, Rosenow's liver broth, or Holman's were satisfactory culture media.

Buchbinder was concerned with elevating root canal therapy from the realm of pure empiricism to the status of a surgical science. This was necessary because of the rise of the concept of focal infection. He felt the bacterial culture of the root canal, as a final proof of sterility, was an essential step in this progressive approach. He described the use of brain heart infusion broth in small screw cap tubes as a suitable media, but did express some concern for the anaerobes.
In 1940, Brewer\textsuperscript{95} described a thioglycollate medium, for the growth of anaerobes, that did not require elaborate anaerobic cultivation equipment. Crowley\textsuperscript{96} compared Brewer’s medium to the standard aerobic media. There were 143 cultures taken of teeth in various stages of treatment, 92 were negative in both, 24 positive in both, 10 positive only in the aerobic medium, and 12 positive only in the anaerobic medium. Despite these figures she felt the differences in the two media did not seem significant and concluded that strict anaerobic conditions are not necessary for the routine cultivation of bacteria from infected root canals.

Shay\textsuperscript{97} extended Crowley’s experiment a little further and compared six media, trypticase dextrose at pH 7.2, brain heart infusion, serum dextrose broth, brain agar, Brewer’s thioglycollate, and trypticase dextrose at a pH 5.5. He cultured 709 root canals and found 184 positive cultures. Trypticase dextrose at pH 7.2 revealed the highest percentage (90.6\%) of positives and brain heart infusion the next highest (86.1\%). However these two media produced positive results in 97.8\% of all the positive cultures. Therefore these two media were deemed sufficient for practical purposes. This resulted in the use of two media in some circles.

Slack\textsuperscript{98} became interested in the anaerobic flora of root canals. He used Robertson’s meat medium which was the anaerobic culture medium of Britain. He found 61 anaerobes out of 514 positive cultures.

Leavitt, Naidorf, and Shugaevsky\textsuperscript{99} were able to obtain five positive cultures out of 35 cases that had already given two consecutive negative
cultures when tested with routine aerobic culture media. They felt that these undetected anaerobes might be the cause of some of the unexplained failures in root canal therapy. They tested trypticase soy broth to which they had added 0.1% agar and found it to be more sensitive for aerobic growth than the other media tested. In addition, it was about 1000 times more sensitive for anaerobic growth than Brewer's thioglycollate with agar. Their hope was that this medium might eliminate a small percentage of endodontic failures.

Cobe, Chilton, and Kaufman\textsuperscript{100} did some further work on the trypticase soy broth with agar. They modified the amount of agar to 0.2% and decided that it was not only the most suitable for aerobic and anaerobic growth, but also for growth of yeast forms. They felt the use of this was preferable to the use of two separate media.

Not only were anaerobes of special concern but other more fastidious organisms were also detected in root canals. Mazarella, et. al.\textsuperscript{101} detected spirochetes in 13 of 21 samples examined. Brown and Rudolph\textsuperscript{102} were able to show that microbial forms were present in 90% of the unexposed canals of the pulp-involved teeth that they examined using phase contrast and dark-field microscopy. When they used direct stained smear technique, they found only 71% of the teeth had organisms. Spirochetes which were not cultivable were observed microscopically. They also showed the incidence of obligate anaerobes to be 24%. They concluded that the culture methods currently used were not entirely adequate to reveal the complete
bacterial flora of pulp canals. Hampp\textsuperscript{103} working with Brown and Rudolph, found spirochetes in 21 of 38 samples examined. Out of these he was able to cultivate ten pure strains of the small type of treponemes. Cran\textsuperscript{104-106} had been undaunted by the possible presence of anaerobes in previous studies but finally added an anaerobic medium, Robertson's cooked meat medium, to his study in 1956. He found 18\% of the bacteria isolated to be anaerobes.

MacDonald, Hare, and Wood\textsuperscript{107} studied only the flora of intact teeth devitalized by trauma. They used two media for their initial cultures, Connaught penicillinase dextrose broth incubated aerobically and Difco thioglycollate broth incubated anaerobically in a Brewer jar. They found 32\% of the organisms isolated were obligate anaerobes, and postulated that anachoresis was the route of infection.

Van Amerogen, et. al.\textsuperscript{108} compared various media. They found brain heart infusion with 0.05\% agar in screw-capped bottles was quite satisfactory and permitted the growth of most common aerobic and anaerobic organisms. Two years later they added 0.1\% and found that they could increase the percentage of lactobacilli growth from 2 to 8\%.

After noting from previous studies that the percentage of anaerobes was much higher than had been originally thought, Leavitt, et. al.\textsuperscript{109} were anxious to test their trypticase soy agar broth in a clinical situation. They found it disclosed anaerobes in percentages comparable to those
shown by strict anaerobic technique, that being approximately 33%, and at the same time it was particularly sensitive for most aerobic growth.

Despite all these previous studies Hobson did another study on the types of organisms isolated from the necrotic root canal before any treatment had been given. She took two initial cultures, one in nutrient broth and the other in Robertson's meat broth. If growth occurred she subcultured it on to horse blood agar and incubated these both aerobically and anaerobically. She concluded that anaerobic cultivation does not increase the number of organisms found. However, a year later, Shovelton and Sidaway using the same anaerobic medium found 25 of the 110 positive cultures they studied had only anaerobic organisms. An additional 50 cultures contained both aerobic and anaerobic organisms. They felt that the practice of incubating cultures from root canals aerobically only must be viewed with grave suspicion.

Sciaky and Sultzen expressed feelings similar to Shovelton but arrived at their conclusion by using two totally different media, brain heart infusion and thioglycollate containing 0.1% agar and 1% glucose.

Engstrom and Frostell limited their study to the cultures taken on the initial opening from 36 intact non-vital teeth. They used various media and cultured organisms from 21 of the teeth. In every instance where growth was observed, there was growth in Brewer's thioglycollate medium. An additional five cases showed bacteria on smears. Although no figures were given, they stated that most of the strains isolated were
anaerobic. Consequently they stressed the importance of an anaerobic technique. It should be noted here that the thioglycollate medium used had been modified from the one that Brewer initially proposed in 1940. They added a 20% cystine solution, dextrose, a protein hydrolyzate, and yeast extract which greatly enriched the medium allowing for better growth potential.

Crawford and Shankle point out the efficacy of the enriched thioglycollate medium in an extensive bacteriologic study of the root canal flora of open and closed teeth as compared to the flora of the oral cavity. They placed their initial culture samples in a transport media. Then each sample was streaked on the three different enriched agar plates and incubated anaerobically for 2 to 7 days. It was also transferred to an enriched thioglycollate medium and incubated aerobically for 7 days. Only one of the initial specimens that was negative in the thioglycollate medium was positive on the enriched solid agar, while almost 26% of the total root canal specimens that were positive in thioglycollate were negative on agar plates. In addition to the culture methods they also subjected each specimen to:

1. phase-contact microscopic examination of wet mounts.
2. dark-field microscopic examination of wet mounts.
3. bright-field microscopic examination of gram-stained dry smears.

They too detected microorganisms microscopically which could not be detected by cultures alone. When noncultivable spirochetes were found,
they were always in combination with other cultivable flora.

In still another cultural and microscopic bacteriologic study, Sulitzeanu, et. al. \cite{115} found 22 forms from 101 teeth by the use of direct smears that could not be recovered by cultures. Also 25\% of the organisms they isolated were obligate anaerobes. Their specimens were also taken from closed canals immediately upon opening into the pulp chamber. They actually took three specimens from each tooth. The first point was placed into ascites broth containing 0.1\% agar, the second was used to make a smear, and the third was used to inoculate a blood agar plate which was incubated anaerobically. On the basis of this study, they recommended the combined use of ascites broth culture and direct smears for routine bacteriologic studies of root canals. They concluded that even when negative findings with both of these or with any other method are obtained, this cannot be regarded as proof of sterility since some organisms may be present but not detectable.

Contradictions and inconsistencies seem to be the rule rather than the exception when one reviews the literature concerned with culturing. It is easy to see why there is a controversy.

**The Importance of Bacteria in Pulp and Periapical Disease**

Even though there is a lot of controversy centered around culturing, the fact that bacteria play an important role in the condition of pulpal and periapical tissues cannot be denied. Although this had been postulated and assumed for years as far as the pulp was concerned, it wasn't
until the classical work of Kakehashi, Stanley, and Fitzgerald\textsuperscript{116} in 1965 that this was established as fact. In their experiment, they exposed the pulps in both conventional and gnotobiotic (germ-free) rats and histologically studied the pulp at time intervals varying from 1 to 42 days.

