A HISTOPATHOLOGIC
EVALUATION OF RETAINED ROOT FRAGMENTS

by

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INTRODUCTION

It is the purpose of this investigation to seek methods for determining the effects of fractured infected and non-infected root tips and other foreign bodies in the jaws and other body tissues.

Dentists are often faced with the problem of removal of these root tips and foreign bodies from the jawbones. Roentgenographic examinations will frequently show the presence of foreign bodies, many of which were not suspected. Often one sees fragments of instruments, amalgam or other types of filling materials or root tips in the bone. Are these root fragments and foreign bodies potentially dangerous to the patient? Should they all be removed without exception? Can we predict from radiographs those requiring surgical removal? If viable organisms are present in these fragments, what is the significance to the patient? To what extent are these embedded structures harmless? These are some of the questions facing the dental practitioner.

We must consider the possible untoward results of removal of these foreign bodies. If some were harmless,

their removal has resulted in expense, discomfort, possible inferior alveolar nerve or maxillary sinus involvement, or excessive surgical removal of bone? This last often results in a loss of the bony ridges so essential to the optimum construction and function of prosthetic appliances.

If it were possible to microscopically examine these foreign objects and the surrounding tissue, in situ, probably many would be left in place.

Of the foreign substances, embedded root fragments are probably the most frequent -- is it always necessary that these be removed as is taught in most of our dental schools?

Archer (4, p. 1) lists under indications for the extraction of teeth "(1) roots and fragments" without attempting any classification. He (4, p. 20) also states that "roots are removed to eliminate a focus of infection. Even if the original tooth were non-infected, the root becomes infected at the time of extraction because of the decomposition of contents of the root canal plus the invasion of oral bacteria. Remaining roots or fragments of root structures act as mechanical irritants and may set up an inflammatory reaction which may give rise to neuralgic pains of obscure origin and difficult of diagnosis." These statements seem to reflect the thinking of most oral surgeons and may represent personal experience.

In reviewing the literature one finds considerable controversy but little sound experimental evidence which

would aid the practicing dentist in making decisions regarding embedded root fragments.

The present investigation is concerned with infected and non-infected root fragments and the associated histologic and bacteriologic changes. Simpson (39,40,42) summarizes the problem in discussing pre-existing infection by stating "A distinction must be drawn between infected and uninfected roots." In his experimental work using human and Macacus Rhesus material he indicates that non-infected root tips showed no tissue change except for the finding that the larger fragments had a tendency to become infected since they were more likely to project into the superficial part of the extraction wound and mechanically interfere with closure of the wound. Basically the surrounding tissues are able to deal with non-infected large root fragments as easily as with small ones.

If the root tip were infected, size was immaterial and the patient's symptoms would persist with chronic discomfort and with the potential for acute exacerbations, cyst formation, or development of a sinus to the mouth or skin.

Simpson (41) observed epithelial proliferation around the fragments and at first thought that the rests of Malassez had been stimulated to multiply but wherever it was possible to trace the epithelium satisfactorily on serial sections a connection was found with the surface epithelium.

Most text books ignore the subject of retained roots completely, thus leaving the practitioner to rely on his own clinical experience, which may account for the diversity of opinion.

Shafer, Hine and Levy (38) state that surprisingly little sound scientific study of the periapical tissues of infected teeth has been reported. One of the factors which has made this a difficult area of investigation is the inability of the dentist to extract a tooth without bacteriologically contaminating the periapical tissues. Many investigators have suggested that the granuloma is a sterile lesion. In this study, utilizing known infectious agents, it may be possible to determine whether the granulomas observed are infected or sterile. It has not been possible to associate particular types of micro-organisms with specific periapical lesions based upon either clinical or histologic evaluation.

Boulger (9) describes bone, secondary cementum and pulp with viable odontoblasts and changes comparable with aging in a histologic examination of the teeth of a 45 year old woman who suffered injury to her teeth at age 12. He points out that odontoblasts were previously thought to be destroyed by slight trauma yet in these teeth the pulp showed no inflammatory changes. The significance of the presence of a vital pulp leads to the conclusion that injury does not always impair the vitality of the organ, in spite of the fact that the odontoblastic layer is very

susceptible to any form of trauma.

Boulger (10) in studying results of root canal therapy used apices from human teeth which were extracted "without contamination"[1] and were embedded in muscle tissue of the leg of a rat. He found that rat connective tissue replaces the pulp of these normal non-infected human root tips. When infected apices were used there was marked inflammation in the surrounding rat tissue with abscess formation. He also demonstrated that the apices filled with gutta percha were well tolerated and should be as well tolerated in human jaws. He further states that we are led to believe that every radiolucent shadow indicates a possible source of infection, while it may have but a temperary significance, indicating the formation of scar tissue or repair without inflammation.

Allen and Gardner (1) point out the importance of periapical infections and their contribution to the origin and course of numerous diseases. They state "the absence of teeth does not exclude dental infection. In fact, in more than 30% of instances in which all teeth have been extracted, roentgenographic examination will disclose retained roots. These roots are usually infected."

Glickman, Pruzansky and Ostrach (22) studied the healing of retained root remnants in 18 young adult male and female albino rats. The maxillary first molar on the

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[1] Author's quotes.

right side was removed in 14 rats and both right and left first molars were removed in 4 animals. The animals were sacrificed at varying time intervals ranging from 1 to 108 days and each histologic preparation is described in detail. No attempt was made to deliberately infect any of the teeth prior to the fracture of the roots. Some of the descriptions include root fragments located deep to the surface and others at the surface. Their findings document the healing of extraction wounds with retained root fragments and the complications which interfere with the postextraction healing process.

Their discussion includes an analysis of each tissue involved in the healing process and shows that the surface epithelium may heal over an infected root fragment, leaving an undesirable clinical problem. Many of the retained root remnants are well tolerated by the adjacent tissues without discomfort to the patient after the surface of the wound is healed.

The implications of pathologic changes at the apices of retained root remnants are not diminished by the fact that such remnants are often so well tolerated that they become covered by a cementum-like peripheral layer. Glickman, et al (22) recommend the removal of any bone fragments at the time of operation and stress the need for wound surface hygiene, showing that surface debris interfered with epithelial continuity.

Burket (14) utilized post-mortem material for

correlation of the periapical bacteriologic findings with the clinical conditions of the teeth and the individual roentgenological lesions. Teeth were carefully removed after sterilizing and streptococci were most commonly recovered. Members of the Streptococcus viridans group have been recovered in most cases by a number of authors, (14,17,29,49) and seem to be associated more than other micro-organisms with periapical cystic and granulomatous lesions. In most of the studies based on material secured from extracted teeth a high percentage of positive cultures were obtained from radiolucent periapical areas with the Streptococcus viridans group predominating. No attempts were made to control the bacteriological methods by examination of clinically and roentgenographically normal periapical areas, which raises the question of whether the periapical findings were due to organisms actually present in the periapical region or were contaminations from the mouth flora. Later investigations showed wide variation in bacteriological findings in normal teeth.

A method for obtaining rat molar pulp contents aseptically was developed by Smith and Tappe (43) to enable them to study infection in pulp canals. The teeth were isolated with rubber dam and ligatures, then swabbed with phenol and then alcohol. After this preparation, the pulp canals were entered and the contents obtained by use of small sterile burs. The end of the bur was cut off with sterile wire cutting pliers and dropped directly into

sterile medium, which was then incubated at 37 C. No growth occurred in the medium containing normal pulp contents. When the bur was contaminated with bacteria before entering the canal, growth occurred in the medium, demonstrating that aseptic conditions were achieved and that the bactericidal effect of the phenol was neutralized by the alcohol.

Burket (14) made roentgenographic examinations of the mouths of cadavers and then removed blocks of bone containing teeth. The defects were repaired and the sections removed and again examined roentgenographically. The surface areas were sterilized and periapical cultures obtained using sterile knives and burs. Streptococcus viridans was isolated as a pure culture in 28 per cent of the cases and in mixed and pure cultures in 61.2 per cent.

Table 1: Percentages of Positive Cultures Obtained from the Different Clinical Groups. (Burket)

Group	Number of teeth	Per Cent Positive
(1)	(2)	(3)
Normal	92	43
Carious	144	38
Restorations	115	50
Exposures	50	68
Pulpless	28	72

These post-mortem findings and clinical findings of other investigators are mutually confirmatory. The highest percentage of pure cultures was obtained from teeth with no periapical roentgenological change.

Helsham (27) made a clinical survey of 2000 patients referred for the removal of retained roots. The patients were classified by age, symptoms and pathology, extraction date, position of the root in relation to the crest of the ridge and their relation to systemic conditions. He found that pain and acute infection is only rarely experienced in connection with retained roots and then mostly with roots exposed to the mouth. The most striking result of the survey is that such a small percentage (22.5%) of retained roots have caused symptoms or any demonstrable pathological change. This includes those showing sclerosis of bone which he feels should not be considered. If sclerosis of bone were excluded then the percentage with signs or symptoms would only be 16.2%. Only 40 roots wholly in bone showed any pathology and of this group only 12 cases with granulation areas and five definite cases with cysts were of more than two years duration. Helsham concluded that the danger period with retained roots is either soon after extraction is attempted or many years later when resorptive processes have brought the root to the surface.

Retained roots from 60 consecutive patients which were wholly in bone, were sectioned and stained with haematoxylin and eosin. All patients were without symptoms. Five of the supposed roots were actually sclerotic bone. Eight were fragments of cementum only. One root had an associated area of granulation tissue. "The remaining 46 roots each contained a vital pulp. While recognizable as

pulp, there were differences from normal pulp tissue. The average pulp contained a nerve bundle, an arteriole and two veins, although up to four vessels were seen. Not one section contained a layer of odontoblasts lining the dentinal surface, nor were any cells seen resembling odontoblasts.

Not one section contained stellate pulp cells. The pulp in each case was, with the exception of its nerve and vessels, indistinguishable from connective tissue. The pulps were relatively acellular and the cells were fibroblasts and fibrocytes with their nuclei roughly parallel to the pulpal walls."

This was of great interest since one of the specimens in our series of seven totally embedded human root tips, which had been present for many years, showed a relatively normal pulp with a well formed layer of odontoblasts.

Helsham indicates that the vast majority of roots that are retained are vital when fractured and remain vital. Those that are infected tend to be exfoliated and granulation tissue in association with a retained root can always be identified roentgenographically. The granulation tissue is an indication of a non-vital root and it should be removed; however, he feels that there is no justification for the removal of vital root tips.

Riddet (37) also feels that many small buried roots appear to do no harm and without x-ray evidence of damage deeply buried roots are best left alone, although pressure of a denture will cause trouble over a buried root.

Claflin (16) studied the healing of disturbed and undisturbed extraction wounds in dogs and gives detailed descriptions of changes at various time intervals. In some wounds he placed a grain of barley, in others gauze, and infected some with streptococci and staphylococcus. Some were treated and others left untreated with no difference between them in healing or sequellae except that gauze interferred with epithelialization. Harvey (25) points out that healing of a wound is a form of growth that, under normal and uncomplicated circumstances, is primarily auto-catalytic and proceeds at a given velocity. The process takes precedence in the body economy and cannot be materially expedited but can be delayed or stopped by many complicating factors.

Anderson et al (3) studied the changes in molar teeth of rats and their supporting structures following extraction of the upper right first and second molar. One group was fed an adequate diet, the second placed on a diet dificient in inorganic salts. (7) Certain pathological changes were observed in all the rats in which the teeth were extracted, irrespective of the diet consumed and these changes were not seen in any of the intact animals previously studied. This indicates that the changes are associated with the extraction of teeth and emphasizes the need for adequate controls in any experiment involving extraction of teeth and the far-reaching effects of alterations in masticatory stress and strain.

It is obvious from the conflicting findings that there is need for further investigation concerning the tissue changes and responses to infected and non-infected root fragments. This study is primarily a preliminary investigation concerned with the development of suitable techniques for producing infected and non-infected root fragments and observing the local tissue reactions in the surrounding tissues.

MATERIALS AND METHODS

Selection of animal.-- Human material would be the most desirable in a study such as this, but for obvious reasons it would be difficult to find human teeth which could be used in an experimental investigation of this kind. There are many differences between rat and human tissues; however, as long as science utilizes culture mediums and as long as certain changes occur consistently and uniformly in these mediums under the same given set of conditions, we are justified in using them for our studies and within reasonable limits, of drawing cautious conclusions. The rat was selected for many reasons -- it is the most universally used experimental animal, is economical to maintain, adjusts to laboratory procedures easily, and a wealth of material exists concerning its reaction to various nutrients, pharmacologic agents and physical conditions. It is indeed fortunate that this animal is susceptible to one of man's most prevalent diseases, dental caries.(45) Some of the disadvantages encountered were the small size of the teeth and oral cavity and the inability of the animal to express subjective symptoms.

The histology, physiology, and anatomy of the dental

and periodontal tissues of the rat molar are quite similar to those of man.(23) The dental formula of the rat is I 1/1 C 0/0 P 0/0 M 3/3 which shows a difference in incisors, cuspids and premolars from man; however, the presence of three molars is similar. There is only one set of teeth, and hence the dentition is monophydont. The incisors are permanently growing teeth, while the molars have a definite period of development and aquire roots. A wide diastema separates the incisors from the molars. The life span of three years in the rat is taken as equivalent to ninety years in man and it is found that the growth changes in the nervous system occur within the same fraction of the life span (i.e., at equivalent ages) in the two forms. The age of the rats in this study were comparable to young adults between the ages of 20 and 25 years of age. All rats used were of the same genetic background.

The rats were sorted according to sex, numbered and then randomly selected so that four groups were formed.

Group I.-- Normal Histology Controls.

These animals received the same diet and handling as the other groups but were not subjected to any surgical procedures or deliberate injections. They were sacrificed on the same time schedule as the other groups and their tissues were processed in the same way.

Group II.-- Control for fractured roots which were not infected with the test organism but were exposed to the

normal oral flora during healing.

This group demonstrates the histologic changes around root fragments when the pulps were not deliberately infected.

The upper first molar on one side was fractured and the animals sacrificed on the same schedule as the other groups. The opposite molar served as the internal control for this group.

Group III. -- Controls for "sham-operated" teeth (mimicking deliberate infecting procedures of the pulp)- including fractured and non-fractured teeth.

This is a control group for histologic changes around root fragments associated with aseptically exposed pulps which were subsequently filled. Both upper first molar pulps were exposed and entered traumatically with sterile broaches in a manner similar to the method used to infect the animals in the experimental group, (IV).

Approximately one month after this procedure, the rats were again anesthetized and the previously "sham-operated" upper first molars were fractured.

The "sham-operated" upper molars on the opposite sides were the non-fractured controls.

Approximately one month after the teeth were fractured the animals were sacrificed and an autopsy performed.

Group IV.-- Experimental group infected with Streptococcus sanguis.

The upper first molars were prepared, drilled into and inoculated in the same manner as the Group III animals, except for the fact that the pulps of the Group IV animals were deliberately inoculated with Streptococcus sanguis.

After approximately one month the operated molars on one side were fractured and a month later all of the animals were sacrificed and autopsied. The opposite molars acted as a control for infected but non-fractured roots.

Arrangement of cages.-- The cages were arranged in five tiers with six cages on each level. The individual cages are constructed of galvanized sheet metal with the sides and back solid and the floor and front of ¼" wire mesh. The front supports a water bottle and food was placed within the cage. The assignment of cages to groups, rats to groups and rats to cages was accomplished by utilizing random numbers.

By assigning and distributing the rats in this manner the variation in temperature, circulation of air currents, and lighting will be randomly distributed among groups. The rats used are of the Sprague-Dawley strain from Northwest Rodent Company; Pullman, Washington.

The rats were assigned to groups using a randomized block design. Each animal was numbered as it was removed from the box with males numbered one to twenty-four and

females numbered one to twenty-four. The random numbers table (20 p. 366) was entered randomly and the four Groups selected by assigning usable numbers to each group, the first number, Group I; second number Group II; etc., until each group had the correct number of rats. (See Table 2.) Cages were assigned to groups in a similar manner. (See Table 3.) The rats which had been assigned group numbers were then placed in the designated cages. Males and females were alternated in each group. The final arrangement is shown in Table 4.

Bacteria selected for study.-- The bacteria used were American Type Culture 10556, a Streptococcus sanguis White isolated from a case of subacute bacterial endocarditis. (2)

White and Niven (36,47) studied 42 cultures of this organism from 36 cases of subacute bacterial endocarditis. A few were duplicate cultures, one of which had been isolated from the patient's blood or heart vegetation after death, a few weeks after the original blood culture. In all instances the duplicate cultures were found to be identical. A summary of their findings concerning this streptococcus and a comparison with some others will be found in Table 5.

The streptococcus isolated was of the alpha or viridans-streptococci with production of a greenish zone of partial hemolysis due to the formation of methemoglobin surrounding the colony on blood agar. This reaction is

Table 2. Random Numbers Designation for Group Assignments of Rats.

Sex of Animal	Group Number			
	I	II	III	IV
(1)	(2)	(3)	(4)	(5)
	5	15	20	2
	3	10	4	23
Male	16	21	11	1
	13	7	24	14
	12	17	18	19
	8	9	22	6
	24	21	1	13
	12	10	23	17
Females	20	3	9	5
	6	22	4	18
	7	19	11	15
	14	8	2	16
Males	6	6	6	6
Females	6	6	6	6
Group Total	12	12	12	12

Table 3. Random Numbers Designation for Assignment of Cages to Groups.

Group Number			
I	II	III	IV
(1)	(2)	(3)	(4)
1	21	31	11
17	35	14	7
47	27	37	10
22	12	42	15
4	13	40	5
28	34	36	45
29	26	41	20
19	24	16	8
6	39	48	2
43	18	30	23
44	46	38	9
32	33	3	25
57	50	51	55
56	49	53	52
58	59	60	54

Table 4. Cage Arrangement by Groups; Animal Numbers and Sex. Arabic Numeral = cage number; Roman Numeral = group number; X - males; O - females, followed by rat number in parenthesis.

SIDE A					
1 I X (5)	2 IV X (2)	3 III X (20)	4 I O (24)	5 IV O (13)	6 I X (3)
7 IV X (23)	8 IV O (17)	9 IV X (1)	10 IV O (5)	11 IV X (14)	12 II X (15)
13 II O (21)	14 III O (1)	15 IV O (18)	16 III X (4)	17 I O (12)	18 II X (10)
19 I X (16)	20 IV X (19)	21 II O (10)	22 I O (20)	23 IV O (15)	24 II X (21)
49 II	51 III	53 III	55 IV	57 I	59 II

SIDE B					
25 IV X (6)	26 II O (3)	27 II X (7)	28 I X (13)	29 I O (6)	30 III O (23)
31 III X (11)	32 I X (12)	33 II O (22)	34 II X (17)	35 II O (19)	36 III O (9)
37 III X (24)	38 III O (4)	39 II X (9)	40 III X (18)	41 III O (11)	42 III X (22)
43 I O (7)	44 I X (8)	45 IV O (16)	46 II O (8)	47 I O (14)	48 III O (2)
50 II	52 IV	54 IV	56 I	58 I	60 III

Table 5. Physiological Characteristics of Streptococcus s.b.e. (10, 39)

	Typical Characteristics	Per Cent Having Typical Characteristics
Greening of blood agar	+	98
Growth at 10 C	−	100
Growth at 45 C	±	55 (−)
Growth on 40% bile blood agar	+	72
Growth in 6.5% sodium chloride	−	100
Strong reducing action	−	100
Final pH in glucose broth	4.6-5.0	100
Arginine	+	100
Sodium hippurate hydrolyzed	−	100
Starch hydrolyzed	−	72
Slime synthesis, 5% sucrose broth	+	95
Arabinose	−	100
Xylose	−	100
Maltose	+	100
Lactose	+	100
Sucrose	+	100
Trehalose	+	98
Raffinose	−	67
Inulin	+	81
Glycerol	−	100
Mannitol	−	100
Sorbitol	−	98
Salicin	+	100
Esculin	+	86

present in 98 per cent of the strains. Of possible significance was the fact that 30 of the 42 cultures were able to grow on blood agar which contained 40 per cent bile. The two recognized viridans streptococci from human sources, Streptococcus salivarius and Streptococcus mitis, do not possess this ability.

