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SCHOOL OF DENTISTRY
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# STUDIES OF FRACTURE HEALING

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# FOREWORD

The research described in this report was accomplished during the period April 1966 - April 1967.

In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

## SUMMARY

A method was developed to produce mandibular fracture in an experimental animal which closely simulates the human injury. The fracture Model was followed by predictable healing and was labile to induced variables.

Eighteen Macacca Mulatta Rhesus monkeys were studied in two groups: An adolescent group with mixed dentition and a young adult group with full permanent dentition. Utilizing clinical, radiographic, histologic, and tetracycline fluoroscopic methods of analysis, it was found that fracture healing in these two groups differed. Fracture "unions" in the young adult monkeys following an arbitrary six weeks of fixation, were similar in many respects to that seen in human situations. The fracture healing proved to be influenced by alterations in therapy, such as the absence of immobilization or the inclusion of foreign material into the fracture site. In the younger adolescent group, very few differences could be observed from these same variables upon fracture healing.

The study of healing variables in trauma turned to isobutyl cyanoacrylate monomer incorporated into the soft tissue and osseous segments of three additional monkeys. The use of the adhesive in the soft tissue flaps was clinically effective and adequately tolerated. In osseous tissue, the adhesive produced inconclusive results. Its use with stable bone fragments such as onlay bone grafts found clinical control of the parts and minimal foreign body response. In a mandibular fracture with bone segment distortion, the application of the adhesive was technically difficult and did not stabilize the fragments. In this setting, foreign body respone was more evident.

In a more basic inquiry of bone repair phenomena, tissue samples from healing mandibular defects in the rabbit were analyzed for LDH activity after 6, 9, 12, and 15 days of healing by means of quantitative spectrophotometric methods and by acrylamide gel electrophoresis. Fibrous bone filled the defects after 15 days of healing. The highest LDH activity occurred in the 6 day samples with a continual decrease in activity through 15 days. Healing defects in rabbit bone contained higher levels in the fastest migrating isozymes $LD_1$, $LD_2$, and $LD_3$. As healing progressed, the isozyme pattern shifted to a pattern in which $LD_3$ and $LD_4$ increased. In the later stages of healing, the pattern again shifted to a pattern in which $LD_1$ and $LD_2$ predominated.

The decrease in LDH activity may be related to a decrease in cellular metabolic activity as healing progresses. The changing patterns of LDH isozymes during bone healing suggest that the metabolism in healing bone changes from an aerobic to an anerobic type and the pattern may be related to tissue maturity. The metabolic implications of this enzyme assay may promote further knowledge of healing phenomena and target areas for therapy.

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# PART I

## INTRODUCTION

Many aspects of human jaw fractures are unique. Some of the complexities are of bacterial environment, presence of teeth, complex musculoskeletal anatomy,and forced alteration of nutritional habits. Although experimental fractures have been studied rather thoroughly, most investigators have utilized small animals and fractures of the long bones. Studies of mandibular fractures on larger animals have been scarce and in many cases plagued by infection, nonunions, and surgical and anesthetic morbidity and mortality. The problems are challenging. Anesthesia must be safe and effective without hindering the operator's work in the mouth and jaws. The anesthetic should provide a safe and quiet recovery without spastic behavior which would jeopardize the fracture reduction. The surgical fracture must create a predictable standard before experimental variations can be tested. Immobilization techniques must be effective and tolerated by the animal. The model fracture must be flexible to experimental change. The experimental fracture which will heal regardless of treatment variations is as poor as one which will never unite.

The monkey was ideal for this fracture investigation. The study was designed: (1) to simulate the human mandibular fracture with a model which would follow a predictable course and be reactive to induced clinical variables, (2) to standardize the clinical, radiographic, histological, and biochemical characteristics of this fracture model, and (3) to use this model to evaluate treatment methods and bone healing phenomena.

On the basis of previous investigations (April 1966), the extraoral surgical approach offered the most predictable control. Several techniques which were tried for immobilization, such as transosseous wires, circummandibular or circumzygomatic wires, acrylic splints or combinations, were not tolerated by the animals and were associated with a degree of the early nonunions. Variations in treatment time did not influence the ultimate result; a nonunion at six weeks would not change appreciably at the end of ten.