In the conventional rats, after 8 days there was complete pulp necrosis with chronic inflammation and abscess formation in the apical areas. In no instance was there evidence of repair. However, in the germ-free animals there were no completely devitalized pulps, pulpal inflammation was minimal and not a single abscess was found. In the latter cases, dentinal bridging was evident at fourteen days.

Using the same experimental model, Balick\textsuperscript{117} studied the additional effects of mechanical irritation on the periapical tissues. His findings were in agreement with Kakehashi, and in addition he found that the conventional rats, as compared to the germ-free animals, showed a progressively worsening inflammation in the periapical tissues when the root canal had been overinstrumented. He concluded that this was due to the presence of microorganisms.

Torneck\textsuperscript{118} used polyethylene tubes sealed at one end to simulate root canals. He filled the tubes with either sterile autoclaved muscle or autoclaved muscle contaminated with a gram-negative cocci, and implanted them into the dorsal subcutaneous tissue of Wistar rats, and studied their effect on connective tissue repair. He found that repair was least favorable around the contaminated tubes.
Another enlightening study on the relationship of bacteria to pulp and periapical disease was done by Korzen, Krakow, and Green, in 1974. They used monoinfected, otherwise, gnotobiotic rats and compared their reaction to the conventional rat. In their study, they exposed the pulp and overinstrumented the palatal canal of an upper molar with a number 10 file contaminated with saliva from that animal. Half were left open to the salivary fluids and the other half were sealed with amalgam. The tissue responses in the early specimens followed a similar pattern, however the differences were significant at later intervals even though microorganisms were found in all of the experimental teeth. They found that repair by the pulp was inversely related to the severity of the infection. The periapical tissue reaction seen could be directly related to the bacterial invasion of the root canal. Where microorganisms were found along the entire length of the root canals, the tissue reaction was severe; when the inoculum was limited so was the reaction. They also found that the inflammatory response to a monoinfection is much less severe than that to a mixed infection. Their conclusion to all of this was that the severity of the inflammatory response of the pulpal and periapical tissues can be related to the quantity of microorganisms within the root canal and the length of time of exposure to them.

Anachoresis

Anachoresis is defined as that phenomenon by which blood-borne bacteria, dyes, pigments, metallic substances, foreign proteins and other
materials are attached to, and fixed in, circumscribed areas of inflammation. This concept relates well to the effect that bacteria have on the health of pulpal and periapical tissues, and helps to explain how these tissues become infected when there is no apparent route of infection.

Csernyei\textsuperscript{120} was able to demonstrate in dogs that areas of chronic periapical inflammation were able to collect and maintain the vitality of the Bang bacilli when it was injected intravenously. He concluded that treatment of teeth after devitalization should be carried out in such a manner as to prevent chronic periapical inflammation.

Robinson and Boling\textsuperscript{121} made 27 deep cavity preparations in the teeth of cats and applied croton oil to irritate the pulp. This was followed by an intravenous injection of a suspension of bacteria from a few minutes to 20 days after cavity preparation. They were able to recover these bacteria by cultural methods from the pulps of 22 of the 27 experimental teeth, (82\%) and from only one of the 30 control teeth, (3\%). They suggested that since irritation of the pulp is known to result from various procedures and stimuli, and since transient bacteremia may occasionally be present in healthy individuals, some cases of postoperative idiopathic pulpitis may be the result of anachoresis. These two investigators\textsuperscript{122} had shown earlier, that self strangulation of the pulp was not feasible and that inflammation alone would not cause pulpal death.

Burke and Knighton\textsuperscript{123} and Smith and Tappe\textsuperscript{124} were able to recover intravenously injected organisms from the pulp of traumatized teeth in
rats. Gier and Mitchell\textsuperscript{125} used the dog as an experimental animal. They cut deep class V cavities and left half of them open to the oral fluids. In the other half, they sealed in croton oil. Immediately afterwards, they injected their test organism. They were able to identify the test organisms in Brown and Brenn stained tissue sections of these teeth, and also by means of fluorescent antibody-stained sections. In addition, they were able to recover and identify the test organisms from cultures taken from the pulps of these teeth. Bacteria were present to some degree in all stages of pulp inflammation from slight leukocytic infiltration to total necrosis. The greater the degree of injury, the more readily was infection demonstrated. The bacteria were absent from the uninjured control teeth. They assumed that any organisms involved in the pathosis of the unexposed pulp could be transported there by the blood circulation.

The fact that blood-borne bacteria can localize in inflamed periapical tissue was shown by Lio\textsuperscript{126} in 1974. He recovered, by culture, a labeled strain of \textit{Streptococcus faecalis} which had been injected intravenously, from the areas of periapical inflammation produced by endodontic over-instrumentation. He further substantiated his culture results by histologic sections of these areas stained with the Brown and Brenn stain. In these sections, darkly stained gram-positive cocci were evident in macrophages giving indication of their fixation in the area of inflammation.

On the bases of these studies it is evident that the phenomenon of anachoresis holds as true for the dental tissues as it does for other tissues.
of the body. It remains a force to be reconed with in the clinical disciplines of operative dentistry and endodontics.

The Effects of Root Canal Procedures on the Periapical Tissues

No study of the periapical tissues involved with root canal therapy would be complete without mention of the effects of these procedures on the periapical tissues, but it is another area that has caused considerable debate among endodontists. The basis of the argument is a lack of agreement on where the root canal stops and the periapical tissues begin.

Blaney,127 Grove,128 and Coolidge129 all felt that the ideal place to terminate the root canal preparation and filling was at the dentinocemental junction. Coolidge operated on the principle that in a canal containing vital pulp tissue free from infection, he tried to sever the pulp close to the apical foramen and then extend the fill to the pulp stump. He illustrated that conditions were more favorable for repair if this was accomplished. All these men were able to show a higher percentage of success in those cases in which the amputation was near the dentinocemental junction. Their cases were remarkable in that they exhibited a lack of inflammation and healing by way of cementum deposition. They also found that overfilling acted as a foreign body and sometimes led to failure. These findings were substantiated by Strindberg130 and Seltzer, et. al.77

Kuttler131 did a microscopic investigation of 402 apices of teeth in an effort to determine the location, direction, form, diameter, and
thickness of the cementum at the apical foramen. He found that most
endodontists were laboring under an erroneous concept because the
minor diameter of the root canal, the apical constriction, was usually
found in the dentine just before the cementum portion began, and from
that point it gradually widened taking on a funnel shape widest at the
apical foramen. He also found that the thickness of the apical cementum
varied from 0.5 to 1 mm. depending on the age of the patient. Because of
the funnel shape of the cementum canal, he reasoned that that portion
could not be hermetically sealed. Therefore he felt justified in filling
the root canal only as far as 0.5 mm. before reaching the foramen. How­
ever there are others, notably Schilder, 132 that still insist on preparing
and attempting to fill to the radiographic apex.

Nygaard Ostby 133 was interested in determining the role of the blood
clot in periapical healing. Consequently he instrumented past the apical
foramen in an effort to develop a blood clot, filled the root canals short,
and then studied the results histologically over a period ranging from
13 days to 3.5 years. He found his results were not influenced by the
original state of the pulp provided that debridement had been successful,
sterility of the root canal obtained, and a bleeding had been produced
prior to the insertion of the root filling material. The injury suffered
by the periodontal tissues healed in a short time. After 13 days there was
an inflammation in the periodontium around the clot, but after 35 days
the periodontal membrane above the apex showed complete healing in all
but one of 17 cases. However resorption of the root surface as a result of the reactive inflammation in the apical periodontium was regularly observed, and the repair of this seemed to proceed slowly. In some cases there was little or no indication of a deposition of cementum even though the blood clot had organized by an ingrowth of granulation tissue, and that the granulation tissue was gradually transformed into fibrous connective tissue.

Nyborg and Tullin[^134] studied the healing process histologically in the pulpal and periapical tissues after vital pulp extirpation. They considered these tissues to be healed if they were free of inflammation. They had only 17 teeth in their study and of these two pulps were completely extirpated and the rest were partially extirpated anywhere from 1.5 to 6 mm. from the radiographic apex. The two cases of complete extirpation, in which there were periapical wounds, were not healed. The periapical tissues in these teeth contained excess root filling material, bordered by granulation tissue, which was surrounded by a fibrous tissue capsule. Healing occurred in 10 of the 15 teeth in which partial extirpations were performed. The periapical tissues were not studied in these cases of partial extirpation. In no instance was it shown that the pulp wound had a better chance of healing if the wound was placed near the apex.