Characteristics of Streptococcus s.b.e. -- Streptococcus s.b.e. is differentiated from the enterococci by the temperature limits on its growth, its inability to grow in a broth containing 6.5 per cent sodium chloride and its failure to show strong reducing action in litmus milk. None of the cultures was able to hydrolyze sodium hippurate. The final pH produced in a 1 per cent glucose broth varied from 4.6 to 5.0 with an average of 4.8. A test of primary importance for the identification of Streptococcus s.b.e. is the hydrolysis of arginine, since the established species of the viridans group do not characteristically attack this amino acid (31); however, a number of strains of an organism, Streptococcus mitis, which occurs in the normal human throat, do hydrolyze arginine, thus causing difficulty in differentiating them from Streptococcus s.b.e..

Niven, White and Kiziuta (35) reported that thirty-two of the thirty-four strains of Streptococcus s.b.e. which were recovered from cases of subacute bacterial endocarditis, synthesized large quantities of a poly-saccharide from sucrose in broth culture as determined

by an increase in viscosity or by actual solidification of the medium. The sucrose broth contains 1 per cent tryptone, 0.5 per cent yeast extract, 0.5 per cent K_2HPO_4, and 0.5 per cent sucrose with a final pH of 7.4. The cultures were incubated for three days at 37°, both aerobically and anaerobically, and were considered positive if there was any increase in viscosity.

These cultures synthesized little or no polysaccharide when streaked on sucrose agar. The sucrose cultures presented striking appearances, becoming extremely viscous after twenty-four hours incubation, and many cultures actually solidified upon further incubation. The test is simple and is of great assistance in identifying Streptococcus s.b.e. but it is not perfect since two cultures of Streptococcus mitis and seven Streptococcus bovis strains isolated from subacute endocarditis have also been found to synthesize a polysaccharide from sucrose in broth culture. These appeared very similar to the Streptococcus s.b.e. strains. It was very disappointing to find that on repeated cultures Streptococcus sanguis, ATTC 10556, grown both aerobically and anaerobically on different sucrose broth formulas continually failed to produce even a slight change in viscosity and rendered this method of tracing useless. Two other tests of significant aid in identifying Streptococcus s.b.e. are the fermentation of inulin and lack of fermentation of raffinose by the majority of strains. This ability to

ferment inulin but not raffinose is a unique combination of fermentation characteristics among the recognized species of streptococci. Streptococcus sanguis (10556) did ferment inulin but did not ferment raffinose.

The Streptococcus s.b.e. grows more slowly than most streptococci on ordinary laboratory media and tends to die out rapidly in liquid media, requiring frequent transfers when broth cultures are used.

In studying the immunological characteristics of Streptococcus s.b.e. it was found that 37 of the 42 cultures studied fell into one serological type, the remaining 5 strains fell into a second type. The strain selected for this study belongs to the most common strain, Type I.

The cells are gram positive, spherical or ovoid, measuring 0.8 - 1.2 micron in diameter and occur in medium or long chains.

According to Breed, Murray and Smith (12 p. 515) the present strains of Streptococcus sanguis which was originally isolated as Streptococcus s.b.e. produce narrow-zoned beta hemolytic colonies on blood agar and have a tendency to throw off non-hemolytic variants. Colony forms are of the matt or glossy type and usually are 0.7 - 0.9 mm in diameter on blood agar after 48 hours at 37° C. Streptococcus sanguis react with group H sera, but attempts to prepare group H sera from them have been unsuccessful. The original group H strains were isolated from human

throats and were not believed to be associated with any serious infection. Streptococcus sanguis originally was isolated from so-called vegetations on heart valves from cases of subacute endocarditis and appeared to be one of the most common streptococci associated with this disease. The bacteria are occasionally isolated from infected sinuses and teeth and from house dust. (12) Although White and Niven (47) failed to isolate Streptococcus s.b.e. from 820 Streptococcus cultures from human throats, they did isolate one culture from an infected sinus and another was recovered from an extracted tooth of a patient suffering from subacute bacterial endocarditis caused by Streptococcus s.b.e.

Hehre (26) examined cultures from the throats of 18 healthy young adults with no history of recent respiratory illness and isolated 28 dextran formers with taxonomic features like those previously described for the dextran-forming streptococci from endocarditis. He concluded that although present apparently in much smaller numbers than Streptococcus salivarius, streptococci of the dextran synthesizing variety do occur in the throats of healthy persons and, hence, may be regarded as a part of the normal flora of the human throat as well as one of the important agents of subacute bacterial endocarditis.

As a possible means of preventing the entry of Streptococcus s.b.e. into the blood stream with subsequent infection of the heart valves, it would be of great

interest to determine the usual habitat of this organism. The fact that most strains of Streptococcus s.b.e. are relatively tolerant to bile would suggest that these organisms could survive, and possibly grow, in the human intestine.

Most authors agree that the Streptococcus viridans is a common inhabitant of the mouth and has been recovered from blood cultures by a number of authors; however, they lump all viridans streptococci together which neither helps prove or disprove the presence of Streptococcus s.b.e. as an inhabitant of the mouth.

Boyd (11 p. 21) states " the portal of entry of the streptococci is almost certainly the mouth, although this is difficult to prove. Streptococcus viridans is present in blood culture in about 75 per cent of cases immediately after tooth extraction from infected gums. Even simple pressure on a tooth with an apical abscess will force bacteria into the lymphatics and blood, and mastication produces the same effect."

Fortunately, the bacteremia is not enough to maintain itself and will be effectively eliminated if the heart valves have not been previously damaged by rheumatic endocarditis or some congenital malformation.

Winkler and van Amerongen (49) in a study of 1,141 positive root canal cultures out of a total of 4,186 reported a total of 285 Streptococcus faecalis, 283 Streptococcus mitis, 20 Streptococcus salivarius, 55

Streptococcus hemolyticus, 69 anaerobic Streptococcus and 28 other streptococci, plus various numbers of other bacteria. Fifty per cent of the pure cultures were Streptococci and one wonders how many of the indifferent streptococci and other streptococci were of the Streptococcus sanguis type.

Cobe (17) in a study of blood cultures taken after various dental procedures such as exodontia, brushing, prophylaxis and chewing gum showed that Streptococci were recovered in more cases than any other bacteria. Of 397 cases studied Streptococcus alpha was recovered 91 times or 22.8 per cent, Streptococcus beta 74 times or 18.6 per cent and Streptococcus gamma 82 times or 20.7 per cent.

Vender and Pressman (8) in a study of a series of blood cultures taken immediately after extraction of from two to ten teeth reported finding Streptococcus viridans 82 times, Staphylococcus albus 11 times, Pneumococcus 8 times, Streptococcus hemolyticus once and non-hemolytic streptococcus 4 times.

Hopkins (29) reported 18 positive blood cultures out of a little more than a hundred cases. In fourteen of the cases with positive blood cultures, the organism was Streptococcus viridans and in the other four cases, Staphlococcus aureus (hemolytic)was cultured.

Findings such as these and particularly the isolation of Streptococcus s.b.e. from a tooth in a patient suffering from subacute bacterial endocarditis

prompted an experimental trial utilizing this bacterium. It was necessary to determine whether or not the Sprague-Dawley strain of rats was susceptible to the microorganism and whether an inflammatory response could be produced. As a preliminary procedure four rats were selected and each was injected in the leg muscle with different bacteria and the same amount of sterile water was inoculated in the leg muscle on the opposite side. The bacteria used were Streptococcus sanguis (10556), Streptococcus pyogenes, a streptococcus isolated from the heart blood of a dog that died from endocarditis and a Streptococcus Beta I stock culture from an unidentified source. One week after the injection the animals were again anesthetized, the hair over the site clipped, the skin bathed in alcohol and then opened with a small incision and a wedge of muscle tissue removed. Grossly the tissue injected with Streptococcus sanguis showed the greatest amount of swelling, discoloration and edema. This observation was further confirmed in the histologic sections which showed more inflammatory reaction than did the other tissues.

Normal oral flora of rats.-- In order to obtain some idea of the normal oral flora of the rats used in this experiment, samples were taken with sterile swabs from the oral cavity and throat and plated on blood agar. Representative colonies were then streaked on slides and stained by Gram's method. (15 p. 69) Those showing hemolysis were inoculated into 5 per cent sucrose broth and analyzed for

production of sliming or gelation. The results are tabulated in Table 6.

A second culture was made at the time of sacrifice to determine any changes in the normal bacterial flora as a result of the artificial infection and to check on the possibility of cross contamination of the non-infected groups. As far as could be determined there was no change from the original types of bacteria which were similar in all groups.

Pneumonia in rats.-- The room temperature, humidity and lighting were controlled as closely as possible since middle ear infection and pneumonia are common among rats. (34) In order to determine how many rats in this study had pneumonia three representative sections were taken from the lungs of each experimental animal at the time of autopsy. Approximately twelve hematoxylin and eosin sections and three to six tissue gram stains were studied for each animal. Two cases of mild pneumonia involving less than one-fourth of a 10x field were found in a Group II and Group III animal. One moderate case involving a little less than a 10x field was found in a Group II animal and two severe cases involving areas larger than a 10x field were found in a Group II and Group IV animal. Bacteria were not positively identified in any of the sections; however, in the severe cases there appeared to be a few organisms within cells. No Group I animals demonstrated pneumonia.

Table 6. Bacteria Recovered from Rat Mouths and Grown Aerobically.

Bacterial Forms Gram Stain Hemolysis	Coccus + b	Dip - -	Strep + a	Cocci + -	Strep + a	Dip - -
Fermentation:						
Xylose	+	-	+-	-	+-	-
Mannose	+	-	+	-	+	-
Glucose	+	+	+	+	+	+-
Fructose	+	-	+	+	+	+-
Galactose	+	-	+	+	+	-
Lactose	+	-	+	-	+-	-
Maltose	+	+	+	+	+	+
Sucrose	+	+	+	+	+	-
Raffinose	-	-	+-	-	+	-
Inulin	-	-	+	-	+	-
Trehalose	+	-	+	+	+	-
Glycerol	-+	-	-	+-	-	-
Mannitol	+	-	+-	+-	-	-
Sorbitol	-+	-	+	-	+	-
Dulcitol	-	-	-	-	-	-
Action on: Milk	Acid Curd	Acid Ppt	Acid Ppt	Alk Curd	Acid Ppt	Alk
Starch Hydrolysis	-		-	-	-	-
Indole Production	-	-	-	-	-	-
H_2S						
Peptone Iron Agar	-	-	-	-	-	-
Kligler Iron Agar	-	-	-	-	-	-
Growth in 5% Sucrose	Heavy Turbid	Floc-culent	Heavy Viscid	Diff-use White	Heavy ppt.	Stringy White
Final Identification	Staph aureus	Neiss sicca	Strep bovis 1	Staph epid	Strep bovis 2	Neiss flava

ANESTHESIA

Method of administering anesthetic.-- The rat was placed in an ether jar and as soon as consciousness was lost, was picked up, gently held in the left hand, head down, so that the viscera were displaced backward and cephalad. Care was taken not to cause pressure on the trachea and the body was not squeezed. An intraperitoneal injection of Nembutal was then made, the 25 guage needle being inserted with a quick thrusting motion just above Poupart's ligament and directed deeply, care being taken not to pass much posterior to the abdominal wall, so that vital organs, such as the liver, were not injured. The anesthesia used was Veterinary Nembutal 50 mgm. in 10 cc. which was diluted with sterile water to a strength of 15 mgm. per cc.

During the initial phases of the experiment, the considerable difficulty encountered with anesthesia required numerous trial and error methods before arriving at an adequate working dosage. On many occasions it was found that two rats of the same age, sex and body weight would react quite differently to the same amount of Nembutal.

The rats produced copious amounts of salivary secretions which tended to clog their respiratory passages and interfere with normal breathing. Hemorrhage was also a problem during the fracturing procedures. To overcome this an aspirator was constructed from plastic tubing and a chip blower nozzle. As an added precaution against aspiration and subsequent pneumonia the animals worked upon were

periodically suctioned until they regained consciousness and were able to cope with the situation.

Dosages.-- Adequate anesthesia was obtained for preparing fillings by administering 2.75 mgm. per 100 grams of body weight for females and 3.5 mgm. per 100 grams of body weight for males. For fracturing of the crowns sufficient anesthesia was produced by 2.25 mgm. per 100 grams of body weight for females and 3.00 mgm. for males. Three milligrams per 100 grams of body weight is often recommended for both males and females, for routine surgical procedures. This dosage was found adequate to fracture teeth in males but not to do fillings. It seemed that the manipulation of materials and instruments within the mouth stimulated tongue and muscle movements to the extent that restorative procedures were impossible. By increasing the initial dose 0.5 mgm. to a total of 3.5 mgm. per 100 grams of body weight this difficulty was overcome.

Duration.-- When this solution was given intraperitoneally, anesthesia was complete in 5 to 15 minutes and lasted for about 45 minutes which allowed adequate time for the experimental procedures.

In the event that more anesthesia was required, a second injection, approximately one quarter of the first dose was given after a minimum wait of 15 minutes after the first injection; otherwise the probability of lethal dosage was too great.

PREPARATION OF EQUIPMENT

A routine procedure was followed in an attempt to standardize the positioning and handling of animals and equipment in the laboratory, the preparation of materials, bacteria, solutions, etc., and in sterilization techniques.

Packs containing operating gowns, rubber gloves, masks, towels, paper towels, ligatures, string, suture materials, tongue blades, rubber dam, and syringes were labelled, autoclaved and stored ready for use. The laboratory bench was prepared by a thorough scrubbing with Roccal 1:1250. Sterile towels were placed on the surface of the bench, then the animal operating tray which had also been scrubbed with Roccal was centered under the light and covered with a sterile towel. The operating tray was made from a rectangular piece of brass which was notched along the sides to receive and hold a small chain. Various types of restraining hooks attached to the chain could be used to hold the animal in the desired position. Sterile packs were placed at convenient points around the tray, opened and covered with sterile towels. Metal instruments, burs, broaches, air syringes and aspirator tips were stored in 1:1000 Zephiran chloride solution which was the only concentration used in this experiment. Sterile instrument forceps were placed within easy reach in Zephiran chloride. Before proceding the operator and assistant were clothed in sterile gowns and masks.

Placing rubber dam.-- After the bench layout was completed the assistant placed the anesthetized rat on the animal tray, attached the restraints, and draped the rat with a special towel which exposed the head only.

A slight modification of the method developed by Smith and Tappe (43) for entering rat molar pulps aseptically was used in Groups III and IV. They passed ligatures between the teeth and through the jaws of the animals. This worker felt that the added trauma might easily disturb the normal relations of the apical areas since the contacts were extremely tight and the teeth had to be moved slightly to permit passage of a ligature. The possibility exists that small ducts may be severed when ligatures are passed through the cheeks and this procedure also exposes a number of areas to infection which might confuse the findings in the present study.

The maxillary first molars were exposed through holes punched in a 3 x 5 inch piece of rubber dam. The dam was held in place by a specially designed cast retainer and by anterior and posterior ligatures attached to the retainer and passed under the rat's head to the corner posts of the animal tray.

The teeth were painted with 5 per cent phenol which was neutralized with 70 per cent alcohol. The operator put on sterile rubber gloves and swabbed the entire exposed rubber dam with Zephiran chloride on a cotton swab. The surfaces of the first molars were scrubbed with

Zephiran chloride and allowed to dry.

Cavity preparation and restoration.-- A 33½ bur was held in a sterile forceps and fitted into the hand-piece which had been cleaned and then wrapped in guaze soaked in 70 per cent alcohol. An occlusal cavity was prepared and the coronal pulp exposed. A small sterile broach was introduced into the pulp and root canals of the Group III animals and the pulp traumatized by sterile pseudo-inoculation procedures. Similar broaches inoculated with Streptococcus sanguis were used in the Group IV animals. Of twelve burs cultured at 37° C, which should have been sterile after use, one showed bacterial growth and two supposedly sterile broaches out of sixteen proved to be positive. The broaches used to inoculate bacteria all gave positive results. Small squares of tin foil were molded into the tooth cavities so that the borders extended occlusalward and the pulps were entirely covered. Soft alloy fillings were then placed and carved to occlusion. Earlier, tin foil, a number of different waxes and amalgam had been tested for bactericidal effect on Streptococcus sanguis on blood agar and all were found to be negative except for amalgam which definitely inhibited bacterial growth. These materials were tested in a manner similar to that used for routine antibiotic sensitivity tests. The results of these tests are shown in Tables 8 and 7.

The rubber dams were removed, the airways cleared by suction and the rats returned to their cages.

Table 7. Aerobic and Anaerobic Sensitivity Interpretation for Streptococcus sanguis on Blood Agar.

Drug tested	Aerobic growth	Anaerobic growth
Aureomycin	Resistant	Very sensitive
Chloromycetin	Very sensitive	Very sensitive
Dihydro-streptomycin	Sensitive	Sensitive
Erythromycin	Sensitive	Very sensitive
Novobiocin	Sensitive	Sensitive
Penicillin	Very sensitive	Very sensitive
Terramycin	Sensitive	Sensitive
Tetracycline	Resistant	Resistant
Elkosin	Very sensitive	Very sensitive
Gantrisin	Very sensitive	Very sensitive
Sulfadiazine	Very sensitive	Very sensitive
Sulfamerazine	Very sensitive	Very sensitive
Sulfathiazole	Very sensitive	Very sensitive
Thiosulfil	Very sensitive	Very sensitive
Triple Sulfa	Very sensitive	Very sensitive

Fracturing of teeth. -- The procedures used prior to fracturing of the teeth were very similar except that the teeth were not sterilized and the rubber dam was not placed. Approximately one-half of the fractures were performed on the right first molar, the remainder on the left first molar. The contra-lateral non-fractured tooth was the control.

Table 8. Bactericidal Action of Amalgam, Waxes and Tin Foil.

Material Tested	Bactericidal Action
Amalgam.....	Positive
Waxes:	
Black boxing.....	Negative
Red boxing.....	Negative
Green relief.....	Negative
Beeswax.....	Negative
Tin Foil.....	Negative

Table 9. A Comparison of the Average Number of Days Between Treatments.

Group Number	Sex	Operation to Fracture	Fracture to Sacrifice	Operation to Sacrifice
I	Females	0	0	0
	Males	0	0	0
II	Females	0	40	0
	Males	0	34	0
III	Females	36	34	70
	Males	36	34	70
IV	Females	31	37	68
	Males	31	38	68

Number of days between treatments.-- A comparison of the average number of days between treatments for the females and males of each group is shown in Table 9. Operation to fracture refers to the time interval between the opening of the pulp, either with or without infecting it, and the time of fracturing the crown. The next column refers to the time lapse between fracturing of the crown and sacrifice of the animal and the last column refers to the overall time lapse from operation to sacrifice. The time intervals are approximately equal between females and males of each group except for the Group II animals. A fifty day interval for one animal in this group considerably raised the average time. It is felt that the time lapse between groups does not vary enough to materially affect the results and the overall time lapses of Group III and Group IV are very close.