Using the mesial root of the lower first molar of the rat, Frausquin, et. al.[^135] studied the histologic effects of overfilling the root canal with
various materials. Regardless of the material used the response was essentially the same. Within 24 hours there was necrosis of the periodontal ligament, the adjacent alveolar bone, and the outer layer of cementum covered by the necrotic periodontal ligament. Within 4 to 7 days the entire thickness of the cementum was necrotic. The repair process took place at different rates depending upon the severity of the damage. Repair started with resorption of necrotic bone and removal of the involved periodontal ligament. This was replaced by highly vascularized tissue which resorbed the necrotic cementum. Later on, the cortical alveolar bone became newly formed, new layers of cementum were laid down, and the periodontal ligament returned to normal. By a vascular injection of India ink jelly they were able to show that the vessels of the periodontal ligament originate in the network of the alveolar fundus. They felt that the necrosis of the periodontal ligament in cases of overfilling may have been correlated to an infarct due to the obstruction of the vessels in the apical area.

Seltzer and his associates published an interesting series of articles dealing with the periapical tissue reaction to the various endodontic procedures. The first one in 1967 dealt with just pulp extirpation. They found that acute inflammation occurred in the remaining pulp tissue below the plane of severance. This was followed in one week by periapical tissue changes in the form of slight infiltrations of polymorphonuclear leukocytes into the periodontal ligament, and cemental and bone
resorptions in the vicinity of the root apex. This reaction was reduced considerably in canals that had been narrowed by secondary dentine and in those canals blocked by pulp stones.

The next study\textsuperscript{137} dealt with the effects of instrumentation. In one series the instrumentation was confined to the canal and in another series the cases were instrumented beyond the apices of the teeth. They found that for optimum results in cases of vital pulp extirpation, instrumentation should be confined to the root canal. This would maintain the vitality of the pulp stump in most cases, and this in turn would elaborate a cementum-like tissue which would repair the resorptions which occurred initially. Repair occurred in an orderly manner up to about 6 months. After longer periods, in the absence of a root canal filling, repair was impeded.

However when the canals were overinstrumented the vitality of the apical pulp stump was lost, and the periapical inflammatory reaction was much more severe. Large periapical granulomatous lesions invariably developed within a few weeks after instrumentation. These lesions persisted and the resorptions of the root were not usually repaired. In many lesions the inflammation stimulated the proliferation of the cell rests of Malassez and there was a profuse growth of stratified squamous epithelium, representing initiation of cyst formation.

In their third report\textsuperscript{138} they filled cases that had been instrumented short of the apex. They found that the presence of the root canal filling
appeared to enhance repair in most of the cases. The results were not as favorable when the filling material was forced beyond the apical foramen, although some repair was still evident in all cases in which this was done. They concluded that the best results are obtained when the apical pulp stump retains its vitality and no foreign material is impinged on the pulp or periapical tissues.

Bhaskar and Rappaport\textsuperscript{139} devised a histologic study to examine the effects of poor endodontic therapy on teeth with pre-existing periapical pathosis. They used the dog as their experimental animal and overfilled and underfilled with both silver and gutta percha. Some were just debrided and left with only an occlusal seal. They found a much more severe reaction in the teeth that were overfilled. These teeth showed bone destruction, root resorption, and a denser and wider area of inflammatory infiltrate. The silver overfills seemed to perpetuate the existing periapical lesions. The underfilled teeth and those not filled at all gave a similar histologic appearance. They concluded that underfilling is usually preferable to overfilling the pulp canal. An important finding of this study was that root resorptions following pulp extirpation occurred consistently but were seen only rarely in radiographs.

Davis, et. al.\textsuperscript{140} also did a histologic study on dogs to determine the reactions of the periapical tissues when canals were underfilled, filled to within 1 mm. of the radiographic apex, and grossly overfilled following vital pulp extirpation. Here again the most severe reaction was
found in the overfilled cases. The excess material was associated with advanced destruction of surrounding tissue and liquefaction necrosis. Lymphocytes, macrophages, and plasma cells abounded. However they found proliferating epithelium in only one case. An interesting observation of this study was made in the cases that had been prepared to or beyond the apex but filled approximately 3 mm. short. Healing was excellent in this group and was of three basic types. Where dentine had been plugged into the apices during preparation there was no inflammation in the periodontal tissues, the bone was intact, and new cementum had been deposited over the dentine filings. In the second type of healing, bone had been deposited within the unfilled portion of the canal and there was no evidence of inflammation. The third type of healing consisted of the formation of a complete attachment apparatus within the unfilled portion of the canal, including alveolar bone, periodontal ligament, and secondary cementum. Again there was no evidence of inflammation or bone destruction.

In spite of this favorable response to the widely opened apical foramen, they felt that this procedure would be clinically unfeasible due to the considerable post-operative discomfort it would cause.

**Rationale for the Use of Streptococcus faecalis**

The choice of *Streptococcus faecalis* as the bacteria for contamination of the root canals in this experiment was based upon reports in the literature. It has been well documented by numerous investigators that this
strain of bacteria is particularly troublesome in root canal therapy.

Houston\(^{141}\) was one of the first to report on the isolation of enterococci from septic root canals. Also Thompson and Megrai\(^{142}\) list a septic tooth as the source of one of the strains of enterococci which they studied. Later Evans and Chinn\(^{143}\) studied the same strain of enterococci that had been isolated by Thompson and Megrai. However none of these studies reported the incidence and persistence of this bacteria in root canals.

Williams, et. al.\(^{144}\) identified the microorganisms of positive cultures obtained over a period of one year at the endodontia clinic of the University of Pennsylvania. These cases had been treated with topical application of a combination of penicillin and streptomycin in high concentration. They found that 14.2% of the microorganisms belonged to the enterococcus group of streptococci, principally \(S.\ faecalis\), and noted that in many instances these microorganisms were cultivable simultaneously from the saliva of the same patient.

In a study designed to test the effectiveness of various antibiotics and combinations of antibiotics and fungicides used to sterilize the root canal, Bender and Seltzer\(^{145}\) confirmed the persistence of the enterococci especially when penicillin and streptomycin were used as intracanal medicaments. Once more it demonstrated the resistance of these bacteria to these two drugs. However when chloramphenicol was added to the combination, the enterococci were eliminated. Guthof\(^{146}\) found that enterococci were also resistant to electrolytic medication.
Winkler and Van Amerongen\textsuperscript{147} did an exhaustive bacteriologic study on the results of 4,186 cultures. They found \textit{S. faecalis} in 20\% of the positive cultures from both vital and necrotic teeth. They developed a ratio by taking the total number of times each organism appeared and divided it by the number of teeth from which it was isolated. This ratio was then a measure of the number of times the same organism was isolated from one canal. \textit{S. faecalis} possessed the highest ratio, which indicated that it was able to persist longer in the canals than any of the other bacteria isolated. They also showed evidence of its tenacity by the higher frequency it exhibited in later cultures, and from the long series of consecutive cultures from one canal. Their conclusion was that group D streptococci were difficult to eliminate from the root canal. The drugs they used to sterilize the canals were chlorophenol-camphor-methanol and polyantibiotics.

Melville and Slack\textsuperscript{148} used a variety of agents to sterilize the root canal and found 8\% of 695 microorganisms isolated from 392 root canals in the process of treatment to be fecal streptococci. In that same year, Engstrom and Frostell reported finding \textit{S. faecalis} in 2 out of 21 positive cultures, (9.5\%), taken from intact pulpless teeth on the initial visit.

Engstrom\textsuperscript{149} also concluded that enterococcal infections of the root canal constituted a treatment problem because they were difficult to eliminate and caused the period of treatment to be greatly prolonged. He used a quaternary ammonium compound or iodophor (0.04\% iodine) as an
irrigant during biomechanical instrumentation, then washed the canal with alcohol, chloroform, and Dakin solution, and sealed in a dressing of 5% iodine in 10% potassium iodide. Still 65% of those canals which contained enterococci remained infected at the second visit.

In yet another study, Engstrom and Frostell\textsuperscript{150} reported that teeth which had been subjected to conservative root canal treatment, with 5% iodine in a 10% potassium iodide solution used as the intracanal medicament, showed a high frequency of enterococci among those cases which were not bacteria free after treatment. In their third culture the enterococci predominated.

Grahnen and Krasse\textsuperscript{151} examined the bacteriological results after flushing infected root canals with either saline, a quaternary ammonium compound, or a polyantibiotic mixture. Then reported that 2 cases treated with the saline remained positive after ten treatments and in both instances were enterococci. Similarly, there were three other cases in which enterococci occurred, two in the group treated with a polyantibiotic, and these took one and four treatments respectively to eliminate the enterococci. The final case was in the group treated with a quaternary ammonium compound, and it required six treatments to eliminate the bacteria.

With intentions of investigating the effect of the presence of persisting infection, at the time of root filling, on the prognosis of such treatment, Engstrom, et. al.\textsuperscript{69} studied positive root canal cultures that had been
taken at the fill appointments, and followed the cases over a period of four to five years. Out of 107 cases which showed positive cultures, 19 (17.7%) were infected by enterococci. Of these 19, five were determined failures, and four of those five showed enterococci in pure culture.

It is also interesting to note that they had no failures out of the 15 cases where Staphylococcus albus was the contaminant. However, where there was contamination by streptococci, the failure rate was the highest.

These results would tend to support the contention that S. albus is not pathogenic in the root canal whereas all types of streptococci are pathogenic. Although the number of cases in which the enterococci were involved was small, one can safely say that these bacteria are capable of producing periapical pathosis.

Fox and Isenberg were interested in the antibiotic sensitivity of organisms, isolated from root canals, and in the process they found that 84 of the 381 isolates (22%) they obtained were enterococci. Their cultures were intratreatment cultures and the medicament they used to sterilize the root canal was eugenol for vital cases and PBSC for all others.

Goldman and Pearson cultured root canals immediately following debridement with files used in conjunction with sodium hypochlorite. Of the resulting positive cultures, enterococci were found in 36% of the vital cases and 27% of the non-vital ones. They used comphorated paramono-chlorophenol as an intracanal medicament and the enterococci were still the most persistent organisms they encountered, sometimes persisting
after three or four treatments.

Myers, Marshall, and Rosen\textsuperscript{154} investigated which bacteria might be responsible for the high percentage (25.9\%) of culture reversals which they experienced. They were able to isolate \textit{S. faecalis} in 20.8\% of these cases.

Mejare\textsuperscript{155} published a report on the results he obtained when he differentiated the species of enterococcal isolates that were obtained from cultures taken at the time of fill. Here again the previous culture had been negative. He was able to get 29 enterococcal isolates from 27 of the 92 positive cultures they studied, (31.5\%) with a preponderance for \textit{S. faecalis} and its subspecies. He also found that the colony forming units of enterococci in 0.1 ml. of the sample suspension which he used, were more often at least \(10^3\) times more than that of other identified microorganisms. He felt that this finding suggests that the enterococci easily recover and multiply in the root canal in spite of antimicrobial treatment and explains why they are difficult to eliminate. He also suggests that this high incidence and prevalence of enterococci in infected root canals at the time of filling, may help to explain the significantly higher rate of failures, reported by other authors, when the fill culture has been positive versus negative.

One can see from all of these reports that the evidence incriminating \textit{S. faecalis} as a major problem and potential pathogen in root canal treatment is overwhelming. It not only exists as an initial invader but is particularly difficult to eliminate regardless of the irrigant or intracanal
medicament used in the process of treatment. Even when the culture shows that the canal has been disinfected, *S. faecalis* is often the culprit in culture reversals. So considering all these facts, *S. faecalis* became the obvious choice for use in this experiment.
CHAPTER III

MATERIALS AND METHODS

Isolating the Microorganism

The design of this study was to contaminate root canals and periapical tissues immediately prior to filling the root canal. For reasons already discussed in the literature review, the microorganism chosen was Streptococcus faecalis. However it was important that the strain used be a virulent organism capable of producing a periapical lesion. Since stock organisms often become attenuated because of prolonged storage, it was decided not to use this source. Instead organisms cultured from clinical patients in the Endodontic Clinic at Loyola University were used.

The initial cultures were taken in a sodium thioglycollate broth with .5% agar added, as described by Crawford and Shankle. This is the media routinely used in the Endodontic clinic and it was the media used throughout the experiment whenever a root canal was cultured.

The microbiologic tests used to differentiate and isolate S. faecalis from positive endodontic cultures were based on the work of Hajna and Perry and Sherman. Hajna and Perry developed SF broth which is specific for the fecal streptococci, however Streptococcus faecium will grow on it as well as S. faecalis. Sherman determined the growth criteria for S. faecalis. He found it would grow in a medium which contained 6.5% sodium chloride, that growth would initiate at a pH of 9.6, and also
that it would survive a temperature of $60^\circ$ C. for 30 minutes. The additional tests were derived from data found in Bergey's Manual of Determinative Bacteriology and were used to help differentiate between the two species, *S. faecalis* and *S. faecium*.

In preparation for the necessary microbiologic procedures the following media were prepared:

1. SF agar plates.
2. Nutrient Broth with 6.5% sodium chloride.
3. Brain heart infusion broth adjusted to a pH of 9.6 with sodium hydroxide.
4. Brain heart infusion broth (pH 7.4).
5. CTA agar with sorbitol.
6. CTA agar with mannitol.
7. Litmus milk.
8. Sheep's blood agar plates.

Positive cultures resulting from the intratreatment cultures were struck for isolation on to SF agar plates and incubated for 24 hours at $37^\circ$ C. A colony was selected from those plates that exhibited the characteristic color change to yellow and was gram-stained to verify that it was a pure culture of a gram-positive cocci. Once this was verified the colony was struck onto a sheep's blood agar plate so that the hemolytic reaction of the bacteria could be noted. After incubation the bacteria growing on this plate were

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\*All available from Baltimore Biological Laboratories, Cockeysville, MD
inoculated into the remaining six media previously prepared. These along with a control were then incubated for 24 to 72 hours.

The brain heart infusion culture (pH 7.4) was taken at 24 hours and placed in a hot water bath at $60^\circ C$ for 30 minutes. After the 30 minutes elapsed a loopful of the culture was inoculated into another tube containing 10 cc of brain heart infusion broth and incubated for 24 hours at $37^\circ C$. If growth occurred this was gram stained to verify that the culture was still a pure gram-positive coccus culture. This was the culture used for storage if the isolated strain exhibited the characteristic responses of *S. faecalis* on all of the media. These tests were performed until four separate strains were obtained.

Because of the varied time intervals at which the bacteria would be needed throughout the experiment, a method of storage was chosen which would ensure an equal amount of pathogenicity regardless of the time interval at which it was used. The method chosen was to freeze the bacteria in sterile whole sheep's blood. This was accomplished by placing 1.0 cc of the blood into sterile ampules, a adding to it 0.1 cc of the 24 hour brain heart infusion culture, and then sealing the ampules with a specially designed torch. b Twenty-five ampules of each strain were prepared in

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a Scientific Products, McGaw Park, Illinois

b Type 3A Blowpipe, National Welding Equipment Company, San Francisco, California
this manner. After all the ampules were filled and sealed they were frozen by rotating them in a bath of dry ice and acetone. Upon freezing they were transferred to the Revco freezer\textsuperscript{a} for storage at $60^\circ$ C.

**Pilot Study**

Once the bacteria had been isolated and stored it was necessary to determine if the microorganisms were indeed virulent. Ideally it would have been nice to use a rhesus monkey for the pilot since this was to be the ultimate experimental model of this exercise. But, because of the prohibitive cost of the monkeys it was decided to use a 50 lb. male mongrel dog instead. Also, Neach\textsuperscript{159} and Mazukelli,\textsuperscript{160} in previous studies, had used the same strain of *S. faecalis* and found it to be pathogenic in both the monkey and the dog. So it was felt that if the bacteria would exhibit pathogenicity in the dog, it would do likewise in the monkey.

Twenty-four hours prior to the start of the pilot experiment a vial of each frozen bacterial strain was removed from the freezer, thawed, inoculated into 10 cc's of brain heart infusion broth, and placed into the incubator. On the day the pilot procedures were to be performed the dog was anesthetized by injecting 4 cc's of sodium pentobarbital,\textsuperscript{b} (65 mg./ml.)