Control groups were coordinated so that their times of sacrifice and ages correspond to the infected group. Individual animals from each group were processed together to help standardize procedures.

Sacrifice of animals.-- The animals were sacrificed by placing them in an ether jar until their respirations and heart beat were no longer detectable. They were then transferred to the autopsy table and the body cavity opened by a midline incision from the sternum to the lower abdomen with a #15 Bard Parker. The incision was extended and further opened utilizing sharp scissors.

The thoracic cavity and mediastinum were opened and as much blood as possible was withdrawn from the heart. The blood was placed in sterile tubes, sealed with Parafilm and allowed to clot. After twenty-four hours the tubes were centrifuged and the serum drawn off with a pipette, placed in labeled tubes and frozen for future study of antibody reactions.

Following removal of the blood, the heart and lungs were removed and the lungs examined for gross evidence of pneumonia. Next the liver, spleen, kidneys and portions of the pancreas were removed and placed in cold 10 per cent formalin.

Perfusion of formalin.-- Perfusion of a small sample of animals was tried to aid in determining the quickest satisfactory method of fixation. The perfusion was accomplished using PE 10 and PE 50 Intramedic Polyethylene tubing made by Clay-Adams Co. The tubing was attached to adaptors and then to stopcocks so that either saline or formalin could be injected. Plastic tubing was inserted into the right and left carotid arteries and outflow tubing into the jugular veins. Blood in the vessels was forced out by the saline rinse and then cold 10 per cent formalin was injected.

The procedure was time consuming and it was almost impossible to keep a closed system. It was also suggested that the perfusion of saline and formalin may effect the concentration and distribution of bacteria around the

apices and within the pulps. Any benefits derived from the method were probably lost due to the time required to assemble the system and if the possibility of changing the distribution of bacteria were real then this method should not be used. It seems obvious that this method of fixation should be tested and would provide an interesting problem for study.

Table 10. Number of rats by groups available for histologic study at the conclusion of the experimental procedure.

GROUPS	MALES	FEMALES	TOTAL
I	5	4	9
II	5	6	11
III	4	5	9
IV	6	5	11
Total	20	20	40

Dixon and Massey (20, p.181) state that the computations for the analysis of variance and the computations for the test for homogeneity of variances are simplified if all samples are of the same size. Also an additional reason for selecting samples of equal size is an expected improvement in our estimate of the variance of the difference of two means.

Since there was considerable variation in the number of rats remaining in each group (Table 10.) it was decided to equalize them by randomly selecting four males and four females from the remaining members of each group, except

in those with only four male and four females, which must obviously be included. (See Table 11.)

Table 11. Random numbers used to select final samples for study in order of occurrence as found in Random Numbers Table, page 368, Middle block, 3rd column, 27th row, #12 first number. (20) Random number listed in parenthesis with cage number selected to the right.

GROUP I		GROUP II	
Male	Female	Male	Female
(2) 6	17	(1) 12	(5) 33
(3) 32	22	(12) 39	(6) 21
(10) 19	29	(20) 24	(8) 26
(19) 28	43	(23) 27	(11) 46

GROUP III		GROUP IV	
Male	Female	Male	Female
3	(7) 41	(4) 25	(15) 5
37	(13) 14	(9) 20	(16) 15
40	(17) 36	(14) 11	(21) 23
42	(24) 38	(18) 9	(22) 10

After the body organs were placed in formalin, the head was severed from the body, partially skinned and the mandible disarticulated and placed in formalin. The maxillary molars were examined under the dissecting scope and photographs were made at this time.

A number of small root fragments and some of the surrounding area were removed from one of the infected animals jaws for bacteriologic culture. This could be done aseptically by first searing the palate with a hot spatula and then surgically entering through this area. It was possible to remove the root fragment in this manner; however, the remainder of the specimen was rendered useless for histopathologic study. It was decided not to attempt to culture apical material from animals in this study but to concentrate on the histopathologic alterations in the apical areas and utilize the culture method for future studies.

Radiographs. -- After completion of the gross examinations each rat head, less the mandible, was radiographed using a standard dental x-ray machine set at 65 KV and 10 M.A. The rat head was placed on a standard sized dental film so that the plane of occlusion of the maxillary arch was angled approximately 30 degrees from horizontal. The long cone was utilized and exposures were made for 1½ and 2 seconds with the central ray directed at right angles to the film. Following this, the heads were divided along the midline and two more cuts parallel to the first cut were made just ahead of the first molars and distal to the third molars. After the excess facial tissue was trimmed away each half of the maxilla was placed in cold 10 per cent formalin and stored in the walk-in cooler. It was hoped that the cooling of the tissue would reduce autolytic

changes and yet allow penetration of the formalin.

After fixation each half of the maxilla was radiographed on Eastman Industrial X-ray Film, Type M, using a Westinghouse Portable X-ray Machine at 65 KV and 15 M.A. for 6½ seconds. Type M film was very fine grained in comparison with standard dental films and enlarges much better.

Weights of rats. -- The weights of the rats in each group were recorded weekly and are presented graphically in Figures 1 - 5. All rats show a gain in weight from the beginning to the sacrifice date with the exception of two Group III rats which progressively lost weight after their teeth were fractured and finally expired. With such a small number of animals these two deaths materially affect the total weights for Group III. The Group I rats showed an overall increase in weight with many alternating periods of gain and loss. The Group II rats were not seriously affected by the fracturing of one maxillary molar with five showing an increase in weight following this procedure.

All of the Group III and IV rats, with one exception in Group IV, increased in weight after the initial operative procedure. Following the fracturing of one molar, many rats experienced a temporary period of weight loss but soon recovered, others continued to gain weight. No definite conclusions from this small preliminary study can be made regarding weight gain or weight loss; however, it appears that the procedures used do not permanently affect the expected weight increases.

Figure 1. Weights of Group I Females and Males.

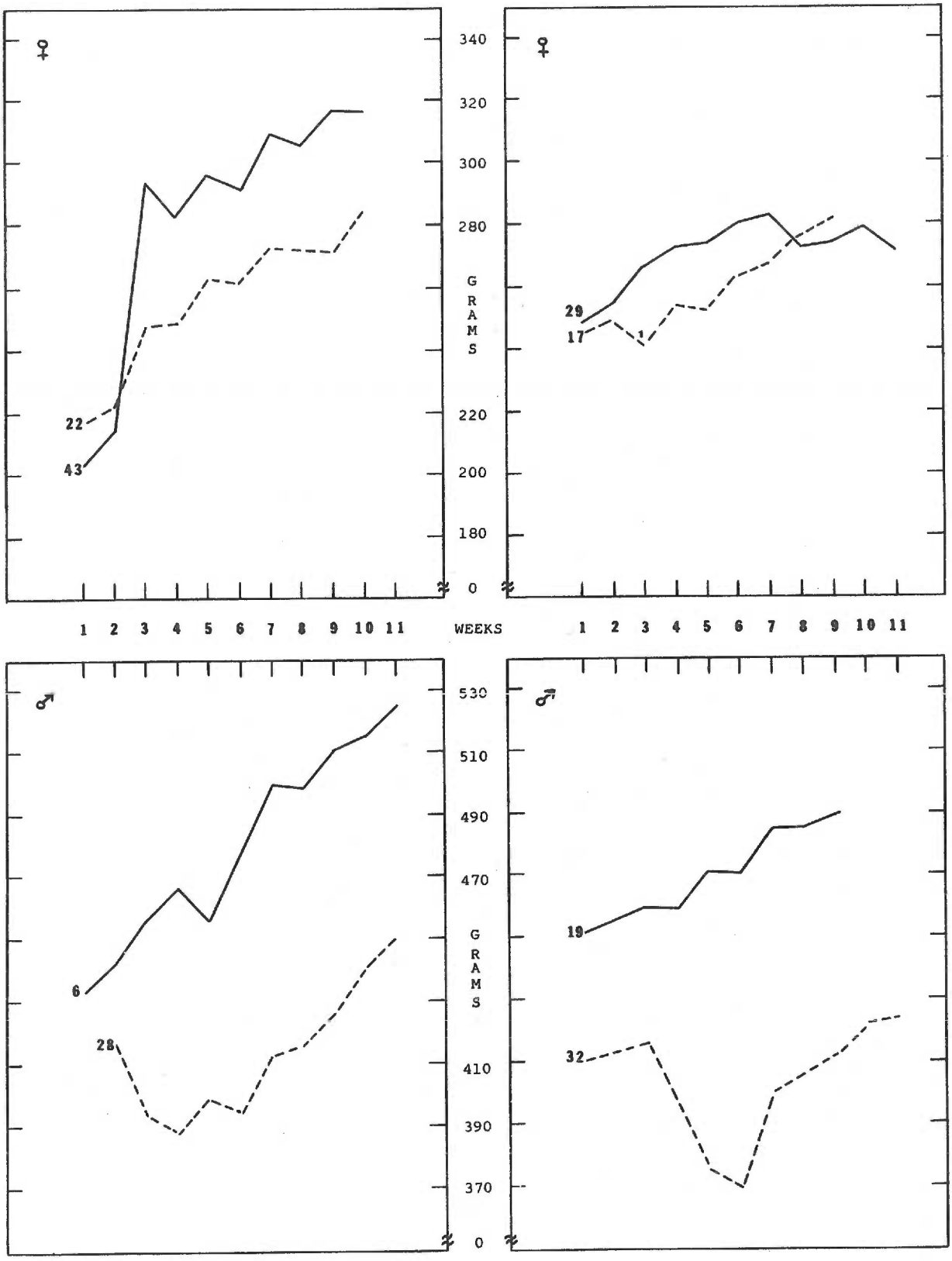


Figure 2. Weights of Group II Females and Males.
1 = Time of Fracture.

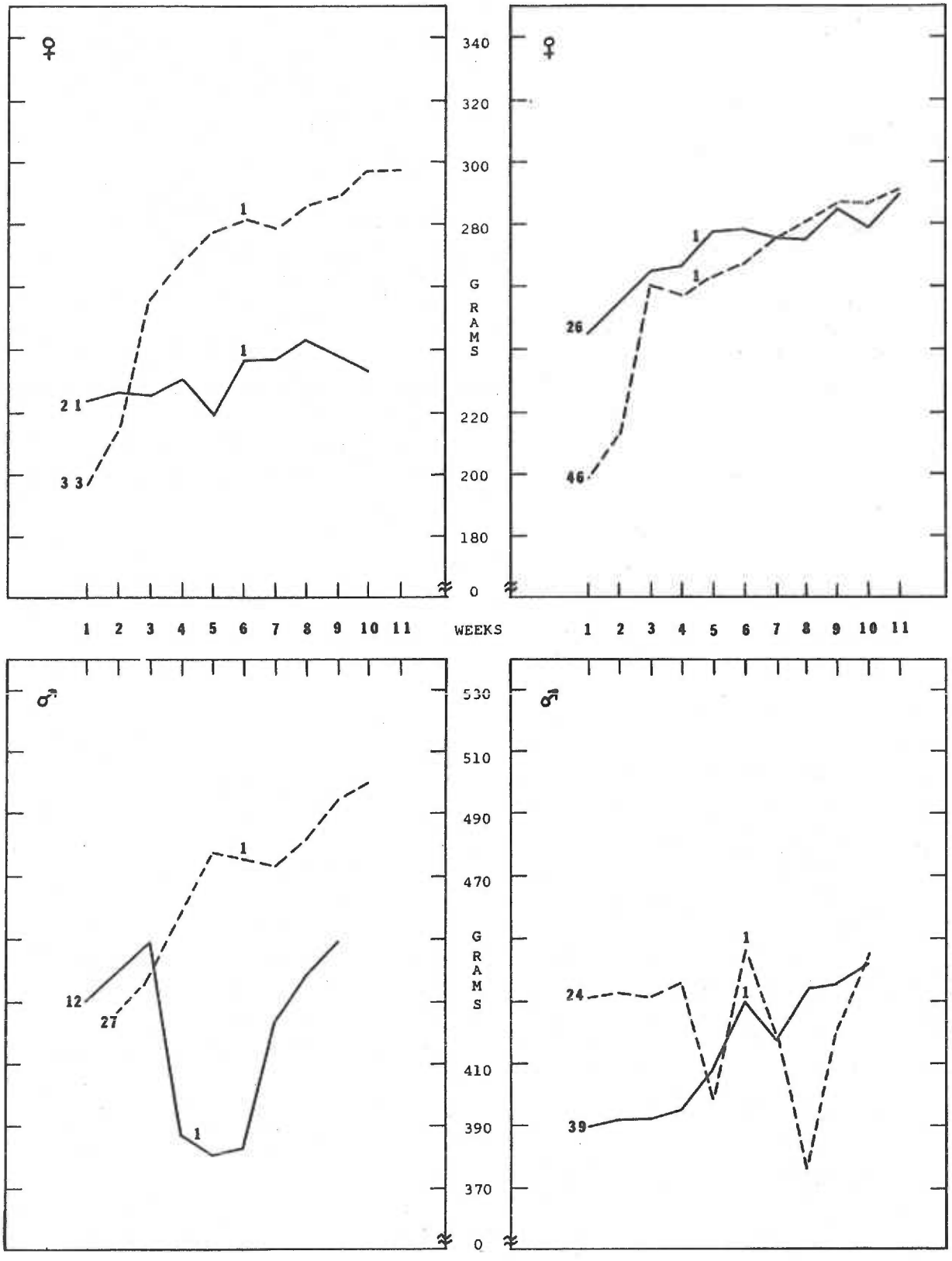


Figure 3. Weights of Group III Females and Males.
0 = Time of Operation.
1 = Time of Fracture.

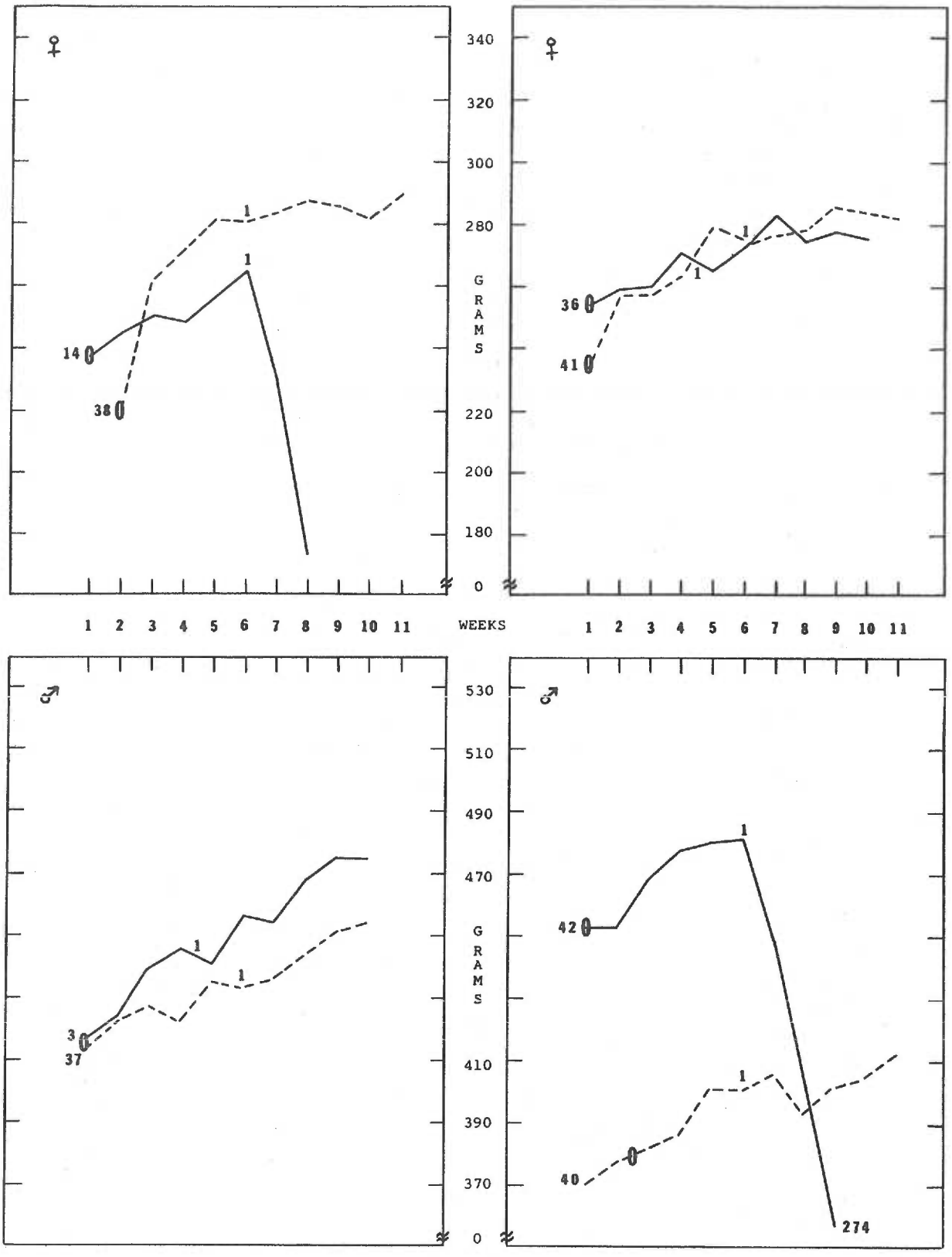


Figure 4. Weights of Group IV Females and Males.
0 = Time of Operation.
1 = Time of Fracture.