\textsuperscript{a}Revco Inc., Industrial Products Division, Deerfield, Michigan

\textsuperscript{b}Sodium Pentobarbital Injection, W. A. Butler Company, Columbus, Ohio
intravenously. The bicuspid teeth of all four quadrants were examined clinically and radiographically. After this examination was completed each tooth was isolated separately under a rubber dam, disinfected with Bactine, a and the pulp exposed with sterile diamonds and burs. The canals were located and prepared by the use of the giromatic handpiece b and sodium hypochlorite, c 5.25%, as an irrigant, up to a size 8 giromatic file. Then an engine-driven reamer in a slowspeed handpiece was used to penetrate the apex. After the apices were penetrated the canals were flushed with sodium hypochlorite and dried with paper points. d

The canals were then contaminated by introducing with a 3 cc disposable syringe, e the 24 hour culture of bacteria previously prepared. A different strain was used in each quadrant and properly recorded. After contamination the chamber was dried with a sterile cotton pellet. The remainder of the culture in the canals was sealed in with a double seal of Intermediate Restorative Material (I.R.M.) f

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a Miles Laboratories, Inc., Elkart, Indiana  
b Medidenta Corp., Woodside, New York  
c Clorox Company, Oakland, California  
d Johnson & Johnson, New Brunswick, New Jersey  
e Sherwood Medical Industries Inc., Deland, Florida  
f L. D. Caulk Company, Milford, Delaware
All 12 teeth were operated on in the same day. The dog was anesthetized for 8 hours and required an additional 7 cc's of sodium pentobarbital to maintain an anesthetic level throughout the procedure.

Radiographs were taken and a clinical examination was performed at 6 and 10 weeks post-operatively, to determine if any pathologic changes had taken place. On the basis of the 10 week post-operative radiograph, a strain of bacteria was selected to be used in the main experiment. The dog was sacrificed by an overdose of sodium pentobarbital\textsuperscript{a} injected intravenously. Histologic sections stained with hematoxylin and eosin were used to confirm the radiographic evidence.

The Experiment

Four adult male rhesus monkeys ranging in weight from 5.1 to 8.2 kilograms were selected for the experiment. Prior to any experimental procedures being performed the monkeys were anesthetized with .6 cc of phencyclidine hydrochloride\textsuperscript{b} (20 mg. per cc). One ml. of atropine sulfate\textsuperscript{c} (.5 mg. per ml.) was also given to control their salivation. Their upper and lower anterior teeth were radiographed to determine their suitability for endodontic treatment. A number was tattooed on the animal's

\textsuperscript{a}Barb-Enthol, Haver-Lockhart Laboratories, Shawnee, Kansas
\textsuperscript{b}Sernylan, Bio-Ceutic Laboratories, Inc., St. Joseph, Missouri
\textsuperscript{c}Atrosol, Burns-Biotec Laboratories, Inc., Oakland, California
chest for identification purposes. After determining that the animals would be suitable, the experiment was scheduled.

On the initial day of the experiment each animal was anesthetized as before. Their upper anterior teeth were isolated under a rubber dam, two teeth at a time, the central and lateral incisors. They were then disinfected using Bactine. An access cavity was prepared with sterile burs and number 10 endodontic files were placed into the root canals to a length predetermined from the pre-operative radiographs. A radiograph was taken at that point to determine the working length. Once the working length was determined the tooth was instrumented with a size 15, to a length 2 to 3 mm. longer than the working length. The canal was then prepared up to a size 30 or 35 instrument, using the technique of canal preparation as described by Weine, so that a good dentin stop was defined at the working length. The canals were also flared, but a solid dentin matrix was not built up because the apex was always kept patent by going back through the apical foramen after each instrument with the size 15 file. Sodium hypochlorite was used as an irrigant. After the canal preparation was completed the canal was flushed, dried, and a sterile cotton pellet without any medication was sealed into the canal with I.R.M.

Four days after the initial procedure the monkeys were again anesthetized, their teeth isolated as before, and the temporary fillings removed. A

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aStar Dental Manufacturing Company, Conshohocken, Pennsylvania
culture was taken of each tooth at this time by placing a paper point in the canal for one minute before placing it into the culture media. The first paper point was used in all instances and all cultures were read at 2, 3, and 7 days. After the culture was taken, the patency of the apical foramen was confirmed by passing an instrument 2 to 3 mm. past the foramen into the periapical tissues. The canal was then irrigated, dried, and again closed as before. All the cultures were incubated for one week.

Two days after the teeth were cultured the cultures were checked for growth by visual means. Also at that time an ampule of the selected bacterial strain was removed from the freezer, and a loopful was inoculated into 10 cc's of brain heart infusion broth and incubated at 37°C. The following day the cultures were checked again, the monkeys anesthetized, the teeth isolated, and the field sterilized. The seal was removed with sterile burs and another culture was taken of each canal using the same technique as before.

After the cultures were taken the teeth were prepared for filling of the root canal. That was the point at which the variable was introduced. In the experimental animal the canal was flooded with the 24 hour culture of S. faecalis and this was worked past the apex 2 to 3 mm. with a #15 file. The culture was allowed to stay in the canals for 3 to 4 minutes. The canals were then dried with paper points and the master gutta percha points were adapted to the working lengths of the respective teeth. Once

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Premier Dental Products Company, Philadelphia, Pennsylvania
this was satisfactorily accomplished the canals were filled using a lateral condensation gutta percha technique. Wach's Paste\textsuperscript{a} was used as the root canal sealer. After the root canal filling had been adequately condensed a double seal of I.R.M. and amalgam was placed, and a post-operative radiograph was taken.

In the control animal the procedure varied in as much as there was no culture of \textit{S. faecalis} introduced into the tooth. Instead sodium hypochlorite was placed in the canal. The apices of these teeth were also violated by passing a \#15 file 2 to 3 mm. past the working length, after which the canals were dried and filled in the same manner as were the experimental teeth. That completed the first series of treatments on each animal.

Five weeks after the upper anterior teeth had been filled the series of treatments was started on the lower anterior teeth. They were treated in exactly the same manner as the uppers, with the exception that all four teeth were isolated under the same field. Also the experimental and control animals were alternated. The animal which had served as the control in the previous series of treatments was used as the experimental animal in this series and vice versa.

By following this sequence of treatments the filling dates of the canals in the lowers lagged exactly 6 weeks behind the filling date of the uppers.

\textsuperscript{a}King's Specialty Company, Fort Wayne, Indiana
Consequently by choosing the proper sacrifice dates observation periods of 24 and 18 weeks were obtained in two of the animals, while observation periods of 12 and 6 weeks were obtained in the remaining two animals.

Preparation of the Sections

After the proper amount of time had elapsed the animals were sacrificed by an overdose of sodium pentobarbital. A clinical examination of the teeth and soft tissues was performed and the observation recorded. Then the soft tissues of the maxilla and mandible were dissected away, and block sections of the anterior teeth were made in each instance. The sections were immediately placed in 10% formalin. After the sections were adequately fixed they were radiographed, and the excess tissue was trimmed off by the use of a dental high speed air rotor\(^a\) with a water spray. The trimming process was monitored throughout by taking dental radiographs. The maxillary sections were divided into a right and a left half.

After an adequate amount of excess tissue had been removed, the sections were placed in 5% formic acid for 2 weeks for decalcification. When the process of decalcification was complete as determined by radiographs, the sections were cut into smaller sections, each section containing one tooth and its corresponding periodontal and periapical tissues. Each section was labeled accordingly and then embedded in paraffin for sectioning. Six to eight micron central sections were made through the apex

\(^a\)Quiet-Air, Midwest American, Melrose Park, Illinois
of each tooth and its surrounding periapical tissues. The sections were
stained alternately with Hematoxylin and Eosin and Brown and Brenn
stains.

The histologic sections were then studied under the light microscope
with the aid of an oral pathologist to determine the status of the periapical
and periodontal tissues. The sections were coded so that the examiner was
not aware of whether he was examining a control or an experimental specimen.
Five specific areas were examined on each slide, the periapical tissue proper,
the alveolar bone, the tooth root, the periodontal ligament away from the
periapex, and the status of the root canal tissue or root canal filling.

The periapical tissue proper was specifically examined for the presence
of proliferating epithelium, cyst formation, bacterial colonies, necrotic
debris, polymorphonuclear leukocytes, lymphocytes, plasma cells, multi-
nucleated giant cells, endothelial proliferation, red blood cells in the
capillaries and extravasated, and fibroblastic proliferation of the connective
tissue background. The alveolar bone was examined to check on the osteo-
blastic and osteoclastic reaction, osteoid formation, the type of bone marrow
present, and the presence of necrotic bone. The root end was examined
for cementum resorption, cementum opposition or hypercementosis, and
ankylosis. The periodontal ligament away from the periapex was viewed
to see if its fibers, connective tissue reaction and vascularity were normal.
The presence of inflammatory cells was also noted. Finally the root canal
and the root canal filling were examined. Gutta percha and/or sealer in
the periapex, necrotic and viable tissue remaining in the canal, and
accessory canals were all noted.