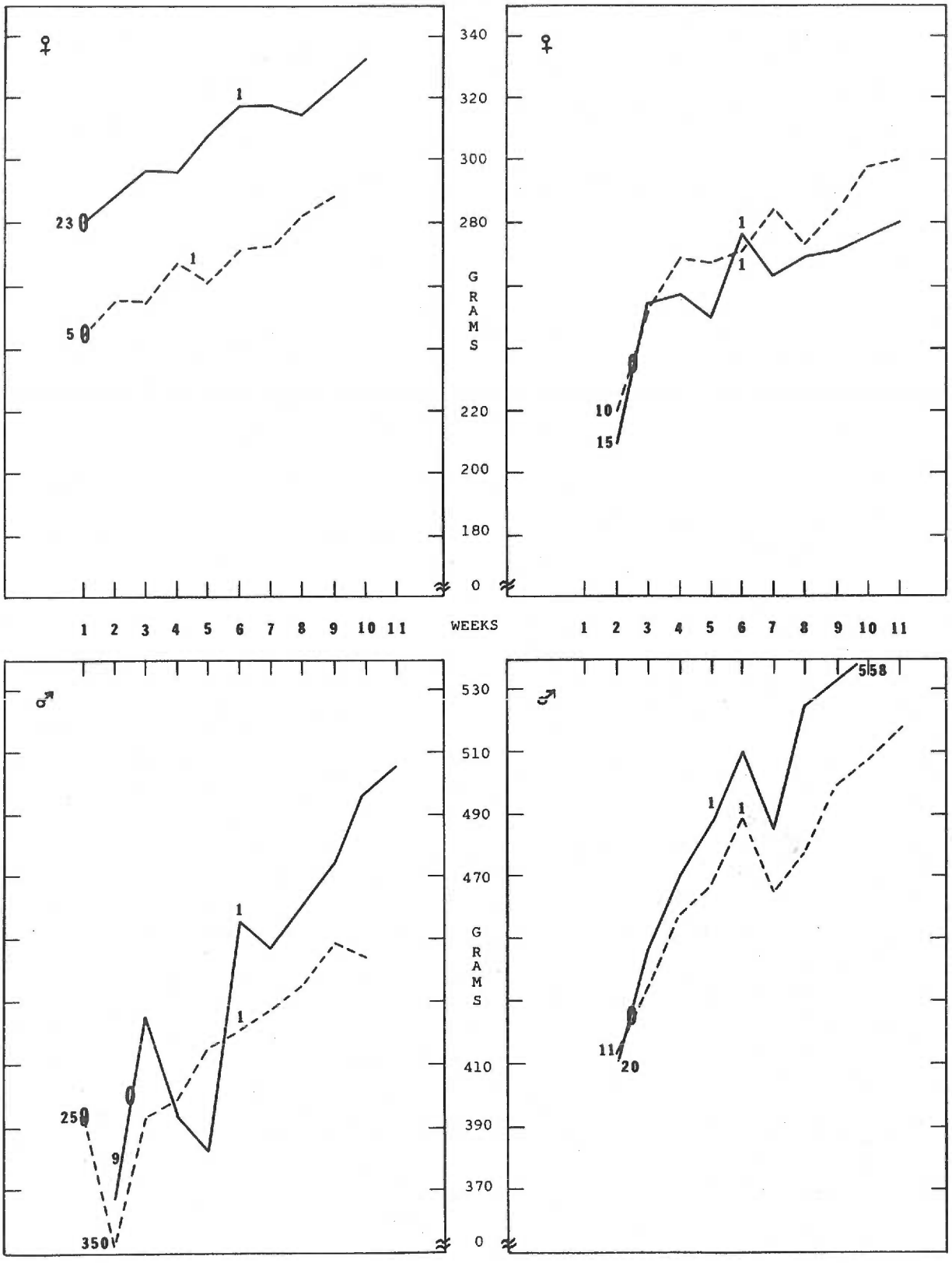
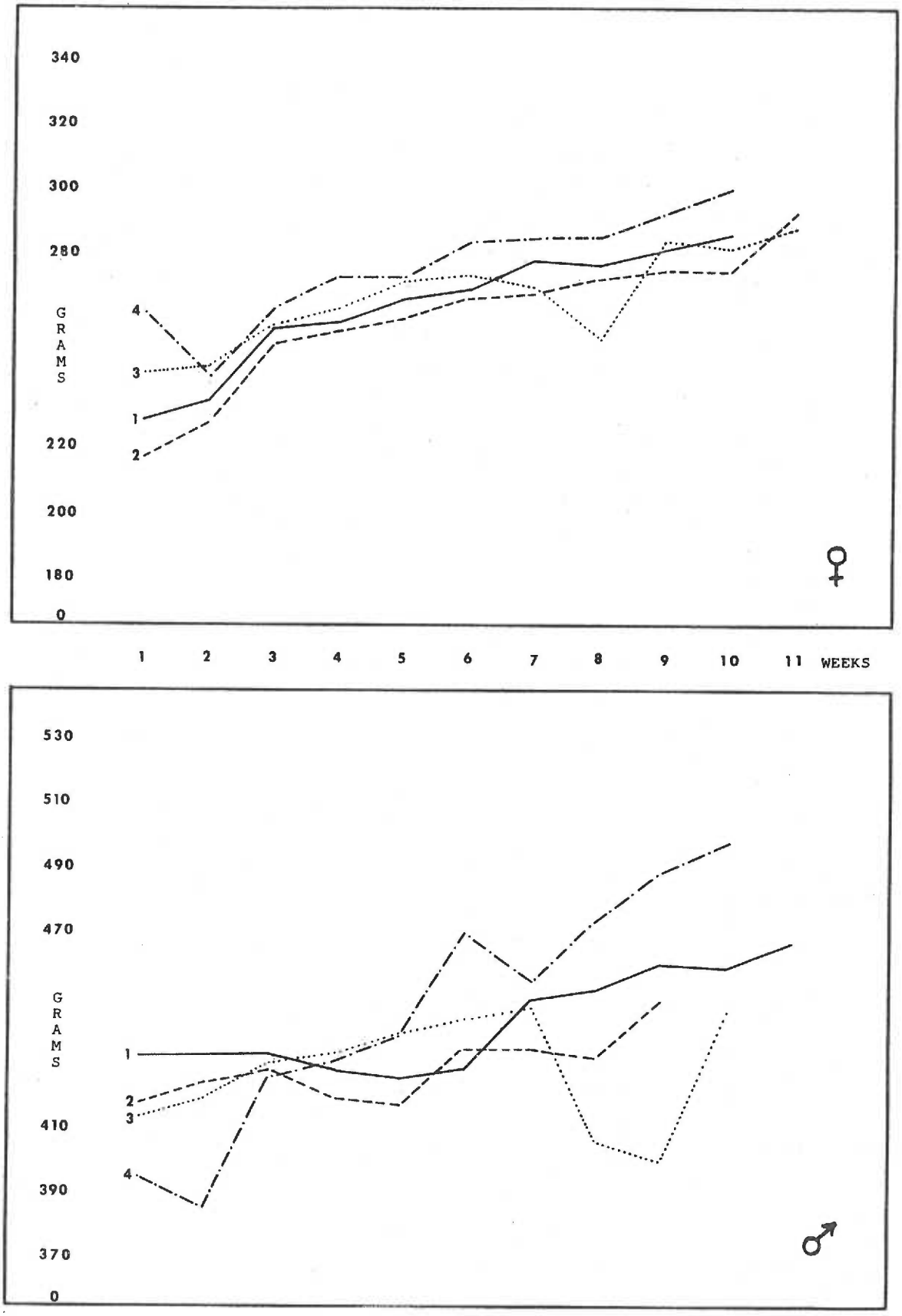


Figure 5. Comparison of Weights of Females and Males of Groups I, II, III and IV.



HISTOLOGICAL PREPARATION

Fixation.-- In any critical histopathologic study attempts are made to preserve fine cellular detail by rapid fixation, or by reducing the temperature concomitant with perfusion of the tissue by some acceptable method of fixation. One of the perplexing problems in oral pathology is how to fix pulpal tissues which are surrounded by cementum, dentine, enamel and dense alveolar bone. Most of the fixative reaching the pulp probably must diffuse through the apical foramen and as a result of this slow process, the central portion of the pulp undergoes varying autolytic changes. In this study, the root fragments should show better fixation since the fixative can penetrate the pulp from two directions, the apical foramen and the exposed pulp at the line of fracture. The uninvolved teeth, and other controls, should also show good fixation near the apical end and should compare favorably with the cellular detail of the root fragments.

A number of methods (19 p. 188, 28, 49) have been suggested to increase rapid penetration of fixative into the pulp, based on the idea that the thinner the area through which fixative must pass the more rapid the fixation. To my knowledge there still is no method of fixation which will eliminate all of the artifacts.

There are objections to the use of formaldehyde (32) but it still is excellent general fixing solution and allows the subsequent use of many staining reagents,

particularly those essential to this study. All specimens were fixed in buffered aqueous 4 per cent formaldehyde solution (10 per cent formalin) for approximately 72 hours and were agitated several times each day.

Following fixation, specimens were placed in a large beaker with running fresh tap water entering at the bottom and then were washed for five to twelve hours, depending on size.

Decalcification.-- Specimens were then placed in formic acid-sodium citrate reagent until the chemical test for calcium was negative for three days in the same solution. The method of decalcification was described by Morse (33) and is a modification of a method used by the Department of Pathology at Tufts College Medical School. In order to fully utilize the chemical test for calcium the decalcifying solutions were mixed and changed daily. The chemical test for calcium as outlined by Arnim (6) was used and when the same solution showed negative results for three days the tissues were washed in running water for 20-24 hours to eliminate any reagent which would interfere with subsequent staining, especially nuclear detail.

In order to be certain that the chemical test for the presence of calcium was working as it should and was producing a qualitative result, a positive control and a mixture of the basic chemicals (negative control) were tested at the same time. Many solutions were tested both

with the pH papers and pH meter and it was found that the average pH was 3.5.

Jaffe (30) states that formic acid causes great swelling of the collagenous fibrils; however, others (21) suggest that the citrate counteracts the swelling caused by the formic acid. Morse (33) states that the "tissues run through the combination (Formic acid-sodium citrate) showed only slight swelling of the cytoplasm and collagenous fibers and the nuclei stained more brilliantly than those of similar untreated material." Fungal and bacterial growth in the sodium citrate was kept at a minimum by the addition of a crystal of thymol.

Dehydration.-- After washing, the tissue was placed in 10 per cent formalin for 24-48 hours, then in 50 per cent ethyl alcohol, with one change in the first twenty-four hours, and then dehydrated through increasing concentrations of alcohol up through absolute alcohol.

The tissues were placed in Xylene until clear and then infiltrated with three changes of 56° - 58° C. melting point paraffin. The third paraffin bath was placed in a vacuum oven at 60° C. with 15° mercury for one-half to one hour or until bubbling stopped. The tissue was then embedded in the usual manner.

The paraffin around the specimen was trimmed so as to have about one-fourth inch at both bottom and top, but having almost no paraffin around the sides, to help prevent wrinkles. 50-75 grains of powdered gelatin were

mixed in the water bath at 45° C. After mounting the block, the specimen was soaked by applying cotton moistened with water and the right and left half of the maxilla were then sectioned along an anterior-posterior plane attempting to follow an imaginary line passing through the uninvolved roots of the second and third molar and the residual root fragments of the first molar. In two rats there was gross evidence of infection with swelling, fistulae and draining abscesses on the face. Only one side of the face was involved and these were sectioned in a buccal-lingual direction so that the soft tissue was also included in the preparation.

If the paraffin was cut in several places on both sides of the tooth prior to picking up, less difficulty was encountered in removing wrinkles. The slides were allowed to drain, eliminating excess water and then a small brush was used to flatten the section. The slides were dried on a warming table or in the oven for 20-30 minutes and were then returned to room temperature. The sections were deparaffinized in two changes of Xylene, each three to five minutes duration and transferred to absolute alcohol. (5)

METHOD OF SECTIONING TISSUES BY GROUPS

Group I. -- A total of 360 sections were made of the right and left maxilla of one rat with every 10th slide stained for bacteria, every 11th slide stained for fungi, and the others stained with hematoxylin and eosin.

The remaining Group I tissues were sectioned as follows; every 21st, 22nd, 23rd and 24th section was stained with hematoxylin and eosin, the 25th and 26th tissue gram stained, the 27th and 28th saved for Gomori stain and the process repeated until the teeth had been completely sectioned. The unstained sections were saved, unmounted, on paper in boxes. There were no major differences noted between tissues of the control animals. Therefore, it was decided not to mount and stain the remaining tissues.

Group II. -- The side on which the tooth was fractured was sectioned as follows; 1st to 4th sections stained with hematoxylin and eosin, 5th and 6th tissue gram stain, 7th and 8th saved for Gomori stain, 9th through 16th sections were saved and the process repeated. The non-fractured side was treated as follows; one tissue was sectioned the same as the fractured side, the remaining tissues were sectioned in the same manner with the exception of 24 sections which were cut and saved following each 8th section, before repeating the process.

Group III.-- The tissues of the Group III animals were sectioned in the same manner as in Group II.

Group IV. -- The tissues of the Group IV animals were sectioned in the same manner as the fractured side of the Group II and Group III rats.

The tissues from one animal of each group were processed together to further insure uniformity in cutting and staining techniques. All of the sections which were

not mounted and stained were saved for further study of critical areas. In a few cases it was necessary to complete the mounting and staining of sections to fully evaluate the pathologic process involved.

SUMMARY OF HISTOLOGICAL PREPARATION

The tissues on the slides are:

1. Either perfused with cold formalin for 24-48 hours and then placed in cold formalin for 24 hours or fixed in 4 per cent aqueous formaldehyde solution (10 per cent formalin for 72 hours.)
2. Washed in running tap water for two to five hours.
3. Decalcified in formic acid-sodium citrate reagent until the chemical test is negative for the presence of calcium.
4. Washed in running tap water 20-24 hours.
5. Replaced in 20 per cent formalin 24-48 hours. Then passed through....
6. 50 per cent ethyl alcohol. One change in 24 hours.
7. 80 per cent ethyl alcohol. Two changes in 48 hours.
8. 95 per cent ethyl alcohol. Three changes in 48 hours.
9. Absolute ethyl alcohol. Three changes in 48 hours.
10. Xylene until specimen is clear.
11. Placed in 56° - 58° paraffin.

    First bath - two hours.

    Second bath - two hours.

    Third bath - one hour using the vacuum oven.

12. Embedded
13. Sections were cut on a standard microtome.
14. Collected on slides.
15. Baked in an oven 20 to 30 minutes.
16. Transferred to Xylene, two changes, three to five minutes each.
17. Transferred to absolute alcohol.
18. Transferred through graded alcohols, absolute, 95, 80, 50, 30 per cent each three minutes.
19. Hydrated in tap water one or two minutes.
20. Stained

FINDINGS

GROSS EXAMINATION.

The gross examination consisted of observation of the general appearance of the teeth, the presence or absence of abscesses, the appearance of the fracture site, how well it had healed, how many root fragments were grossly visible, and the appearance of the control teeth. Details of all findings may be seen in Appendix "H" tabulated in IBM punch card form, together with the interpretive key.

There appeared to be no definite pattern within groups in relation to the staining of teeth and packing of debris in and between the molars, with twenty-three animals showing stains and packed debris and nine with packed debris only. All of the molar teeth showed comparable wear patterns with well-faceted occlusal surfaces.

Facial abscesses. -- Two animals presented large facial abcesses, one a Group II female, the other a Group IV male. Both had large swellings approximately one centimeter in diameter located in the lateral maxillary jaw region which were draining a thick yellow-green purulent material. Cultures of this material revealed a mixed infection with predominance of Staphylococcus aureus. A large number of animals showed small amounts of puriform material around the root fragments mixed with food and

other debris. These were not counted as abscesses.

Exposed root fragments.-- Exposed root fragments were found fourteen times in the fracture sites with one root visible in seven Group II, four Group III and two Group IV rats. One Group IV rat had two exposed roots. Complete healing of the fracture site occurred in three Group III and four Group IV rats. Seven healing fracture sites showed openings less than one-half millimeter, two less than one millimeter, five less than two millimeters and three less than three millimeters. These were the largest openings noted. The areas of healing were wrinkled, folded and uneven in all animals examined except 23 - IV - 15 which showed a completely normal appearance and one could not tell where uninvolved tissue stopped and the fracture site began. Testimony to the tremendous resistance of the rat mucous membrane to infection was seen in the gross examination. Of all animals examined not one showed any indication of inflammation grossly, there were no color changes and no evidence of edema in the area of fracture or elsewhere, even though roots were exposed and debris was packed around them.

Loss of fillings.-- It was rather disappointing that all but three fillings in the control teeth had come out and two of those present were loose. Nearly all fillings were in place at the time the teeth were fractured. The added stress and forces placed on the control tooth may

have been partly responsible for the splitting of the control tooth crown and subsequent loss of the filling. Probably the major reason for their splitting was associated with the manner in which they were prepared. It was incorrectly assumed by the experimenter that a well extended Class I occlusal cavity preparation should be made and this procedure was followed in all cases in which the crowns fractured or the fillings were loose. The proper method of preparing the tooth was discovered quite by accident. During cavity preparation on a Group IV control rat in which the filling stayed, considerable difficulty was experienced with anesthesia and to speed up the procedure a very small, non-extended cavity was prepared. This small centrally located cavity left thicker, stronger supporting walls and provided the tooth with the necessary support to maintain a filling. In one control tooth the crown was intact but the filling was out. All other control teeth were split open and had one, two, or three cusps fractured off. Three of these teeth had an ingrowth of epithelium partly covering and filling the open pulp chamber.

RADIOGRAPHIC FINDINGS.

Fistulous tract.-- None of the radiographs examined exhibited clear cut evidence of the presence of a fistulous tract. In twelve rats there was a radiolucency suggestive of a sinus; however, these could not be differentiated from possible bone fractures. One of the rats with a large facial abscess presented evidence of a fistula and this was

confirmed microscopically.

Root fragments.-- Root fragments were present in all fracture sites and no tooth had been completely removed.

Metal fragments.-- Metal fragments were not seen in any Group I or II rats. Radiopaque objects, some extremely small, were noted in seven rats from Group III and seven from Group IV.

Lamina dura.-- The Group I and II control teeth showed a complete lamina dura. All fracture sites presented marked deviations from normal or were questionable. The apical area(s) of one Group II, four Group III and five Group IV rats showed complete absence of the normally well-defined bony margins. Two Group II, three Group III and four Group IV rats showed irregularities of the lamina dura in the apical area but not total destruction. The lamina duras were partially destroyed in all areas in two Group II, two Group III and three Group IV rats and was entirely absent in one Group II and two Group IV animals. In each of the categories examined the Group IV rats showed the more severe reactions.

Root resorption.-- No pathologic root resorption was seen in control teeth in Groups I and II. Pathologic resorption of roots was apparent in six Group II fracture sites, thirteen Group III and twelve Group IV rats and questionable in one Group II, three Group III and three Group IV rats. In the Group III and IV rats, pathologic

root resorptions occurred in both "control" and fracture sites.

Bone fractures.-- No fractures were present in Group I or Group II control teeth; however, one Group II control area was questionable. Fractures appeared to be present in two Group II and one Group III rat and questionable in two Group II, three Group III and seven Group IV animals. The small size and delicate bone structure of the maxilla make positive diagnosis of fractures very difficult.

Radiolucency.-- Pathologic radiolucencies were associated with roots in all Group II fracture sites and in none of the Group I and II control teeth. The Group III and IV rats each had fourteen radiolucent areas associated with root fragments or foreign bodies, which were generally in the same area when present. One Group III showed a radiolucent area around a foreign body and two Group IV rats had the second molars involved as well as the first molar fracture site.

MICROSCOPIC FINDINGS

Number of tissue sections examined. -- A total of 3,628 sections were examined which represented 2,469 hematoxylin and eosin sections and 1,159 tissue gram stains for bacteria. In addition 72 slides were stained by Grocott's modification of Gomoris' Methenamine Silver Nitrate Stain for fungi. This staining technique is difficult and time consuming and did not materially add to the study so was dropped in favor of the tissue gram stain. (See Appendix C for staining procedures.)

Major categories studied.-- The microscopic analysis was divided into three major categories; the first comprised a general survey of the surface epithelium over the fracture site and notations as to changes in the periodontal membrane, pulp tissue, cementum and dentine; the second involved the type of inflammatory reaction, its size and associated hyperemia; the third analyzed the presence or absence of bacteria, their type, microscopic location and concentration.