All of the categories were graded according to the following scale:

0 = None or no reaction
1 = Slight reaction
2 = Moderate reaction
3 = Severe reaction

Following the grading, the scores of each reaction for each time
period were added together and divided by the total number of samples in
that time period for both the experimental and the control groups. In
addition, a composite average was arrived at by taking the averages com­
puted earlier in each time period for a particular reaction, adding them
together, and dividing by the total number of time periods. In this way
each reaction could be compared to see if there were any significant dif­
ferences in the experimental and the control groups in each time period,
and also as an overall view of the entire experiment by comparing the
composite average scores.
CHAPTER IV

RESULTS

Microbiologic Results

All of the four strains of bacteria used in the pilot study gave results compatible with the organism *Streptococcus faecalis* when the microbiologic tests were performed. That is they grew on SF agar plates, in broth which contained 6.5% sodium chloride, at a pH of 9.6, and after having been subjected to a temperature of 60° C for 30 minutes. They also fermented mannitol and sorbitol, reduced litmus milk without curdling it, and produced gamma hemolysis on blood agar plates.

When, after thawing, a loopful of the stored bacteria was inoculated into a test tube containing brain heart infusion broth, a very turbid growth with settling was obtained within 24 hours.

Pilot Results

The clinical examination of the dog done before the clinical procedures were performed revealed a very healthy periodontum with minimal pocket depth and no signs of pathosis. The clinical crowns of the teeth were caries free and intact. Radiographic examination revealed good periodontal bone and no periapical pathosis.

At the 6 and 10 week post-operative time interval, the supporting tissues of the teeth remained in good health. No periodontal pockets or chronic draining sinus tracts developed as a result of the infecting
procedure. The periapical radiographs at 6 weeks showed minimal periapical bone destruction. However at 10 weeks multiple lesions had developed. It was on the basis of these radiographs that the strain of bacteria was chosen for the monkey experiment. The strain in the lower right quadrant produced the largest lesions and consequently it was chosen. Later it was confirmed by histopathologic examination of the periapical tissues, that periapical granulomae did indeed develop on the teeth of the lower right quadrant.

**Culture Results**

All of the cultures taken on the monkey teeth, both the intratreatment and the preobliteration cultures, turned out negative for growth, even when read on the seventh day of incubation.

**Clinical Results**

The clinical examination of the monkey teeth and supporting tissues prior to the experiment revealed caries free teeth, but periodontal disease in the form of gingivitis in all four monkeys. Otherwise, all the tissues were in a state of good health. The exam at sacrifice time showed no appreciable change in any of the tissues. However the two long term monkeys had fractured off their lower anterior teeth at or near the gum line in the post-operative time interval. This did not interfere with the results.
Radiographic Results

The preoperative radiographs of all four monkeys showed normal healthy teeth and surrounding bone. The post-operative radiographs showed well condensed and well placed root canal fillings. Some did show sealer and gutta percha protruding past the apex though. Those radiographs taken immediately post-operatively did not differ appreciably from those taken at the time of sacrifice. Those teeth that were contaminated did not show any greater degree of periapical breakdown than did those teeth which had not been contaminated.

Histologic Results

A. Loss of Specimens

One specimen in the 18 week experimental group was lost in the laboratory due to technical problems. A specimen in the 24 week control group was unusable because a good central section was not obtainable.

B. Periapical Tissues

Proliferating epithelium and pseudo-cyst formation were found in the 12 week experimental and the 6 week control periods. However this was the same animal in both periods. One pseudo-cyst was also found in the 24 week experimental group. Epithelial proliferation was evident in two additional cases, the 24 week experimental and the 12 week control. The term pseudo-cyst was used because these cysts lacked the severe chronic inflammatory elements and the epithelium surrounded the excess filling material rather than a fluid filled cavity.
It was impossible to distinguish between bacteria and sealer in the Brown and Brenn stained sections. Therefore nothing definite could be concluded as to the presence or absence of bacteria. Slightly more necrotic debris was found in the periapex of the experimental teeth than of the controls.

Polymorphonuclear leukocytes were found very infrequently and usually in slight amounts. The controls did not demonstrate any and the experimental specimens displayed them only in the 6 week and 12 week periods.

Lymphocytes were a consistent finding in nearly all of the specimens. They were distributed equally in both the experimental and the control groups and usually in slight concentrations. The only time their concentrations were judged as moderate was in one specimen each, of the 6 and 12 week control groups. Plasma cells were rarely detected.

Multinucleated foreign body giant cells were found in about 25% of all the cases with no particular dominance in either group. When they were found, they were very few in number.

Endothelial proliferation was evident in all specimens except one, that one being in an 18 week control group. However it was judged as only slight in every case except one. Red blood cells were easily recognized wherever there were capillaries.

There was proliferating fibrous connective tissue in all of the cases. There were two groups, the 12 week experimental and the 6 week control,
that exhibited a degree of proliferation judged as severe. In nearly all of the others there was a slight reaction.

C. Aveolar Bone

The osteoblastic reaction of the alveolar bone was evident in all specimens, and was judged as being anywhere from slight to severe with no particular difference between the experimental or control groups. Osteoclasts could be demonstrated in all specimens except one, that being a 24 week experimental tooth. However in about 25% of the specimens they were more numerous and more easily recognized, so the osteoclastic reaction was judged as moderate. There were two more specimens in the experimental than in the control group that showed this moderate response. A slight osteoid formation was found in all the specimens, however it was not easily demonstrated. The bone marrow was fibrofatty in all of the specimens. There were only two cases in which there appeared to be any necrotic bone, and those were both very small islands, and both were in the 6 week control group.

D. Root

There was very little cementum resorption. On the contrary the cementum reaction seemed to be one of apposition with a very thick layer of cementum being evident at the apex of nearly every tooth. However, ankylosis did not develop in any of the teeth.
E. Periodontal Ligament

The periodontal ligament away from the apex had the same or similar appearance in all of the cases. The connective tissue, vascularity, and ligament fibers all appeared normal. In no case were any inflammatory cells found. Because these responses were the same throughout, they were not included in the tables.

F. Root Canal

Although a concerted effort was made at the time the root canals were filled to confine the filling materials to the prepared canal, a large amount of sealer and gutta percha protruded through the apical foramen. There was more sealer than gutta percha in the periapical tissues in 12 cases of the experimental group and in 8 of the control group. Also a concerted effort had been made during canal preparation to thoroughly debride the canal to the cemento-dentinal junction. Under the microscope it became obvious that this was not accomplished. Necrotic tissue left in the canal was the rule rather than the exception. In at least half of the cases vital tissue could still be demonstrated in the canals. In addition to that, large accessory canals, with viable and necrotic tissue could be seen. Sometimes they were equal in size to the main canal, especially in the lower anterior teeth, indicating that these teeth may have had 2 canals.

The histologic results are summarized in Tables 1 through 3.
TABLE I

HISTOLOGIC PERiapICAL TISSUE RESPONSE

<table>
<thead>
<tr>
<th></th>
<th>6 weeks</th>
<th>12 weeks</th>
<th>18 weeks</th>
<th>24 weeks</th>
<th>Composite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferating Epithelium</td>
<td>0/1.5</td>
<td>1.5/.25</td>
<td>0/0</td>
<td>1/0</td>
<td>.63/.44</td>
</tr>
<tr>
<td>Cyst Formation</td>
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<td>1.5/0</td>
<td>0/0</td>
<td>.5/0</td>
<td>.5/.19</td>
</tr>
<tr>
<td>Bacterial Colonies</td>
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<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Necrotic Debris</td>
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<td>.75/.75</td>
<td>1/0</td>
<td>.75/1</td>
<td>.75/.75</td>
</tr>
<tr>
<td>P.M.N.'s.</td>
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<td>.75/0</td>
<td>0/0</td>
<td>0/0</td>
<td>.31/0</td>
</tr>
<tr>
<td>Lymphocytes</td>
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<td>.75/1.25</td>
<td>1/.25</td>
<td>.75/1</td>
<td>.88/.88</td>
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<tr>
<td>Plasma Cells</td>
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<td>0/.25</td>
<td>0/0</td>
<td>.25/0</td>
<td>.19/.06</td>
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<tr>
<td>Multinucleated Giant Cells</td>
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<td>.25/.25</td>
<td>0/0</td>
<td>.5/.33</td>
<td>.39/.27</td>
</tr>
<tr>
<td>Endothelial Proliferation</td>
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<td>.66/1</td>
<td>.75/1</td>
<td>.85/1.06</td>
</tr>
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<td>R.B.C.'s in Capillaries</td>
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<td>1/1</td>
<td>.66/1</td>
<td>1/1</td>
<td>.92/1</td>
</tr>
<tr>
<td>R.B.C.'s in Extravasated</td>
<td>0/0</td>
<td>.25/.25</td>
<td>0/0</td>
<td>0/.33</td>
<td>.06/.14</td>
</tr>
<tr>
<td>Proliferating Fibrous Conn. Tissue</td>
<td>1/2.5</td>
<td>2.5/1.25</td>
<td>1/1</td>
<td>1.25/1</td>
<td>1.44/1.44</td>
</tr>
</tbody>
</table>

All numbers are average scores derived by adding the score of each specimen and dividing by the total number of specimens. Specimens were initially scored according to the following scale: 0 = no response, 1 = slight, 2 = moderate, and 3 = severe. The numbers are printed as Experimental/Control.
TABLE II

HISTOLOGIC RESPONSE OF ALVEOLAR BONE AND ROOT

<table>
<thead>
<tr>
<th></th>
<th>6 weeks</th>
<th>12 weeks</th>
<th>18 weeks</th>
<th>24 weeks</th>
<th>Composite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoblastic Reaction</td>
<td>1/1.5</td>
<td>2.25/2</td>
<td>1/1.25</td>
<td>1.75/1</td>
<td>1.5/1.44</td>
</tr>
<tr>
<td>Osteoclastic Reaction</td>
<td>1/1</td>
<td>1.25/1</td>
<td>1.67/1.75</td>
<td>1.25/1</td>
<td>1.29/1.19</td>
</tr>
<tr>
<td>Osteoid Formation</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td>Fibrofatty Bone Marrow</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td>Necrotic Bone</td>
<td>0/.5</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/.12</td>
</tr>
<tr>
<td>Cementum Resorption</td>
<td>.5/.25</td>
<td>0/0</td>
<td>.33/0</td>
<td>0/0</td>
<td>.21/.06</td>
</tr>
<tr>
<td>Cementum Apposition</td>
<td>1.25/1</td>
<td>1/1</td>
<td>.33/1</td>
<td>1.25/1</td>
<td>1.21/1</td>
</tr>
<tr>
<td>Ankylosis</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
</tbody>
</table>

Same Legend as Table I
<table>
<thead>
<tr>
<th></th>
<th>6 weeks</th>
<th>12 weeks</th>
<th>18 weeks</th>
<th>24 weeks</th>
<th>Composite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sealer in Periapex</td>
<td>1.25/2.5</td>
<td>.75/.5</td>
<td>1/0</td>
<td>1/1.33</td>
<td>1/1.08</td>
</tr>
<tr>
<td>Gutta Percha in Periapex</td>
<td>1/.75</td>
<td>1/.5</td>
<td>0/0</td>
<td>.5/1.33</td>
<td>.62/.64</td>
</tr>
<tr>
<td>Necrotic Tissue In Canal</td>
<td>.75/1</td>
<td>1/.75</td>
<td>1/.75</td>
<td>1/1</td>
<td>.94/.88</td>
</tr>
<tr>
<td>Viable Tissue In Canal</td>
<td>.5/.75</td>
<td>1/.5</td>
<td>0/.25</td>
<td>.5/0</td>
<td>.5/.37</td>
</tr>
<tr>
<td>Accessory Canals</td>
<td>.5/1.25</td>
<td>.25/.25</td>
<td>0/.25</td>
<td>.25/0</td>
<td>.25/.43</td>
</tr>
</tbody>
</table>

Same Legend as Table I
CHAPTER V

DISCUSSION

This investigation incorporated the violation of two basic principles of endodontics, respect for the periapical tissues and reduction or elimination of bacterial contamination. Because of this, and in light of the results obtained, there is danger that the wrong conclusions might be drawn from this exercise. These principles were violated strictly for experimental purposes and great care should be taken to avoid their occurrence in the clinical practice of endodontics.

In this study it was necessary to produce a periapical inflammation through overinstrumentation so that the bacteria would have a place to localize as demonstrated by Lio. These bacteria trapped in an area of periapical inflammation by a root canal filling was another necessary entity to achieve if the original question was to be answered. It must be stated that the purpose of this study was not to endorse a haphazard technique in endodontics.

The results of this investigation would seem to indicate that the periapical tissues are capable of healing after endodontic therapy even when known viable pathogenic bacteria are present. Very little difference could be seen between the experimental and the control groups, indicating that the bacteria had essentially no effect on the healing. (See Tables I through III.) This observation is in agreement with the work of Kitamura, who
observed that periapical repair of endodontically treated dog teeth was the same, whether or not microorganisms were present.

The implications that the results of this study have on the culture technique are obvious. If the healing of the periapical tissues is not materially affected by the presence of bacteria, why then should it be so essential to obtain a negative culture before filling the root canal? This experiment would justify the speculation by Morse, that factors other than the negative culture have a more important bearing on the success or failure of a case. Since no bacteria could be demonstrated in the periapical tissues it is evident that the defensive response of the hosts were quite adequate to eliminate any bacteria present there, in spite of the preexisting inflammatory condition. Granted only one strain of bacteria was tested in this study so the results cannot be construed as being representative of all bacteria. However a bacterial strain was chosen that has been proven to be one of the most troublesome in endodontics. Consequently the results are very relevant to the routine practice of endodontics and should help the individual operator to weigh the merits of culturing.

The endodontic culture has definite uses. The practitioner who obtains consistently negative cultures probably can be assured that he has maintained an unbroken chain of sterility. This is important because the introduction of any extraneous bacteria provides an additional source of irritation for the body to overcome to gain optimal results. Also, he can be certain that he has done a reasonably good job of debriding the root canal system.
However it is incumbent upon the practitioner to use the very best culture media available to him and to follow all the laws of microbiology as applied to endodontics, so that his results serve as a true indicator of the microbiologic status of the root canal. The culture must be taken before any irrigation or enlargement procedures are performed. The paper points used to culture the canal must reach the apical portion and be kept there for a minimal period of time. The paper points used must be those having the best chance of carrying the microorganisms, e.g. the first one or a combination of the first three. A sufficient period of time must be allowed for incubation before the results are read, at least 72 hours and possibly 96 hours, if slow growing anaerobes are suspected.

Culturing can also be valuable to detect a potent pathogen which could cause a serious problem if the root canal were filled in its presence. Most organisms commonly found are not pathogenic and would cause no problem. However if beta hemolytic streptococci or Staphlococcus aureus were involved the situation could be very different. A serious and debilitating infection could result.

When positive cultures result one must use the information they convey. If one can verify the chain of instrument sterility the most likely source of contamination is a leaky seal. This must be checked and steps taken to correct the problem. Another possibility is an undiscovered canal that has been left unprepared. This is frequently the problem in lower anterior teeth, the mesial buccal root of upper first molars, and the distal root of
lower molars. After the additional canal is found and prepared, the culture will give negative results. Canals prepared short of their apical termination can also contribute to positive cultures because there is still debris in the apical portion. In this situation the working lengths need to be recalculated and the canals prepared to the proper length.

Some fairly severe reactions were noted in the experimental animals, however equally severe reactions were noted in the controls. The severity of the reaction did not seem to be related to the bacteria. Other factors seemed more important. The most impressive of these factors was the degree of overfilling. Without exception there was always more inflammation accompanying the excess gutta percha and sealer in the periapex. This overfilling was also accompanied by epithelial proliferation and cyst formation. On the other hand when there was little or no foreign material in the periapex, there was almost no inflammatory response.

These findings would seem to corroborate the reports of several other investigators. Seltzer, et.al. 77 in a clinical study, found that overfilling of the root canal was definitely less successful (70.6%) than either under-filling (87.7%), or flush filling (86.8%). In that same study the status of the culture had no significant effect on success in teeth which had no areas of rarefaction. Strindberg 130 also observed that teeth which were over-instrumented and overfilled were less successful than those in which the apex was not violated and the canal filled short of the radiographic apex.

In a histologic study on dogs Seltzer, et. al. 78 found the most severe inflammatory responses appeared to occur as a result of overfilling. In
addition severe inflammatory responses occurred around particles of root canal cement forced into the periapical tissues. The culture results in that study had no bearing on the periapical healing. These findings are all similar to those in this study.