Surface epithelium at the fracture site. -- The majority of sections of surface epithelium overlying the fracture site showed openings of varying size generally associated with exposed root fragments or with those lying very close to the surface. Five Group II, five Group III and seven Group IV animals showed these changes. One Group III male and one female and one Group IV female appeared to have an intact epithelial surface with some downgrowth and proliferation attempting to surround root fragments

and presumably remove them to the surface. One Group II male and one female showed extensive epithelial proliferation which had surrounded the root fragment as demonstrated by examination of multiple serial sections; however, the surface epithelium was not intact over the root fragment. Others showed combinations of partial and complete proliferation around fragments. In numerous cases it appeared that the epithelium had extended across the fracture wound and had attached itself to the cementum near the fractured surface of the root and then had proliferated along the root surface. In these cases the periodontal membrane fibers were not discernible and possibly their absence permitted this downgrowth of epithelium. Occasionally a cementum-like substance was found in the pulp canal near the fracture and in at least one case the epithelium appeared to be attached at this point in an attempt to seal off the pulp canal.

Food debris.-- Food debris was found in the interproximal areas, packed in the grooves on the occlusal surfaces of teeth, and in the gingival crevice distal to third molars in nearly all animals and was considered a normal finding. Also large amounts of hair was commonly packed in the interproximal spaces. The Group III and IV control teeth with one exception contained large amounts of food debris and hair in the pulp chambers due to the loss of the fillings and fracturing of most teeth. Food deep in the tissue was seen only once in a Group II fracture site.

NORMAL FINDINGS

Gingiva.-- The surface epithelium of the alveolar gingiva was stratified squamous in type and was covered by a keratin layer which varied somewhat in thickness. Occasionally rete pegs were present but usually the epithelium had a ribbon-like appearance. The connective tissue fibers in the marginal gingiva formed a horizontal band encircling the tooth. Mononuclear cells and polymorphonuclear leukocytes were often present in the subepithelial connective tissue and frequently the adjacent capillaries were dilated and filled with erythrocytes.

Alveolar bone. -- Compact bone normally formed the peripheral areas of the alveolar process and lamellae extended inward to form marrow spaces. In some areas the compact bone was quite thick and showed many reversal lines. Resorption lacunae were noted and some contained multinucleated giant cells. Marrow spaces were numerous and often quite large. Numerous blood vessels were seen passing through the bone to the periodontal membrane and gingivae. The marrow was quite vascular and markedly cellular.

Periodontal membrane.-- The periodontal membrane was generally of uniform thickness, quite cellular and well vascularized. The cervical fibers extended from the cementum obliquely into the marginal gingivae and horizontally through the interproximal gingivae to adjoining teeth. The main body of fibers passed obliquely from the

cementum occlusally to alveolar processes. These were well demonstrated on some of the tissue gram stained sections. The fibers about the apices of roots radiated from cementum to alveolar bone, while those in the bifurcations of roots entered the processes at various angles. The nuclei were oval, large and vesicular with their long axes parallel to the course of the fibers. The vessels were larger and more numerous along the bone surfaces.

Cementum.-- The cementum was thin in the cervical third of the root, thicker and more uniform in the middle third and thickest in the apical third. The cervical third, like the zone adjacent to dentine in the middle and apical thirds, was acellular. The remainder of cementum in the middle and apical thirds contained cells with numerous processes in fairly even distribution. The nuclei were round or oval and had a light halo around them. They appeared to be equal in number to those in adjacent alveolar bone. Their distribution; however, was not so regular or systematic. The periodontal surfaces of the cellular cementum were uneven. Fibers of the periodontal membrane were seen embedded in cementum. Resorption lacunae were more numerous in the cellular regions.

Dentine. -- The dentine was composed of an apparently homogeneous matrix containing the dentinal tubules and their contents. The tubules radiated from the pulp chamber to the outer surface of the dentine. A predentinal layer bordered the pulpal surface of the dentine. Some tubular

secondary dentine was seen capping the pulpal horns in the cusps and opposite the sulci. Secondary dentine which capped the pulpal horns was sparsely tubular and contained occasional cells and minute spaces. Resorption lacunae involving dentine were observed on the cemental surfaces of roots; however, none were seen along the walls of the pulp chambers or root canals.

Enamel.-- The spaces formerly occupied by enamel were well outlined by the dentine on one side and occasionally by debris or epithelium on portions of the other side.

Pulp.-- The pulp was composed of loose, vascular, fibrous connective tissue. The cells had large vesicular nuclei and numerous protoplasmic processes. They were embedded in an intercellular substance containing a fine network of fibrils. Along the border of the pulp there was a dense zone of odontoblasts with large nuclei in a palisade arrangement. Cytoplasmic processes could be seen extending from the odontoblasts into the dentinal tubules. Many large dilated blood vessels were seen filled with red blood cells. Occasionally irregular structures resembling "pulpstones" were encountered. The findings were essentially the same as those of others (7).

MICROSCOPIC APPEARANCE OF TEETH - Control and Fractured Side.

Periodontal Membrane. -- The findings regarding thickening or atrophy of the periodontal membrane of the first molar did not show enough variation to be considered

different. With the stains used it was extremely difficult to fully appreciate the changes in the membrane. Proliferating fibrous tissue often confused the picture. Fifty-six second molars had normal periodontal membranes, three were questionable and one each in Group II, III and IV showed thickening on the fractured side. Two control animals of Group IV, one male and one female showed some thickening.

Pulp. -- Generally, the pulps of all operated or fractured teeth showed degenerative changes. The pulp of those roots connected to the surface was totally or partially necrotic. Often one or two roots would not show inflammatory changes while others in the same animal were necrotic. In some the odontoblasts were absent and the pulp had been replaced with fibrous connective tissue. More Group IV animals showed pulp necrosis than in the other groups. The second molars quite consistently showed no changes unless the crown or root had been traumatized during the operative procedure. The pulp of one Group III female presented most interesting changes, with approximately one-half of the pulp necrotic and the other half apparently normal. An inflammatory reaction separated the two areas. Three second molars in Group IV animals showed degeneration and necrosis, while all of Group I and II were normal.

Cementum. -- The cementum in the fracture sites was undergoing resorption, both in the smaller fragments and

along the larger root surfaces. No appreciable difference was seen in the distribution of small fragments since they were present in Group II, III and IV animals. Three second molars in Group III and IV and two in Group II showed resorption. One Group III control and two Group IV second molar controls had areas of resorption.

Dentine. -- A total of fifty-six teeth had caries with twenty-two occurring in control teeth which had not been operated or fractured. In most cases the bacteria had deeply penetrated the dentinal tubules and secondary dentine had been deposited presumably to protect the pulp. The dentine in all fractured first molars showed resorption as did the control teeth in Groups III and IV. Caries was noted in the second and third molar of one Group I and one Group IV animal and third molar caries in one each in Group I, II and III.

INFLAMMATORY REACTION

Around roots. -- Acute inflammatory reactions either along or associated with subacute and chronic changes were present in the fracture sites in seven Group II, eight Group III and six Group IV animals. The control teeth of the Group III and Group IV rats showed eight and seven acute reactions, respectively. Chronic inflammatory changes showed a similar distribution. The hyperemia associated with these reactions generally involved from one-fourth to one-half of the vessels in the immediate

Table 12. Type of Inflammatory Reaction, Presence, Type and Concentration of Organisms in Pulps of Control and Fractured Teeth.

	Group I	Group II		Group III		Group IV	
	C	C	F	C	F	C	F
INFLAMMATION:							
Absent	16	8	1				
Chronic						2	1
Acute			1	2	2		1
Chronic - Subacute					1	1	1
Chronic - Acute			2		1	1	1
Subacute Acute				1		1	
Acute - Subacute - Chronic						1	
Acute with Necrosis			4	5	4	2	4
ORGANISMS ASSOCIATED							
Absent	16	8	1		1		
Fracture Surface Exposed			2			1	
In pulp -Diffuse			1		1	1	1
In pulp -Focal			1	1	1		1
Focal & Diffuse			3	7	5	6	5
Same + 2nd Molar							1
TYPE:							
Cocci & Strep.						2	3
Mixture - Cocci predominate			7	8	7	6	5
CONCENTRATION:							
Light			1		1		1
Moderate			4	3	1	3	2
Heavy			2	5	5	5	5

Table 13. Type of Inflammatory Reaction, Presence Type and Concentration of Organisms Around Roots and Root Fragments.

	Group I	Group II		Group III		Group IV	
	C	C	F	C	F	C	F
INFLAMMATION:							
Absent	16	8					
Chronic							2
Acute/ Size of Reaction			1/2	1/3		1/1 1/5 1/4 1/3	1/3 1/5
Chronic - Subacute			1			1	
Chronic - Acute			5	4	8	2	3
Subacute Acute			1	1			1
Acute - Subacute - Chronic				2		1	
ORGANISMS:							
Absent	16	8	2	3	1	4	1
Roots				4		4	
Root Frag. Buried					2		1
Surface			2	1			1
Both			4		5		5
TYPE:							
Cocci & Strep.				2	3	3	4
Mixtures			6	3	5	1	3
CONCENTRATION:							
Light			5	3	4	2	3
Moderate			1	1	4	2	4
Heavy				1			

area.

Pure acute reactions were present more often in the Group IV animals in the ratio, six Group IV to one each in Group II and III. This increase in purely acute reactions may possibly be due to the introduction of the Streptococcus sanguis, since the other control teeth and fracture sites were exposed to the same environment with the exception of this particular bacterium.

Involving the pulp. -- Acute inflammatory reactions alone or in combination with subacute and chronic reactions involved seven Group II, seven Group III and six Group IV fracture sites. Necrosis was present in the pulps at the fracture area in four Group II, III and IV animals and in five Group III and two Group IV control teeth. Purely acute reactions were present in the fracture sites of one Group II, two Group III and one Group IV rat. Chronic reactions were more frequent in the Group IV pulps with a total of eight. Five occurred in control teeth and three in fractured teeth. None of the control teeth in Groups II or III had chronic inflammation and two animals of each of these groups showed a chronic reaction in their fractured teeth. The associated hyperemia was of a lesser degree than that seen around roots. See Table 12 and 13.

Around metallic fragments.-- The inflammatory reaction around metal fragments was about evenly divided between chronic, subacute and acute. Seven metal fragments were noted with four present in fracture sites and three

in the area of control teeth with missing restorations.

At the surface. -- Acute reactions either alone or in combination with chronic and subacute inflammation were found in two Group I and II, four Group III and ten Group IV animals. The acute reactions in the Group I rats were associated with traumatically implanted material near the surface. The other reactions were usually associated with extensions from deeper tissues or were present around root fragments at the surface. Practically all animals showed some degree of chronic inflammation associated with impacted hair and debris in the interproximal spaces and in the crevice distal to the third molar. Hyperemia was slightly greater in the Group IV rats.

Size of the reaction.-- The figures represent the number of 10 power microscopic fields covered by the reaction, regardless of the type of inflammation. A comparison of the total size of the reactions in the fractured areas showed Group II - 3.9 plus one rat over ten 10 power fields, Group III - 6.7, Group IV - 5.4 plus one rat over ten 10 power fields. The Group III and Group IV control teeth had 3.6 and 2.7 respectively. One Group II and one Group IV rat (those with reactions over ten 10 power fields) presented extensive involvement with acute abscesses which were visible grossly on the fractured sides. The reaction was traceable to the fractured teeth, although the severe zone of acute inflammation was located just beneath the skin and was connected with the surface through

a draining sinus. Since the type of inflammation was not taken into consideration when measuring the overall reaction it is obvious that the Group IV acute reactions were not as extensive as the more generalized chronic reactions seen in the other groups.

The size of the reaction showed some difference between males and females with a total area of involvement of 3.6 for males and 2.7 for females in the control teeth and 10.5 plus one rat over ten 10 power fields in males compared to 3.5 plus one rat over ten 10 power fields in females. If the males and females of Group III and IV are compared then the males showed an area of 7.5 plus one rat over ten 10 power fields and the females 2.6 in the fracture sites. The control sides were more similar with 3.6 for males and 2.7 for females.

Fibroplasia. -- The findings regarding fibroplasia did not reveal differences between groups and appeared to respond in direct proportion to the trauma inflicted and the resorption of roots. The greatest proliferation was seen in areas where roots had been completely removed.

Bacteria at the epithelial surface. -- Mixtures of bacteria with spherical forms predominating were present in the interproximal areas of all control teeth. Bacteria in varying numbers were associated with the pulpal areas and around the fractured cusps of the Group III teeth. The category of light concentration of bacteria was present in Group I and II with ten each, compared with two in Group III

and one in Group IV. The designation of heavy concentration of bacteria was just the opposite with eleven and ten heavy concentrations in Group III and IV and only two each in Groups I and II. The control tooth in both males and females of Group III and IV had heavy concentrations while the fractured teeth showed only three and two respectively.

Bacteria in tissues.-- This category was included to cover situations in which bacteria were seen in tissues but were not directly found in conjunction with root fragments. These areas probably were adjacent to root fragments but the fragments were not seen, since they correlated fairly well with the findings for organisms around roots and root fragments. The Group IV control and fracture sites showed the same number of areas with organisms and without organisms in both of the categories, that is, in tissues and around roots.

The spherical forms of bacteria were present in the fracture sites in the ratio of two rats in Group II and III and four in Group IV. Mixtures of bacteria with spherical forms predominating were present in four Group II, five Group III and three Group IV fracture sites. The Group I and II control areas were free of bacteria which would indicate that the bacteria present in fracture sites were real and not artifactual.

Bacteria in the pulp.-- Bacteria were found diffusely in the pulps of a single rat in each of Groups II, III, and IV fracture sites and in one Group IV male

control. Focal accumulations were seen in four Group II rats with three of these areas at the exposed fracture surface. One Group III and one Group IV female showed focal areas in the pulps of the root fragments as did one male control tooth in a Group III rat.

Combinations of focal and diffuse accumulations of bacteria were seen more often than either alone, with three in Group II, five in Group III and six in Group IV. The Group III and IV controls had seven and six rats in this classification. The total number of focal and diffuse areas in Group III and IV control and fracture teeth were twelve each. No organisms were found in one Group III female.

The bacteria found in the pulp generally were mixtures, with spherical forms predominating in seven fracture sites in Group II and III and five in Group IV. The Group IV rats showed what appeared to be spherical forms only in three fracture sites and two control areas with none of this category noted in any other group.

A comparison of the location of microorganisms in the pulp of the fractured teeth in female rats of Group II, III and IV show one each with focal, two each with focal and diffuse and one Group II and IV with diffuse dispersion. The Group II males have two focal and one focal diffuse, the Group III one diffuse and three focal and diffuse, and the Group IV with four focal and diffuse.

Bacteria around roots and root fragments. -- No bacteria were seen around the roots of any of the Group I or II control teeth, nor in association with two Group II, one Group III and IV fracture sites, three Group III and four Group IV control teeth, even though the fillings were out and the pulps contained bacteria.[1] The roots of the remaining control teeth in Group III and IV showed bacteria present.[2] Organisms were associated with buried root fragments only in two Group III and one Group IV fracture sites[3] and were found along exposed fractured surfaces in two Group II and one Group III and IV rats. Bacteria were found in association with combinations of exposed and buried root fragments in four Group II, and five in each of Group III and IV animals.[4]

Table 14. Percentages of Periapical Inflammatory Areas Exhibiting Bacteria in the Eight "Control" Teeth in each of Groups III and IV.

Control Teeth:	Group III	Group IV
With inflammation	100%	100%
Bacteria present in inflammatory tissue	62.5%	50%

1. Refer to Table 13, page 76; Organisms.
2. Refer to Table 14, page 82.
3. Refer to Table 16, page 83.
4. Refer to Table 15, page 83.

Table 15. Percentages of Inflammatory Areas Around Root Fragments Exhibiting Bacteria in the Eight Teeth in Each of Groups II, III, and IV.

Fractured Teeth:	Group II	Group III	Group IV
With Inflammation	100%	100%	100%
Bacteria present in inflammatory tissue	75%	87.5%	87.5%

Table 16. Percentages of Inflammatory Areas Around Completely Embedded Root Fragments Exhibiting Bacteria.

Embedded root fragments:	No. of Fragments	Group III	No. of Fragments	Group IV
With Inflammation	2	100%	1	100%
Bacteria present in inflammatory tissue	2	100%	0	0

DISCUSSION AND SUMMARY

The purpose of this investigation was to seek experimental methods suitable for studying the effects of fractured infected and non-infected root fragments and other foreign bodies found in the jaws and other body tissues. The methods employed in this study would be suitable with a few modifications.

Placing a rubber dam on the maxillary first molar is not a simple procedure and skill is gained only by repeated attempts. Many styles of cast retainers designed to hold the rubber dam in place were modeled in wax and then cast in either gold or vitallium. The cast gold retainer finally selected was adequate, however after repeated applications, ideas for further improvement evolved and will be tested in the future.

A more detailed study of the normal oral flora of the rats under study would be advantageous. In the present study the experimenter attempted to gain some idea of the most prevalent forms of microorganisms and realizes that many other bacteria could probably be isolated from the mouths and throats of these animals.

Results would be greatly improved if the mesial roots of the upper 1st molars were routinely removed from

the fracture sites. This root is much larger than the others and tends to fracture near the crown, leaving an undesirable exposed situation. Most fracture sites would have been healed had it not been for the exposed mesial root. This root could be removed with small elevators or specially designed forceps. The second molar might prove very useful in a study in which bacteria were sealed in the pulp with a filling. This tooth would probably not be suitable for a study involving fracturing of roots since the contacts are so tight and the crown size is so small that it is not likely this tooth could be fractured without disturbing the adjacent molars.

It is important that cavity preparations be non-extended in order to preserve the strength of the walls, prevent breakage of the cusps and the subsequent loss of the filling. The smaller cavity size makes it a little more difficult to manipulate broaches but the advantages far outweigh this difficulty.

Many aspects of fixation procedures and histologic preparations could be studied; however, most of these studies would be projects in their own right. If the techniques employed produce results which can be interpreted accurately and are free from excessive artifacts, then they may be considered to be adequate.

The use of labelled antibody would greatly facilitate a study such as this and would help dispel the many doubts which arise concerning the presence or absence of minimal

numbers of bacteria in equivocal tissue sections.

The differentiation of spherical forms from closely allied shapes proves difficult if not impossible and the end view of a rod-shaped form could appear spherical. It is not likely that all rod-shaped organisms would be aligned in one direction only, giving the appearance of coccal forms; therefore, when a number of microorganisms were present and only rounded shapes were seen, they were listed as "pure" spherical forms. If any doubt existed they were classified as mixtures with spherical forms predominating.

Another major facet of this study was to evaluate a bacterium, Streptococcus sanguis (10556). From injection studies into the leg muscles of rats it appeared that this microorganism was pathogenic for rats of the strain being used. This finding appears to be borne out by the results of this study since more pure acute reactions were found associated with roots of the operated and infected (with Streptococcus sanguis) animals than with those in any of the other groups. It is realized that this was an extremely small sample and that no conclusive or significant findings can be predicated for a dichotomous situation such as this.

Very little difference was noted regarding resorption of roots in fracture sites. It would seem that the tissues tend to remove fragments of roots left following extractions, particularly when inflammation is present.

many root fragments were seen which had not elicited an inflammatory reaction and which also showed no resorption.

Since two Group IV rats had radiolucencies around the second molars and each of these animals exhibited acute inflammatory reactions around roots of the first molar, it seems reasonable to suspect that the Streptococcus sanguis influenced this extension. Radiolucencies were not found involving roots of second molars in any other groups.

It is difficult to explain the findings regarding an increased number of chronic inflammatory reactions in animals of Group IV, since these teeth were not only exposed to the oral flora but were also deliberately inoculated with Streptococcus sanguis. One explanation might be that the body defense mechanisms were brought into play at the time of the original operation to infect the pulp, which was not true of the other groups, and for this reason these pulps were better able to cope with the trauma and oral flora at the time of the fracture.

Healing, which appeared to be complete in gross examination in three Group III and four Group IV rats, was not confirmed in all of these cases by microscopic findings. Visual examination utilizing a dissecting microscope is not sufficient to determine whether fracture sites are completely healed or not. Small clefts may exist which can only be evaluated histologically. The normal surface irregularities also tend to make

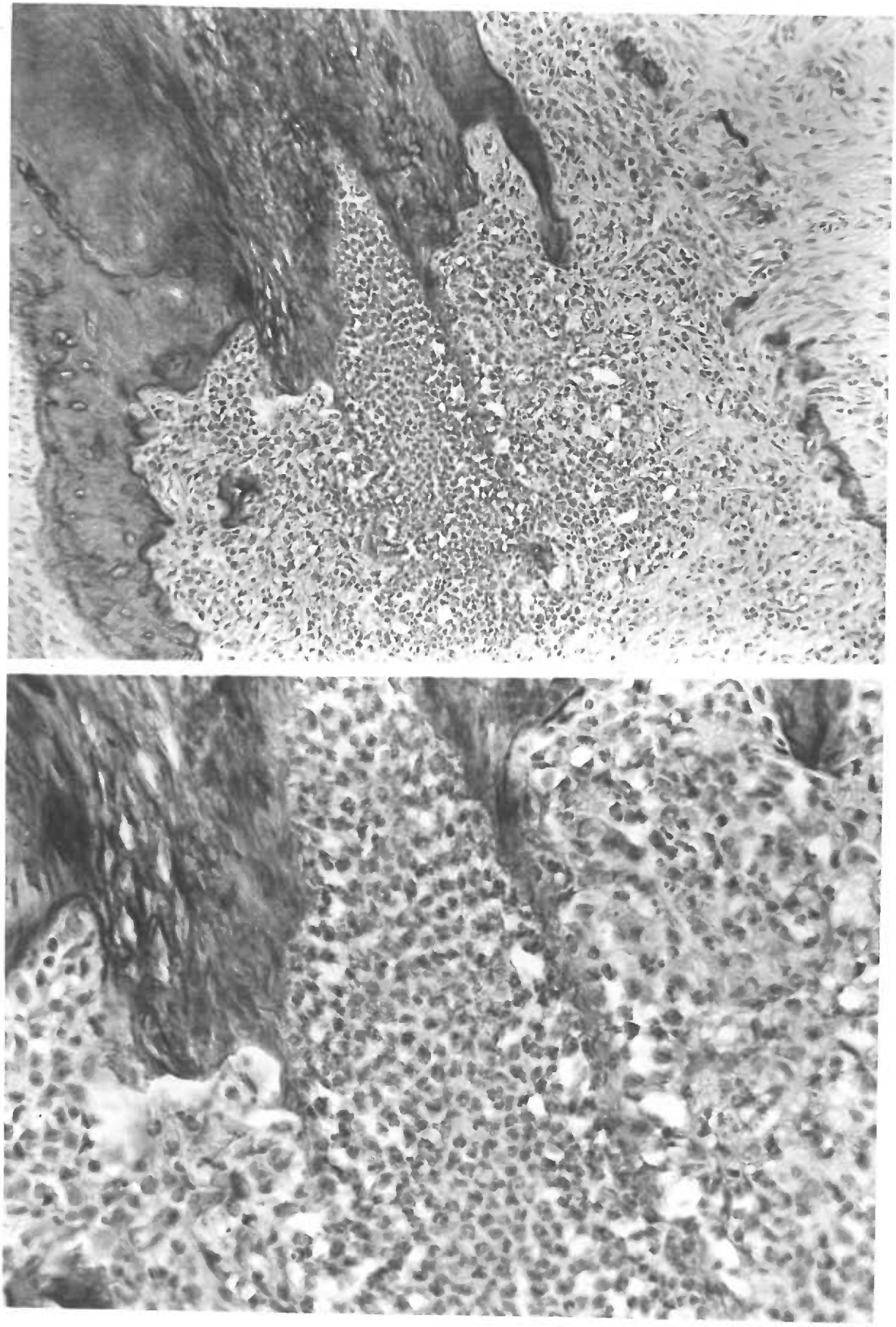
interpretation more difficult. Actually, two Group III and one Group IV animal showed complete healing of their fracture sites when examined microscopically. One of these had bacteria diffusely in the pulp but none in tissues or associated with root fragments, both of the others had microorganisms diffusely in the tissue, both focally and diffusely in the pulp and also associated with buried root fragments. The bacteria present in one of the above cases were spherical forms found diffusely in the tissues, and associated with buried roots, the other Group III rat showed mixtures of bacteria in these locations and in the pulp.

The Group IV rat showed spherical forms of bacteria in the pulp and no organisms in the tissues or around fractured roots. The pulp in this animal was partially necrotic with an acute inflammatory reaction. The area surrounding the roots showed only a mild chronic reaction. This may indicate that the acute reaction was able to cope with the bacteria present in the pulp and that the chronic reaction was an associated peripheral response, possibly occurring in the area surrounding the apex of the root as a result of stimuli other than the physical presence of bacteria.

This same microscopic pattern might appear after bacteria were first present in the tissues and then were destroyed, except in the confined spaces of the pulp canal which limits the overall influence of the inflammatory

Figure 6. Acute Inflammatory Reaction Involving the Apical Area of a Root Fragment with a Surrounding Chronic Inflammatory Reaction. Cementum resorption is also evident. H & E. 240x

Figure 7. A High Power View of the Above Acute Inflammatory Reaction. H & E. 500x



reaction.

The apical tissues of one of the Group III rats showed chronic and acute inflammatory changes and the presence of spherical forms of microorganisms. The pulp which contained a mixture of bacteria had necrosis and an acute reaction. Possibly the fact that a mixture of organisms was present increased their ability to invade surrounding tissues.

The greatest concentration of bacteria were associated at the surfaces of broken control teeth (from which fillings had fallen out) which presented large irregular surface areas which harbored the bacteria. Exposed root fragments in the fracture site also presented these irregular surfaces; however, fewer numbers and smaller areas of tooth surface were exposed.

It was interesting to note that three rats in Group II had focal and diffuse dispersions of bacteria in the pulp in comparison with five in Group III and six in Group IV. This would seem to indicate that the traumatization of the pulp by sham operation and by infection made this tissue more susceptible to bacterial growth in a diffuse pattern since the Group II rats showed a higher tendency toward focal areas. Also, the Group III and IV totals for focal and diffuse pattern were equal.

The type of bacteria present in the pulp showed the Group IV rats to be the only ones with "pure" collections of spherical forms in the pulps of three fractured roots and two control teeth. This may possibly indicate that

these were more dominant organisms or that they were more capable of survival in this particular location. The pulps showed many more mixtures of bacteria than were seen in the tissues, in the ratio of 33:18, almost double. The types in the tissues were divided with fifteen "pure" spherical forms as opposed to eighteen mixtures.

These differences between types of bacteria in the pulp in tissues and around roots would seem to indicate that the surface areas of exposed pulps harbor mixtures of bacteria and that in the deeper tissues the spherical forms are seen more often in "pure" accumulations. It also seems to be the tendency for the Group IV pulps to show the greatest number of spherical forms, nineteen, the Group III considerably fewer, eleven, and the Group II, only two.

There was little difference among groups in the number of root fragments associated with bacteria in both exposed and buried locations; however, their occurrence in relation to the periapical areas of Group III control teeth indicates that the periapical infections were manifested without fracturing the teeth and in a manner not unlike open carious exposures of the pulps.

Considerable disagreement exists regarding the presence or absence of bacteria in radiolucent areas surrounding the apices of roots. This study shows that radiolucent apical areas were found both free from microorganisms in some cases and liberally infected by them in others. This finding does establish, therefore,

Figure 8. A Subacute Inflammatory Reaction With a Mixture of Cell Types Distributed Throughout the Fibrous Connective Tissue. H & E. 240x

Figure 9. A Tissue Gram Stain Showing Mixtures of Bacteria Between Two Root Fragments in the Tissue. B & B. 500x

that not all radiolucent areas are sterile and that histologic examination or bacteriologic culture of the tissue is necessary before one can make a positive statement. It is to be expected that in any series of periapical areas associated with infected pulps, some will be at least temperarily free of bacteria due to vigorous defense activities of the periapical tissues. The interaction of these tissues with the "protected haven" of the pulps and with the rest of the body over varying time periods remains to be investigated in the future.

CONCLUSIONS

With slight modifications the techniques employed are suitable for study of infected and non-infected root fragments and other foreign bodies in the jaws of laboratory animals.

Streptococcus sanguis appeared to be capable of eliciting acute inflammatory response in the pulp canal and around root fragments in teeth which have been operated, infected and fractured, and in other body sites when injected.

Microorganisms were not normally found in the tissues, the pulp or around roots of teeth in the control group, Group I, or in the control teeth in Group II.

Mixtures of bacteria were present in the occlusal grooves of molar teeth, in the interproximal areas and

in the gingival crevices around teeth in all groups.

The rats used in this study were susceptible to dental caries when maintained on an ordinary stock laboratory diet.

Inflammatory reactions were present around root fragments in the absence of microorganisms in some rats; however, bacteria were present in their pulps.

No inflammatory reactions were found in which bacteria were completely absent in the entire associated dental apparatus.

Not all root tips from non-infected teeth became infected at the time of fracture, nor did all of their pulps undergo decomposition and suffer invasion of bacteria. Some of these pulps remained essentially normal and others became fibrotic.

Microorganisms were found in association with inflammatory changes around root fragments in some rats and indicates that not all apical rarefactions and inflammatory tissues are free from bacteria, which has been alluded to by many practitioners.

Microorganisms were found in a significant number of inflammed periapical areas of teeth which had been left in situ after pulp traumatization and deliberate infection in some, while others were left open to the normal oral flora. Bacteria were also found around some completely embedded root tips from fractured teeth, some of which had been deliberately infected prior to fracture, and others of which had not.

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APPENDICES

APPENDIX A

ANESTHESIA RECORDS GROUP II

Cage No.	Rat No.	M or F	Date 1961 Mo.Day	Body Wt. Gms.	Mg/Gm Total Body Wt.	Mg/100 Gm Body Wt.	CC Used	Mg cc	Side Fractured
12	15	M	12 13	383	13.4	3.50	.893	15	R
13	21	F	12 13	267	7.3	2.75	.490	15	L
18	10	M	12 22	440	13.2	3.00	.880	15	R
21	10	F	12 22	237	5.33	2.25	.355	15	L
24	21	M	12 22	446	13.4	3.00	.892	15	L
26	3	F	12 22	278	6.3	2.25	.417	15	L
27	7	M	12 22	476	14.3	3.00	.952	15	R
33	22	F	12 22	281	6.3	2.25	.421	15	R
34	17	M	12 22	417	12.5	3.00	.834	15	R
35	19	F	12 22	274	6.2	2.25	.410	15	L
39	9	M	12 22	430	12.9	3.00	.863	15	R
46	8	F	12 13	268	7.4	2.75	.493	15	L

Group IV Rats Infected with Streptococcus Pyogenes

51		M	11 14	443	15.5	3.50	1.03	15	Inf.
			12 22	433	14.1	3.25	0.94	15	L
52		M	11 14	435	15.2	3.50	1.01	15	Inf.
			12 22	438	14.2	3.25	0.95	15	L
53		F	11 14	270	7.4	2.75	0.49	15	Inf.
			12 22	252	6.3	2.50	0.42	15	L
54		M	11 14	382	13.4	3.50	0.89	15	Inf.
			12 22	398	12.9	3.25	0.86	15	L
55		F	11 14	241	6.6	2.75	0.44	15	Inf.
			12 22	261	6.5	2.50	0.43	15	R
60		M	11 14	385	13.5	3.50	0.90	15	Inf.
			12 22	413	13.4	3.25	0.89	15	R

ANESTHESIA RECORDS GROUP III

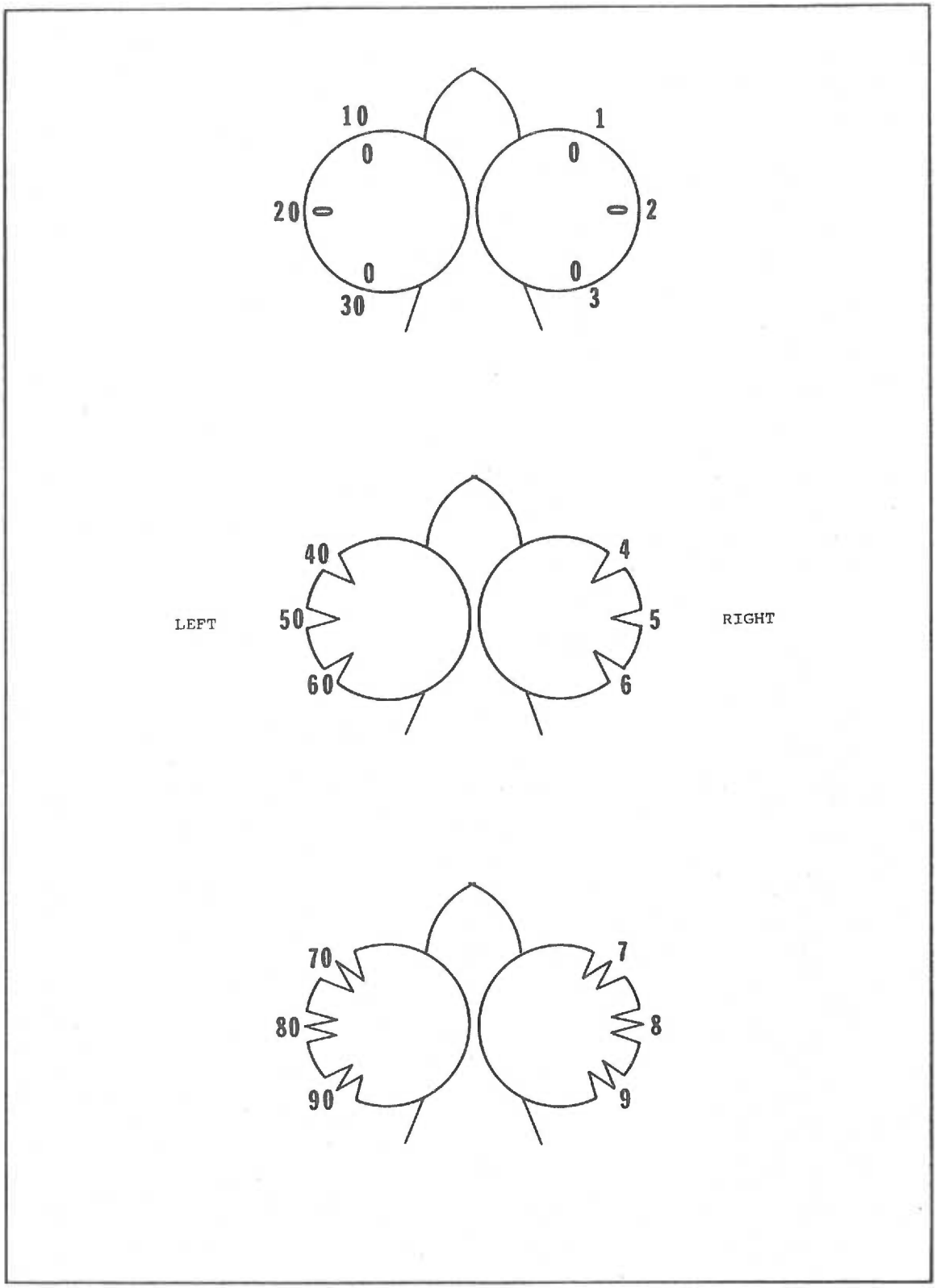
Cage No.	Rat No.	M or F	Date 1961 Mo. Day	Body Wt. Gms.	Mg/Gm Total Body Wt.	Mg/100 Gm Body Wt.	CC Used	Mg cc	Sham Operated or Fract. R or L
3	20	M	11 13	417	12.5	3.00	0.50	25	S
			12 13	456	15.9	3.50	1.06	15	F L
14	1	F	11 15	238	6.5	2.75	0.43	15	S
			12 22	265	5.9	2.25	0.39	15	F L
16	4	M	11 15	391	13.7	3.50	0.91	15	S
			12 22	418	12.5	3.00	0.84	15	F R
30	23	F	11 13	233	7.0	3.00	0.46	15	S
			12 13	246	6.7	2.75	0.45	15	F L
31	11	M	11 15	379	13.3	3.50	0.88	15	S
			12 22	393	11.8	3.00	0.78	15	F L
36	9	F	11 09	254	10.2	4.00	0.41	25	S
			12 13	273	7.5	2.75	0.50	15	F R
37	24	M	11 13	414	12.4	3.00	0.83	15	S
			12 22	434	13.0	3.00	0.87	15	F R
38	4	F	11 24	249	6.8	2.75	0.45	15	S
			12 22	281	6.3	2.25	0.42	15	F R
40	18	M	11 24	376	13.1	3.50	0.87	15	S
			12 22	402	12.1	3.00	0.81	15	F L
41	11	F	11 15	235	6.5	2.75	0.43	15	S
			12 22	274	6.2	2.25	0.41	15	F L
42	22	M	11 13	452	13.6	3.00	0.90	15	S
			12 22	481	14.4	3.00	0.96	15	F R
48	2	F	11 14	274	7.5	2.75	0.50	15	S
			12 22	303	6.8	2.25	0.45	15	F L

ANESTHESIA RECORDS GROUP IV

Cage No.	Rat No.	M or F	Date 1961 Mo. Day	Body Wt. Gms.	Mg/Gm Total Body Wt.	Mg/100 Gm Body Wt.	CC Used	Mg cc	Infected or Fract.
2	2	M	11 24 12 22	415 489	14.5 14.7	3.50 3.00	0.97 0.98	15 15	I F L
5	13	F	11 13 12 13	245 271	7.3 7.4	3.00 2.75	0.49 0.49	15 15	I F R
7	23	M	11 13 12 13	384 412	11.5 14.4	3.00 3.50	0.77 0.96	15 15	I F L
8	17	F	11 13 12 13	239 245	7.2 6.7	3.00 2.75	0.48 0.45	15 15	I F L
9	1	M	11 24 12 22	367 456	12.8 13.7	3.50 3.00	0.85 0.91	15 15	I F L
10	5	F	11 24 12 22	243 271	6.7 6.1	2.75 2.25	0.45 0.41	15 15	I F L
11	14	M	11 24 12 22	413 489	14.4 14.6	3.50 3.00	0.96 0.97	15 15	I F L
15	18	F	11 24 12 22	233 276	6.4 6.2	2.75 2.25	0.43 0.41	15 15	I F R
20	19	M	11 24 12 22	411 510	14.4 15.3	3.50 3.00	0.96 1.02	15 15	I F R
23	15	F	11 13 12 22	280 318	7.7 7.1	2.75 2.25	0.51 0.48	15 15	I F L
25	6	M	11 14 12 22	394 421	13.8 12.6	3.50 3.00	0.92 0.84	15 15	I F L
45	16	F	11 13 12 22	253 273	6.9 6.1	2.75 2.25	0.46 0.41	15 15	I F R

APPENDIX B

EAR PUNCH IDENTIFICATION



APPENDIX C

STAINING PROCEDURES:

A. HEMATOXYLIN AND EOSIN

Staining Procedure: The tissues on the slides were:

1. Deparaffinized through:
   a. Two changes of Xylol for two minutes each.
   b. Two changes of absolute alcohol for one minute each.
   c. Two changes of 95 per cent alcohol for one minute each.
   d. Two changes of 80 per cent alcohol for one minute each.
   e. Two changes of 50 per cent alcohol for one minute each.
   f. Two changes of 30 per cent alcohol for one minute each.
   g. Distilled water for two minutes.
   h. Four changes of tap water for one minute each.
2. Stained in Harris Hematoxylin
   a. Twenty minutes for teeth and bone.
   b. Five minutes for soft tissue.
3. Rinsed in four changes of tap water, a few seconds each.
4. Decolorized with 1 per cent acid alcohol, three to ten dips (check under microscope).
5. Rinsed in tap water, four dips.
6. Passed through six dips of ammonia water.
7. Flooded with three drops of NH_4OH in 1000 cc. of water or saturated aqueous Li_2CO_3.
8. Rinsed in distilled water for 15 minutes.