An interesting observation in this study was the incidence of epithelial proliferation and cyst formation. Seltzer, et. al. noted that one of the periapical tissue responses caused by overinstrumentation was an inflammation that stimulated the cell rests of Malassez. They found a profuse growth of stratified squamous epithelium would sometimes result. This was also found to be true in this study. However it seemed to be more evident in some monkeys than in others. One monkey was responsible for 5 out of the 8 cases of epithelial proliferation that were noted. So it seems quite possible that this tendency varied from monkey to monkey depending upon the amount of epithelial debris left in the periodontal ligament. This feeling was given additional credence by the greater amount of epithelial cell rests which could be found in those monkeys. It can be speculated that the same would be true in human beings.

Also in most instances cyst formation was accompanied by the added irritation of overfilling. Possibly this additional insult provoked the inflammatory response to such a degree that the climate became more favorable for cyst formation.

Another finding that seemed to have an influence on the inflammatory response was the presence of necrotic tissue in the root canal. It was
surprising to find so much tissue left in the canals, both vital and necrotic, in light of the tedious detail that went into the canal preparation of these teeth. Also the radiographs gave the appearance of well prepared and filled canals following the natural curvatures of the teeth, but microscopically this was not very often the case. However this finding should no longer be a surprise. Baker, et. al.\textsuperscript{162} reported that when teeth which had been thoroughly instrumented and irrigated were subjected to an examination with the scanning electron microscope, significant amounts of tissue and debris remained in the prepared root canals. Haga\textsuperscript{163} found that in many instances the endodontic instrument made contact on only three walls, leaving a void in the fourth wall. The phenomenon was seen in some specimens of this study.

As early as 1928 Hatton\textsuperscript{164} found a very high percentage of superficially cleansed root canals with much of the pulp tissue still remaining when he subjected failures to a histologic examination. Reig, et. al.\textsuperscript{165} in another histologic study found that the endodontic procedures recommended in 1952 were not sufficient to produce accepted standards of surgical cleansing in the root canal.

Nevertheless the ego of most operators would want to make them believe that they are superior clinicians aided by the latest techniques, and that they are capable of thoroughly cleansing a canal of all tissue. An examination of some of their own work under the microscope would be a sobering sight for anyone so predisposed. This is not mentioned so that
the endodontist will assume a defeatist attitude. On the contrary, it is hoped that the realization of this deficiency will spur him on to pay more and more detailed attention to his methods of canal debridement, since it is generally agreed by many authors $71,93,161$ as essential to lower the concentration of noxious agents to as low a level as possible to achieve a biologic balance.

A finding that was conspicuous by its absence was the lack of resorption of the apical cementum. It can be surmised that there must have been a tremendous periapical inflammation following the overinstrumentation of these teeth. Reports by Seltzer, et. al.,$^{137}$ and Bashkar and Rappaport$^{139}$ would support the contention that there should have been a considerable amount of cementum resorption in this experiment, yet this was not the case.

However, this points out an area in which this study could have been improved. There was no acute specimen in this series. Consequently there was no way to compare what happened at 3 or 7 days post-operatively to what the picture was at the later time intervals. It is quite possible that considerable cementum resorption did take place in the acute phases and was repaired by the time the first animals were sacrificed. This remains as pure speculation but seems quite likely since there was a large amount of cellular cementum in many of the specimens, so much so that it could be referred to as hypercementosis.
Another reason for having an acute specimen would have been to have a positive slide to demonstrate the bacteria. In the Brown and Brenn stained slides the presence of bacteria very easily could have been masked by the root canal sealer. They give a similar appearance under the microscope. Consequently it would have been advantageous to have a definite positive slide for the bacteria with which to compare the other slides. This could have been accomplished by placing the bacteria into a prepared tooth 3 days prior to the sacrifice time without filling the root canal, so as to avoid confusion with the sealer.

The object of any good research is to limit the variables to just one thing. This investigation attempted to do that but other variables kept being introduced unintentionally, which tended to cloud the results. However if tables I, II, and III are analyzed, it appears that the additional variables were introduced with approximately equal frequency in both the experimental and the control groups and consequently tended to cancel each other out. Therefore the composite averages of the various responses seem to be very true figures which can be used to compare the overall response of the two groups. As can be seen by comparing these two figures, very little difference was noted.
CHAPTER VI

SUMMARY AND CONCLUSIONS

This was an experiment designed to determine if periapical tissues
contaminated with a known pathogenic bacteria could heal after endodontic
therapy.

The first phase of the experiment was concerned with isolating and
storing a strain of Streptococcus faecalis, and verifying its pathogenicity
in a pilot study. Once the former was accomplished the latter was con­
firmed by using the bacteria to produce periapical lesions in dogs. Then
the main experiment was conducted.

The root canals of 32 monkey teeth were prepared for root canal
fillings. At the same time they were prepared, they were also over­
instrumented to produce areas of periapical inflammation. Before filling
their sterility was verified by means of the culture technique to insure
that the controls were indeed controls. Then immediately prior to filling,
the root canals and periapical tissues of half of the teeth were contaminated
with the bacteria prepared in the pilot study. The other half were left
uncontaminated to act as controls, and filled in the same manner as the
experimental teeth. The experiment was timed so that 6, 12, 18, and 24
week post-operative intervals were obtained.

The monkeys were sacrificed and block sections were made of the
operated teeth. Histologic sections of 6 to 8 microns were prepared
and stained with Hematoxylin and Eosin and Brown and Brenn stain. The results were studied under the light microscope. The data obtained indicated that there was no significant difference between the healing in the experimental and the control groups.

On the basis of the evidence accumulated in this study the following conclusions can be drawn:

1. The healing potential of periapical tissue in monkeys following root canal therapy was not appreciably affected by the presence of the strain of \textit{S. faecalis} used in this study.
2. The mere obtaining of a positive culture does not indicate a potential endodontic failure.
3. Complete debridement of all tissue from the root canal system by present day materials and techniques in the monkey is a virtual impossibility.
CHAPTER VII

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APPENDIX
Figure 1: Radiograph of the control teeth taken 6 weeks postoperatively.

Figure 2: Radiograph of the experimental teeth taken 6 weeks postoperatively.
Figure 3: Radiograph of the control teeth taken 12 weeks postoperatively.

Figure 4: Radiograph of the experimental teeth taken 12 weeks postoperatively.
Figure 5: Radiograph of the control teeth taken 18 weeks postoperatively.

Figure 6: Radiograph of the experimental teeth taken 18 weeks postoperatively.
**Figure 7:** Radiograph of the control teeth taken 24 weeks postoperatively.

**Figure 8:** Radiograph of the experimental teeth taken 24 weeks postoperatively.
Figure 9: Photomicrograph of a 6 week control specimen demonstrating epithelial proliferation and pseudo-cyst formation.

Figure 10: Photomicrograph of a 6 week experimental specimen showing excess filling material surrounded by mild infiltration of both acute and chronic inflammatory cells.
Figure 11: Photomicrograph of a 12 week control specimen demonstrating excess filling material in periapex surrounded by mild infiltration of both acute and chronic inflammatory cells.

Figure 12: Photomicrograph of a 12 week experimental specimen showing an overfill surrounded by a mild inflammatory response. Also viable and necrotic tissue are evident in the canal.
Figure 13: Photomicrograph of a 12 week experimental specimen showing pseudo-cyst formation. Note the lack of inflammation surrounding the epithelium.

Figure 14: Photomicrograph demonstrating the numerous epithelial cell rests of Malassez found in the monkey that yielded most of the pseudo-cysts.
Figure 15: A high power photomicrograph of a 6 week experimental specimen demonstrating the acute inflammatory cell response.

Figure 16: Photomicrograph of an 18 week control specimen demonstrating a mild periapical reaction.
Figure 17: Photomicrograph of an 18 week experimental specimen with some sealer in the periapex but the inflammatory response is mild.

Figure 18: Photomicrograph of a 24 week control specimen with an overfill. A mild response is evident and the alveolar bone is in close proximity to the overfill.
Figure 19: Photomicrograph of a 24 week experimental specimen with necrotic debris in the periapex. There is minimal epithelial proliferation with a mild inflammatory response.

Figure 20: Photomicrograph of a 24 week experimental specimen with chords of proliferating epithelium. A moderate chronic inflammatory response is also present.
APPROVAL SHEET

The thesis submitted by Dr. Edward P. Theiss has been read and approved by three members of the Graduate School faculty, Department of Oral Biology.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is, therefore, accepted in partial fulfillment of the requirements for the degree of Master of Science.

\[\text{Signature of Advisor}\]

\[\text{Date}\]