9. Counterstained with eosin from 15 seconds to two minutes.
10. Passed through two changes of 95 per cent alcohol for one minute.
11. Passed through two changes of absolute alcohol for one minute each.
12. Then passed through three changes of xylol for two minutes each.

B. GOMORI'S METHENAMINE - SILVER NITRATE TECHNIC
Grocott's Application to Fungi (24)

Staining procedure: The rat tissues and a control slide were:

1. Deparaffinized through two changes of xylene and through absolute 95, 80, 50, and 30 per cent alcohols to distilled water.
2. Oxidized in 5 per cent chromic acid solution for 1 hour.
3. Washed in running tap water for ten minutes.
4. Rinsed in 1 per cent sodium bisulfite for 1 minute to remove any residual chromic acid.
5. Washed in tap water for five to ten minutes.
6. Washed with three or four changes of distilled water.
7. Placed in working methenamine - silver nitrate solution in oven at 58° Centigrade for 30 to 60 minutes until section turned yellowish brown. Paraffin-coated forceps were used to remove the slide from this solution. Slide was dipped in distilled water and checked for adequate silver impregnation with microscope. Fungi should be dark brown at this stage.
8. Rinsed in six changes of distilled water.

9. Toned in 0.1 per cent gold chloride solution for two to three minutes.
10. Rinsed in distilled water.
11. Unreduced silver was removed with 2 per cent sodium thiosulfate (hypo) solution for two to five minutes.
12. Washed thoroughly in tap water.
13. Counterstained with working light green solution for 30 to 45 seconds.
14. Dehydrated with two changes of 95 per cent alcohol, absolute alcohol, cleared with two to three changes of xylene and mounted.

Results:

Fungi were sharply delineated in black with the inner parts of mycelia and hyphae staining an old rose as a result of toning in gold. Mucin also assumes a rose-red color as a result of toning. This method, using readily available reagents and stable solutions, produced highly photogenic preparations without the use of costly color filters.

C. GRAM STAIN FOR TISSUES (13)

Staining Procedure: The tissues on the slides were:

1. Deparaffinized.
2. Stained in freshly filtered alum-hematoxylin (Harris) for two to five minutes.
3. Washed in distilled water.
4. Washed in ammonia water until blue.
5. Washed in distilled water.
6. Placed horizontally on staining rack then a mixture of five drops of 5 per cent aqueous solution of sodium bicarbonate with about 0.75 cc. of 1 per cent (by weight) aqueous solution of gentian violet

was poured onto the slides. They were then stained for two minutes.

7. Washed quickly with water.
8. Covered with Lugol's iodine solution for one minute.
9. Washed with water and blotted.
10. Decolorized in one part of ether plus three parts of acetone by dropping it onto the slide until no more color comes off.
11. Blotted.
12. Stained for five minutes with basic fuchsin.
13. Washed in water.
14. Blotted but not allowed to dry.
15. Passed through acetone.
16. Decolorized and differentiated by dropping over the section a solution of 0.1 gram picric acid in 100 cc. of acetone until the section becomes yellowish pink. This was the most critical stage of the process and was carried out by holding the slide over a white plate or dish. Most of the Fuchsin should be decolorized from the tissue but the Gram-negative bacteria should remain red.
17. Passed successively through acetone, equal parts of acetone and xylol and then xylol.
18. Mounted in balsam after clearing in xylol. (It is best to work with only one slide at a time.)

Results:

Cell nuclei - dark reddish-brown
Gram-positive bacteria - deep violet to black
Gram-negative bacteria - bright red
Cytoplasm - yellowish
Leukocytes - dusky yellowish cytoplasm
Basophilic granules - red
Striated muscle - yellow, some pink
Fibrin - yellow, some pink

APPENDIX D

PREPARATION OF REAGENTS

1. Formic Acid - Sodium Citrate reagent.

   Solution A

90% formic acid, C.P.	1 part
Distilled water	1 part

   Solution B

Sodium citrate, C.P.	20 grams
Distilled water	100 cc.

   Combine equal parts of Solution A and Solution B. Use large volumes of the reagent and change daily.

2. Test for presence of calcium.

   To 5 cc. of the used decalcifying reagent, add 1 cc. of concentrated ammonium hydroxide, mix thoroughly, then add 0.1 cc. of saturated aqueous solution of ammonium oxalate; a precipitate will form when calcium is present. Repeat the additions of 0.1 cc. amounts of ammonium oxalate at intervals of 15 - 20 minutes until a total of 0.4 cc. has been added. If a precipitate fails to form after the addition of 0.4 cc. of ammonium oxalate, leave the tissue in the same decalcifying reagent for at least 48 hours longer and repeat the test. When the test remains negative for 3 days in the case of a single tooth and for one week in the case of larger tissues, proceed with dehydration and infiltration.

3. Harris's Hematoxylin

Hematoxylin crystals	5.0 gm.
Alcohol, 95%	50.0 cc.
Ammonium or potassium alum	100.0 gm.
Distilled water	1000.0 cc.

Mercuric oxide 2.5 gm.

Dissolve the hematoxylin in the alcohol, the alum in the water by the aid of heat. Mix the two solutions. Bring the mixture to a boil as rapidly as possible and then remove from the heat and add the mecuric oxide. Reheat the solution until it becomes dark purple, about one minute, and promptly remove the container from the flame and plunge into a basin of cold water. The solution is ready to use when cool. Add 2 - 4 cc. of glacial acetic acid to 100 cc. of solution if desired.

4. Acid Alcohol

70% Alcohol	1000 cc.
Hydrochloric acid, concentrated	10 cc.

5. Ammonia Water

Tap water	1000 cc.
Strong ammonia water	2 to 3 cc.

6. Saturated Lithium Carbonate

Lithium carbonate	6.6 gm.
Distilled water	500 gm.

7. Alcoholic Eosin Solution

Eosin Y water soluble	2 gm.
Distilled water	160 cc.
Alcohol, 95%	640 cc.

Dissolve Eosin Y in the distilled water, then add the 95% alcohol. If a deeper shade is desired, add a drop of acetic acid to each 100 cc. of solution.

8. Lugol's Solution (Weigert't modification)

Potassium iodide	2 gm.
Iodine crystals	1 gm.
Distilled water	100 cc.

9. Alcoholic Iodine Solution

Iodine crystals	1 gm.

Alcohol, 95% 100 cc.

10. Sodium Thiosulfate Solution (Hypo)
    Sodium thiosulfate 5 gm.
    Distilled water 100 gm.
11. 5% Chromic Acid
    Chromic acid 5 gm.
    Distilled water 100 gm.
12. 5% Silver Nitrate Solution
    Silver Nitrate 5 gm.
    Distilled Water 100 cc.
13. 3% Methenamine Solution
    Hexamethylenatetramine U.S.P. 3 gm.
    Distilled water 100 gm.
14. 5% Borax Solution
    Borax (photographic grade) 5 gm.
    Distilled water 100 gm.
15. Stock Methenamine - Silver Nitrate Solution
    Silver Nitrate, 5% solution 5 cc.
    Methenamine, 3% solution 100 cc.
    A white precipitate forms but immediately dissolves on shaking. Clear solutions remain usable for months.
16. Working Methenamine - Silver Nitrate Solution
    Borax, 5% solution 2 cc.
    Distilled water 25 cc.
    Mix and add:
    Methenamine-Silver Nitrate, stock solution 25 cc.
17. 1% Sodium Bisulfite Solution
    Sodium Bisulfite 1 gm.
    Distilled water 100 gm.
18. 0.1% Gold Chloride
    Gold Chloride, 1% solution 10 cc.
    Distilled water 90 cc.
    This solution may be used repeatedly.

19. 2% Sodium Thiosulfate (Hypo) Solution

Sodium thiosulfate	2 gm.
Distilled water	100 cc.

20. Stock Light Green Solution

Light green, S.F. (yellow)	0.2 gm.
Distilled water	100 cc.
Glacial Acetic Acid	0.2 cc.

21. Working Light Green Solution

Light green, stock solution	10 cc.
Distilled water	50 cc.

22. 1% Crystal Violet Solution

Crystal Violet	1 gm.
Distilled water	100 cc.

23. 5% Sodium Bicarbonate Solution

Sodium Bicarbonate	5 gm.
Distilled Water	100 gm.

24. Gram's Iodine Solution

Iodine	1 gm.
Potassium iodide	2 gm.
Distilled water	300 cc.

25. Saturated Basic Fuchsin Solution

Basic Fuchsin	approx. 0.25 gm.
Distilled water	100 cc.

26. Working 0.1% Basic Fuchsin Solution

Basic Fuchsin, saturated solution	0.1 cc.
Distilled water	100 cc.

27. 0.1 Picric acid - acetone

Picric acid	0.1 gm.
Acetone	100 cc.

APPENDIX E

BACTERIOLOGIC CULTURE MEDIA

1. Sucrose broth:

   1 per cent tryptone

   0.5 per cent yeast extract

   0.5 per cent K_2HPO_4

   5.0 per cent sucrose

   final pH 7.4

2. Litmus milk

Carnation Instant Non-fat Dry Milk	11 gm.
Water	100 ml.

   Litmus (liquid) sufficient amount to give a light lavender color.

3. Brom Cresol Purple Base

Proteose - Peptone No. 3 (Difco)	10 gm.
Bacto-Beef Extract	1 gm.
Sodium Chloride	5 gm.
Brom Cresol Purple W.S.	0.015 gm.
Water	1000 ml.
Agar	2 gm.

4. The following sugars and alcohols were prepared in tubes with inserts to determine presence of gas using 25 ml. of 10% sugar or alcohol to 250 ml. of broth.

   a. d (+) Xylose
   b. d (+) Mannose
   c. Dextrose (d-Glucose)
   d. d (-) Levulose (d-fructose)
   e. d-Galactose

f. Lactose
g. d (+) Maltose
h. Saccharose (Sucrose)
i. Raffinose
j. Inulin
k. Trehalose
l. Glycerol
m. Mannitol
n. Sorbitol
o. Dulcitol

5. Dextrose Starch Agar

Proteose Peptone No. 3 , Difco	15 gm.
Bacto-Dextrose	2 gm.
Soluble Starch	10 gm.
Sodium Chloride	5 gm.
Disodium Phosphate	3 gm.
Bacto-Gelatin	20 gm.
Bacto-Agar	10 gm.

6. Nitrate Agar

Bacto-Beef Extract	3 gm.
Bacto-Peptone	5 gm.
Potassium Nitrate	1 gm.
Bacto-Agar	12 gm.

7. Reagents to test for nitrates.

Sulfanilic acid reagent

Sulfanilic acid	8 gm.
5 N Acetic acid	1000 ml.

a-naphthylamine reagent

a-naphthylamine	5 gm.
5 N Acetic Acid	1000 ml.

A few drops of each reagent are put into the tube to be tested. A distinct pink or red color indicates the presence of nitrite reduced from original nitrate.

APPENDIX F
ANALYSIS OF RAT DIET

Purina Laboratory Chow manufactured by Ralston Purina Company, St. Louis 2, Mo. was fed ad libitum.

Analysis:

Crude protein	not less than 23 per cent
Crude fat	not less than 4.5 per cent
Crude fiber	not greater than 6.0 per cent
NFE	not less than 44 per cent
Ash	not greater than 9.0 per cent

Meat and bone meal, dried skimmed milk, wheat germ meal, fish meal, animal liver meal, dried beet pulp, corn grits, oat middlings, soybean oil meal, dehydrated alfalfa meal, feeding cane molasses, vitamin B_{12} supplement, calcium pantothenate, choline chloride, folic acid, riboflavin supplement, Brewer's dried yeast, thiamin, niacin, vitamin A oil, D activated plant sterol, vitamin E supplement, 0.5 per cent defluorinated phosphate, 0.5 per cent iodized salt, 0.075 per cent iron citrate, 0.2 per cent manganese sulfate and a trace of zinc oxide.

APPENDIX G

GROUP I

Week		1	2	3	4	5	6	7	8	9	10	11
Month		Nov.(61)			Dec.						Jan. (62)	
Day		14	20	2	9	16	22	30	6	13	20	27
Female	Cage 17	245	248	241	254	253	263	267	276	281	-	-
	22	216	223	247	247	263	262	273	272	271	284	-
	29	248	255	267	273	274	280	283	272	275	279	271
	43	202	214	273	262	276	272	289	286	297	297	-
	Total	911	940	1028	1036	1066	1077	1112	1106	1124	860	271
Avg.		228	235	257	259	266	269	278	276	281	286	271
Male	6	433	442	457	466	455	477	499	498	511	514	525
	19	451	456	460	459	471	470	485	486	489	-	-
	28	-	417	394	389	398	394	411	416	426	441	450
	32	410	413	416	393	375	370	400	407	412	422	424
Total		1294	1728	1727	1707	1699	1711	1795	1807	1838	1377	1399
Avg.		431	432	432	427	425	428	449	452	460	459	466
					GROUP II							
Female	21	224	227	226	231	220	237	237	243	238	234	-
	26	247	255	265	267	278	278	277	276	285	279	290
	33	197	214	256	269	278	281	279	286	289	298	298
	46	199	214	260	257	264	268	276	282	287	287	291
Total		867	910	1007	1024	1040	1064	1069	1087	1099	1098	879
Avg.		217	228	252	256	260	266	267	272	275	275	293
Male	12	430	439	448	387	381	383	423	438	449	-	-
	24	431	433	432	436	399	446	420	376	415	445	-
	27	-	427	438	458	478	476	474	482	494	500	-
	39	390	392	392	395	408	430	418	433	436	442	-
Total		1251	1691	1710	1676	1666	1735	1735	1729	1794	1387	
Avg.		417	423	428	419	417	434	434	432	449	346	

APPENDIX G

GROUP III

Week		1	2	3	4	5	6	7	8	9	10	11
Month		Nov.(61)				Dec.					Jan. (62)	
Day		14	20	2	9	16	22	30	6	13	20	27
	Cage											
Female	14	238	245	251	249	256	265	230	174	-	-	-
	36	254	259	260	271	266	273	283	275	278	276	-
	38	-	220	262	272	281	281	284	288	286	282	290
	41	235	257	257	263	280	274	277	279	286	284	283
Total		727	981	1030	1055	1083	1093	1074	1016	850	842	573
Avg.		242	245	257	263	271	273	269	254	283	281	287
Male	3	417	424	439	445	441	456	454	468	475	475	-
	37	414	422	427	423	435	434	436	444	451	454	-
	40	370	377	382	386	401	402	406	393	402	405	412
	42	452	452	469	477	480	481	449	322	274	-	-
Total		1653	1675	1717	1731	1757	1773	1745	1627	1602	1334	412
Avg.		413	419	429	433	439	443	436	407	400	445	412

GROUP IV

Female	5	245	255	255	267	262	271	272	282	289	-	-
	10	-	220	253	269	268	271	284	273	284	292	295
	15	-	210	254	257	250	276	263	269	271	275	280
	23	280	288	296	296	308	318	318	315	324	333	-
Total		525	973	1058	1089	1088	1136	1137	1139	1168	900	575
Avg.		262	243	264	272	272	284	284	285	292	300	287
Male	9	-	367	425	394	383	456	448	460	474	496	505
	11	-	413	435	458	467	489	464	477	500	507	517
	20	-	411	446	470	488	510	486	524	534	544	558
	25	394	350	393	399	415	421	427	435	449	444	-
Total		394	1541	1699	1721	1753	1876	1825	1896	1957	1991	1580
Avg.		394	385	425	430	438	469	456	474	489	498	526

APPENDIX H.

KEY TO ANALYSIS OF TISSUES

1-12 GENERAL:

1- 2 CAGE NUMBER

3 GROUP NUMBER

4 SEX OF ANIMAL

1 - Female
2 - Male

5- 6 TYPE OF TREATMENT - Right and left first molar.

0 - None
1 - Operated, sham (not infected)
2 - Operated, infected
3 - Sham-operated and fractured
4 - Infected and fractured
5 - Fractured (no other treatment)

7-12 TIME LAPSE IN DAYS

Operation to fracture
Fracture to sacrifice
Total time lapse (Operation to sacrifice)

13-18 GROSS: EXAMINATION AT THE TIME OF SACRIFICE

13 APPEARANCE OF TEETH IN GENERAL - Normal Wear Pattern

0 - Clean
1 - Stained
2 - Stained and packed debris (small amount)
3 - Stained and packed debris (large amount)
4 - Packed debris

14 ABSCESS FORMATION

0 - Absent
1 - Present

14 ABSCESS FORMATION

0 - Absent
1 - Present

TOOTH FRACTURE SITE:

15 APPEARANCE OF FRACTURE SITE AT TIME OF SACRIFICE

0 - Complete healing
1 - Incomplete healing, opening less than 0.5 mm.
2 - Incomplete healing, opening greater than 0.5 mm, less than 1 mm.
3 - Incomplete healing, opening greater than 1 mm, less than 2 mm.
4 - Incomplete healing, opening greater than 2 mm, less than 3 mm.
5 - Incomplete healing, opening greater than 3 mm, less than 4 mm.
9 - Not applicable

16 NUMBER OF VISIBLE ROOT FRAGMENTS

0 - None
1 - One
2 - Two
3 - Three
4 - Four
5 - Five
9 - Not applicable

CONTROL TOOTH:

17 APPEARANCE OF CONTROL TOOTH AT TIME OF SACRIFICE

0 - No filling placed
1 - Filling in place
2 - Filling in place but loose
3 - Filling out, tooth intact
4 - Filling out, tooth split open
5 - Filling out, tooth split open and missing one cusp
6 - Filling out, tooth split open and missing two cusps
7 - Filling out, tooth split open and missing three cusps
8 - Filling out, tooth split open and missing more than three cusps.

18 EPITHELIAL INGROWTH WHEN A PORTION OF THE TOOTH IS FRACTURED AWAY

0 - Absent
1 - Present
9 - Not applicable

19-31 RADIOGRAPHS:

19-20 FISTULOUS TRACT - Control and fractured side.

0 - Not apparent
1 - Yes
2 - Questionable

21 ROOT FRAGMENTS

0 - Absent
1 - Present

22-23 METALLIC FRAGMENTS IN TISSUE - Control and fractured side

0 - Absent
1 - Present

24-25 LAMINA DURA - Control and fracture side

0 - Complete
1 - Questionable
2 - Absent at apex
3 - Irregular at apex
4 - Partially absent (multiple areas)
5 - Entirely absent

26-27 ROOT RESORPTION - control and fractured side

0 - Not apparent
1 - Yes
2 - Questionable

28-29 ALVEOLAR BONE FRACTURES - Control and fractured side

0 - Absent
1 - Present in First Molar area
2 - Present in other sites
3 - Questionable

30-31 PRESENCE OF RADIOLUCENCY - Control and fractured side

0 - Normal
1 - Questionable
2 - Associated with roots or root tips
3 - Associated with foreign body and roots or root tips

4 - Associated with foreign body
5 - Present but not associated with 2 or 4
6 - Associated with 2nd Molar as well as 1st Molar.

1-80 MICROSCOPIC APPEARANCE OF TISSUES:

1- 2 CAGE NUMBER

3 GROUP NUMBER

4 SEX OF ANIMAL

1 - Female
2 - Male

5 SURFACE EPITHELIUM AT THE FRACTURE SITE

0 - Intact - complete healed over, no proliferation
1 - Intact with proliferation around root fragment, partial
2 - Intact with proliferation around root fragment, complete
3 - Opening with no proliferation
4 - Opening with proliferation around root fragment, partial
5 - Opening with proliferation around root fragment, complete
6 - Opening with proliferation around root fragment, partial and complete
9 - Not applicable

6- 7 FOOD DEBRIS

0 - Normal locations
1 - Pulp Chamber
2 - Tissues (deep)

8-11 PERIODONTAL MEMBRANE - control and fractured side, 1st and 2nd molar

0 - Normal
1 - Markedly atrophic
2 - Markedly thickened
3 - Questionable

12-15 PULP - control and fractured, 1st and 2nd molar

0 - Normal
1 - Degenerated including necrosis
2 - Some with inflammation

16-19 CEMENTUM - control and fractured side, 1st and 2nd molar

0 - Normal
1 - Resorption (abnormal)
2 - Fragments
3 - Fragments and resorption

20-23 DENTINE - control and fractured side, 1st and 2nd molar

0 - Normal
1 - Resorption
2 - Caries (Bacteria in tubules)
3 - Resorption and caries
4 - 2nd and 3rd Molar caries
5 - 3rd Molar caries only
6 - 2nd Molar resorption and 3rd Molar caries

INFLAMMATORY REACTION - control and fractured side control side in Group I.

LOCATION OF INFLAMMATION WHEN PRESENT

24-27 ROOTS

Type of inflammation and associated hyperemia

28-31 PULP

Type of inflammation and associated hyperemia

32-35 FOREIGN BODY

Type of inflammation and associated hyperemia

36-39 SURFACE

Type of inflammation and associated hyperemia
(The following would apply to each location, i.e., roots pulp, foreign body, and surface.)

TYPE OF INFLAMMATION

0 - No inflammation
1 - Chronic (Majority of cells are plasma cells, lymphocytes and macrophages with few or no polymorphonuclear leukocytes)
2 - Subacute (Mixture of cell types)
3 - Acute (50 per cent or more polymorphonuclear leukocytes)
4 - Chronic and subacute
5 - Chronic and acute

6 - Subacute and acute
7 - Acute subacute and chronic
8 - Acute with necrosis
9 - Not applicable

ASSOCIATED HYPEREMIA

0 - None apparent

1 - Up to ¼ all vessels involved in area
2 - ¼ to ½ of vessels in area involved
3 - ½ to ¾ of vessels in area involved
9 - Not applicable

40-42 SIZE OF THE INFLAMMATORY REACTION

0 - None apparent
1 - 0.1 of a 10x field
2 - 0.2 of a 10x field
3- to 9- same as above
10 - ONE 10x field
15 - One and one-half 10x field, etc.
99 - Over ten 10x fields

43-44 ASSOCIATED FIBROPLASIA

AGE

1 - Immature
2 - Mature
3 - Mature and immature
9 - Not applicable

AMOUNT

1 - Minimal
2 - Moderate
3 - Extreme
9 - Not applicable

ORGANISMS - Control and fractured side

Location

45-51 EPITHELIAL SURFACE

0 - Absent
1 - Present, generalized
2 - Present, fracture site only
3 - Present, fracture site and interproximals
4 - Present, interproximals
5 - Present, fractured control tooth and interproximals

6 - Present, fractured control tooth, 2nd molar and interproximals

52-59 IN TISSUES

0 - Absent
1 - Present, focal areas
2 - Present, diffuse areas
3 - Present, focal and diffuse areas
9 - Not applicable

60-67 IN PULP

0 - Absent
1 - Present in pulp at fracture surface, exposed
2 - Present in pulp at fracture, buried
3 - Present, diffuse areas
4 - Present, focal areas
5 - Present, focal and diffuse areas
6 - Present in pulp of fragments and also 2nd molar
9 - Not applicable

68-75 AROUND ROOTS OR ROOT FRAGMENTS

0 - Absent
1 - Present associated with roots
2 - Present associated with root fragments, buried
3 - Present associated with root fragments at the surface
4 - Present associated with both exposed and buried root fragments
9 - Not applicable
(The following would apply to each location, i.e., surface, tissues, pulp and around roots)

TYPE OF ORGANISM

1 - Cocci and streptococci
2 - Rods
3 - Fungi
4 - Mixtures with spherical forms predominant
5 - Mixtures with rods predominant
9 - Not applicable

MICRO-LOCATION

I - Intracellular
2 - Extracellular
3 - Both
9 - Not applicable

CONCENTRATION

0 - Light
1 - Moderate
2 - Heavy
9 - Not applicable

76-80 NUMBER OF SECTIONS EXAMINED

76 - 78 Hematoxylin and eosin

79 - 80 Tissue gram stain

Card 1. To Follow Radiographic Examination.

32-34 Weight at start

35-37 One week later.

38-39 Gain or loss of weight after one week

40-42 Two weeks after start

43-44 Gain or loss of weight after two weeks

45-47 Weight at time of fracture of Group II,III, IV.

48-50 Weight after one week

51-52 Gain or loss of weight after one week

53-55 Weight after two weeks

56-58 Gain or loss of weight after two weeks

59-61 Weight at time of sacrifice

62-64 Weight change - Start to Fracture

65-67 Weight change - Start to Sacrifice

68-70 Fracture to Sacrifice - weight change

Card 1. Number Corresponding to Key to Analysis

			General						Gross				Radiographs			
1-2	3	4	5-6	7-8	9-10	11-12	13	14	15	16	17	18	19-20	21	22	23
17	1	1	00	00	00	00	4	0	9	9	0	9	00	0	0	0
22	1	1	00	00	00	00	3	0	9	9	0	9	00	0	0	0
29	1	1	00	00	00	00	2	0	9	9	0	9	00	0	0	0
43	1	1	00	00	00	00	2	0	9	9	0	9	00	0	0	0
33	2	1	50	00	40	00	2	1	3	1	0	9	00	1	0	0
21	2	1	05	00	30	00	4	0	3	1	0	9	02	1	0	0
26	2	1	05	00	41	00	4	0	1	1	0	9	00	1	0	0
46	2	1	05	00	50	00	3	0	1	1	0	9	02	1	0	0
41	3	1	13	37	40	77	2	0	3	1	3	0	20	1	1	0
14	3	1	13	37	26	63	3	0	4	1	7	0	00	1	0	1
36	3	1	31	43	30	73	2	0	4	1	4	0	22	1	0	0
38	3	1	31	28	40	68	2	0	0	0	6	1	20	1	1	0
05	4	1	24	30	35	65	4	0	1	1	5	0	02	1	0	1
15	4	1	42	28	41	69	4	0	0	0	5	0	20	1	0	0
23	4	1	24	39	30	69	4	0	0	0	5	0	00	1	1	0
10	4	1	24	28	41	69	3	0	1	0	2	0	20	1	1	0
06	1	2	00	00	00	00	2	0	9	9	0	9	00	0	0	0
32	1	2	00	00	00	00	4	0	9	9	0	9	00	0	0	0
19	1	2	00	00	00	00	2	0	9	9	0	9	00	0	0	0
28	1	2	00	00	00	00	3	0	9	9	0	9	00	0	0	0
12	2	2	50	00	35	00	2	0	1	1	0	9	00	1	0	0
39	2	2	50	00	30	00	4	0	1	0	0	9	00	1	0	0
24	2	2	05	00	30	00	2	0	2	1	0	9	00	1	0	0
27	2	2	50	00	40	00	3	0	2	1	0	9	00	1	0	0
03	3	2	13	30	39	69	2	0	0	0	4	0	00	1	0	1
37	3	2	31	39	30	69	3	0	0	0	6	1	00	1	0	1
40	3	2	13	39	40	79	3	0	3	1	5	1	00	1	1	0
42	3	2	31	37	26	63	4	0	3	0	5	0	00	1	0	1
25	4	2	24	38	30	68	2	0	0	0	4	0	20	1	0	0
20	4	2	24	28	41	69	3	0	0	0	2	0	00	1	1	0
11	4	2	24	28	40	68	3	1	4	2	1	0	02	1	1	0
09	4	2	24	28	40	68	3	0	1	1	5	0	20	1	1	5

Card 1. Number Corresponding to Key to Analysis

Radiographs					Start	One Week	+ -	Two Weeks	+ -
1-2	24-25	26-27	28-29	30-31	32-34	35-37	38-39	40-42	43-44
17	00	00	00	00	245	248	03	241	04
22	00	00	00	00	216	223	07	247	31
29	00	00	00	00	255	267	12	273	18
43	00	00	00	00	214	273	59	262	48
33	02	01	00	02	197	214	17	256	59
21	04	01	33	02	244	227	03	226	02
26	03	01	00	02	255	265	10	275	20
46	30	01	01	02	214	260	46	257	43
41	21	12	23	32	235	257	22	257	22
14	31	11	00	22	238	245	07	251	11
36	43	11	13	22	254	259	05	260	06
38	11	21	30	22	249	267	18	276	27
05	32	11	03	23	245	255	10	255	10
15	23	11	30	22	233	255	22	253	20
23	41	12	30	32	280	288	08	296	16
10	23	02	30	22	243	261	18	268	25
06	00	00	00	00	442	457	15	466	24
32	00	00	00	00	410	413	03	416	06
19	00	00	00	00	451	456	05	460	09
28	00	00	00	00	417	294	-23	389	-28
12	01	02	01	02	430	439	09	448	18
39	05	01	00	02	390	392	02	392	00
24	04	01	00	02	431	433	02	432	01
27	01	00	00	02	427	438	11	458	31
03	31	11	00	23	417	424	07	439	22
37	22	11	00	22	414	422	08	427	13
40	21	11	00	32	376	384	08	388	12
42	14	21	00	14	452	452	00	469	17
25	44	11	30	26	394	350	-44	393	-1
20	32	11	00	26	411	446	35	470	59
11	25	11	03	32	413	435	22	458	45
09	51	12	30	33	367	425	58	394	-21

Card 1. Number Corresponding to Key To Analysis.

	Frac.	One Week	+ -	Two Weeks	+ -	Sacr.	Start Frac.	Start Sacr.	Frac. Sacr.
1-2	45-47	48-50	51-52	53-55	56-58	59-61	62-64	65-67	68-70
17	263	267	04	276	013	282	018	037	019
22	263	262	-01	273	010	285	047	069	022
29	280	283	03	272	-008	272	025	017	-008
43	276	272	-04	289	013	297	062	083	021
33	281	279	-02	286	004	299	084	102	018
21	237	237	00	243	006	234	-007	-010	-003
26	278	277	-01	276	-002	296	023	041	018
46	268	266	-02	272	004	296	054	082	028
41	274	277	03	279	005	282	039	047	008
14	265	230	-35	174	-091	174	027	-064	-091
36	273	270	-03	278	005	275	019	021	002
38	281	284	03	288	007	291	032	042	010
05	271	276	05	271	000	289	026	044	018
15	276	263	-13	269	-007	283	043	050	007
23	318	318	00	315	-003	334	038	054	016
10	271	284	13	273	002	298	028	055	027
06	477	499	22	498	021	527	035	085	050
32	370	400	30	407	037	428	-040	018	058
19	470	485	15	486	016	490	019	039	020
28	394	411	17	416	022	452	-023	035	058
12	384	382	-01	400	007	453	-046	023	069
39	430	418	-12	433	003	443	040	053	013
24	446	420	-26	376	-070	446	015	015	000
27	476	482	06	494	018	501	049	074	025
03	456	454	-02	468	012	476	039	059	020
37	434	436	02	444	010	456	020	042	022
40	402	406	04	393	-009	413	026	037	011
42	481	449	-32	322	-159	274	029	-178	-207
25	421	427	06	435	014	444	027	050	023
20	510	486	-24	524	014	563	099	152	053
11	489	464	-25	477	-012	520	076	107	031
09	456	448	-08	460	004	508	089	141	052

Number of Microscopic Sections Examined for each Rat.

Cage Number	Hematoxylin and Eosin	Brown Brenn
17	52	26
22	240	60
29	44	22
43	56	28
33	146	73
21	68	34
26	68	34
46	68	36
41	68	34
14	68	28
36	84	42
38	64	32
05	64	32
15	96	48
23	100	50
10	64	42
06	48	24
32	48	24
19	40	20
28	44	22
12	68	34
39	64	32
24	72	36
27	68	34
03	72	36
37	72	36
40	56	28
42	64	32
25	88	44
20	68	44
11	147	42
09	100	50

Card 2. Numbers Corresponding to Key to Analysis.

1-2	3	4	5	6-7	8-11	12-15	16-19	20-23	24-25	26-27	28-29	30-31
17	1	1	9	00	0000	0000	0000	0002	00	99	00	99
22	1	1	9	00	0000	0000	0000	2020	00	99	00	99
29	1	1	9	00	0000	0000	0000	2020	00	99	00	99
43	1	1	9	00	0000	0000	0000	0020	00	99	00	99
33	2	1	4	00	0000	0010	0010	0210	05	92	05	92
21	2	1	4	02	0000	0010	0030	0210	04	11	08	92
26	2	1	4	00	0000	0020	0030	0032	03	01	00	99
46	2	1	5	00	0000	0020	0010	2230	06	02	08	92
41	3	1	4	10	2023	2010	1030	1010	65	22	64	13
14	3	1	1	00	2023	2022	1031	1033	75	22	35	23
36	3	1	6	10	2020	1020	1030	1010	75	12	33	11
38	3	1	4	11	2000	1010	1010	1011	35	12	83	20
05	4	1	4	10	2223	1020	1131	3111	76	22	74	11
15	4	1	4	10	0020	1010	1010	3032	53	22	43	02
23	4	1	1	10	3010	2010	1030	3010	31	30	68	21
10	4	1	4	10	0020	1010	1030	3030	45	22	18	02
06	1	2	9	00	0000	0000	0000	0020	00	99	00	99
32	1	2	9	00	0000	0000	0000	0522	00	99	00	99
19	1	2	9	00	0000	0000	0000	0020	00	99	00	99
28	1	2	9	00	0000	0000	0000	2400	00	99	00	99
12	2	2	4	00	0020	0010	0010	0030	05	92	03	92
39	2	2	5	00	0020	0010	0031	0531	05	92	08	99
24	2	2	6	00	0020	0010	0030	0030	05	92	05	91
27	2	2	4	00	0022	0010	0011	0033	05	92	08	92
03	3	2	4	10	2022	1010	1011	3036	55	22	88	12
37	3	2	1	10	2020	1010	3030	3010	55	22	88	12
40	3	2	4	10	2020	1010	3131	3311	55	22	88	12
42	3	2	4	10	3020	1010	1010	3030	55	22	88	12
25	4	2	4	10	3020	1011	1011	3233	35	32	55	22
20	4	2	4	10	2022	1011	1011	3233	33	22	88	22
11	4	2	4	10	2220	1110	1130	3330	55	22	58	22
09	4	2	4	10	2030	1010	1010	1024	31	20	11	11

Card 2. Numbers Corresponding to Key to Analysis.

32-35	36-39	40-42	43-44	45-46	47	48-49	50-51	52-53	54-55	1-2
0099	1100	000	99	44	4	22	22	00	99	17
0099	1100	000	99	44	4	22	10	00	99	22
0099	1100	000	99	44	4	22	11	00	99	29
0099	4121	000	99	44	4	22	10	00	99	43
0099	1512	099	22	43	4	22	01	03	94	33
0099	1121	002	21	43	4	22	01	02	91	21
0099	1411	002	22	43	4	22	00	02	94	26
0099	4431	005	32	43	4	22	01	00	99	46
6019	4422	102	32	53	4	33	20	13	14	41
0099	4422	302	32	53	4	33	22	33	44	14
0099	4422	803	32	53	4	22	21	20	19	36
0099	5532	304	22	53	4	22	21	22	11	38
0692	7722	609	32	53	4	23	22	23	11	05
0099	3522	203	32	53	4	22	21	22	41	15
6029	5121	300	21	54	4	22	20	00	99	23
0099	1411	102	22	53	4	22	22	02	94	10
0099	1512	000	99	44	4	22	00	00	99	06
0099	1111	000	99	44	4	22	00	00	99	32
0099	1512	000	99	44	4	22	00	00	99	19
0099	1111	000	99	44	4	22	00	00	99	28
0099	1111	002	22	43	4	22	00	91	91	12
0099	1411	008	32	43	4	23	01	03	94	39
0099	1411	006	22	43	4	23	02	03	94	24
0099	1512	014	32	43	4	22	02	00	99	27
0190	1111	720	33	53	4	22	21	21	44	03
0190	1111	210	33	54	4	22	20	12	11	37
0099	1111	820	33	53	4	22	22	22	44	40
0192	5521	406	32	53	4	22	22	11	44	42
0099	4421	105	33	53	4	22	21	13	14	25
0099	7521	505	32	53	4	22	21	02	91	20
0099	5522	599	32	63	4	22	21	23	44	11
4019	5121	410	22	53	4	22	21	23	11	09

Card 2. Numbers Corresponding to Key to Analysis.

56-57	58-59	60-61	62-63	64-65	66-67	68-69	70-71	72-73	74-75
99	99	00	99	99	99	00	99	99	99
99	99	00	99	99	99	00	99	99	99
99	99	00	99	99	99	00	99	99	99
99	99	00	99	99	99	00	99	99	99
93	92	05	94	93	92	04	94	93	90
93	91	03	94	93	91	04	94	93	91
93	91	01	94	93	91	03	94	93	90
99	99	05	94	93	90	04	94	93	90
33	00	55	44	33	10	04	94	93	90
33	22	55	44	33	12	12	44	33	11
39	19	50	49	39	19	00	99	99	99
13	00	44	44	22	22	14	11	33	01
33	12	55	11	33	12	14	11	33	02
33	21	55	41	33	12	13	11	33	01
99	99	53	41	33	10	00	99	99	99
93	91	14	44	33	22	04	94	93	92
99	99	00	99	99	99	00	99	99	99
99	99	00	99	99	99	00	99	99	99
99	99	00	99	99	99	00	99	99	99
99	99	00	99	99	99	00	99	99	99
91	90	05	94	93	91	00	99	99	99
93	91	04	94	92	92	03	94	93	90
93	91	00	99	99	99	04	94	93	90
99	99	01	94	93	91	00	99	99	99
33	01	55	44	33	22	04	94	93	91
33	00	55	44	33	22	12	11	33	00
33	21	55	44	33	22	14	41	33	20
33	10	53	44	33	21	14	44	33	00
33	11	36	14	33	21	14	11	33	10
93	91	55	44	23	21	02	94	93	91
33	22	55	44	33	22	14	44	33	11
33	01	55	44	23	22	04	91	93	90