Experimentation with various treatment techniques resulted in a chosen method yielding predictable results.

## MATERIALS AND METHODS

The Macacca Mulatta Rhesus monkey most closely simulates the unique problems encountered in human mandibular fractures. Its adequate size and characteristic physiological reaction to jaw injury, changes in diet, and nutritional limitations seemed essential to this work.

While it is difficult to produce nonunion of fractures in smaller animals, we soon found that excessive trauma, inadequacy in reduction or immobilization will produce deficient healing in the monkey. The arbitrary period of six weeks of immobilization found the clinical result in the monkey fracture similar to that in the human.

### ANESTHESIA

The intramuscular Phenylcyclidine Hydrochloride (Sernylan) has been used throughout our investigation. Early problems of inadequate anesthesia or convulsions due to overdosage have been eliminated by a dosage range of 2 mg/kg of body weight. Anesthesia occurs 10 minutes after intramuscular injection and lasts approximately two hours. No endotracheal tube has been necessary; the animal can swallow, cough, and clear any obstruction which might occur.

### SURGICAL PROCEDURE

#### Mandibular Fracture (Figures 1-6)

Approximately two weeks following the extraction of the mandibular right second bicuspid or primary second molar and preoperative lateral jaw radiograph, the animal was anesthetized, shaved, and prepared with Hexachlorphene soap. The operators scrup and gown and the animal is draped in the usual sterile manner for the extraoral procedure.

Through a 3-cm incision at the base of the right mandible, the bone is exposed by means of sharp and blunt dissection. A cut is made with a No. 6 carbide bur in the region of the previous extraction. The section is continued vertically through medullary bone up to, but not including, lingual cortex. (This is the Model II method, quite different from the early Model I which included complete sectioning of both cortical plates.) Following adequate sectioning, the fracture is completed through the lingual cortex with a twist of an elevator. The fracture is not compounded openly into the oral cavity. Mobility is demonstrated by manual manipulation. After adequate hemostasis, the soft tissue is closed in layers and the skin sutured with interruped 4-0 braided silk sutures.

### Immobilization (Figures 7-12)

A modified intraoral Risdon appliance is used to immobilize the fractured segments. A 0.020-in. stainless steel wire is applied to each two teeth adjacent to the fracture site. The free ends are adapted to span the area of the fracture site in a Risdon fashion. A self-curing acrylic is applied to the teeth incorporating all of the wire. The teeth are placed in occlusion to check the alignment of the fracture segments and appropriate contour of the acrylic material.

## POSTOPERATIVE MANAGEMENT

After surgery, the animals are placed in their cages with the head hyperextended for recovery from the anesthetic. During the course of healing, all monkeys receive a commercial soft diet preparation. Weights are recorded three times in the experimental course. All animals maintained their weight during the healing period observed.

## EVALUATION METHODS (Figure 13)

### Radiographs

Lateral jaw radiographs were taken: (1) preoperatively following tooth extraction, (2) postoperatively at time of fracture, and (3) at time of sacrifice.

Films were exposed at 65 kv and 0.4 seconds on Kodak Morlite Occlusal Dental Film. Thin horizontal sections taken for tetracycline studies were radiographed at 90 kv and 60 seconds with Kodak-type fine grain industrial Film.*

### Tetracycline Labeling

All animals received 100 mg of tetracycline intramuscularly one week before sacrifice.

## SACRIFICE PROCEDURE

After six weeks, a veterinary lethal solution containing pentobarbital and alcohol vehicle was injected intravenously. The fracture was immediately evaluated for clinical union. The mandible was disarticulated and again a clinical impression of fracture stability was obtained. Following lateral

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*Our appreciation is extended to Professor A. G. Richards, Chairman of the Department of Radiology, School of Dentistry, University of Michigan, for his assistance.

jaw radiography, the specimen was placed in a 10% neutral buffered formalin solution for histologic preparation.

### Histologic Technique

A horizontal section was taken through the fracture area, approximately 1 mm of thickness at a level 5 mm above the inferior border of the mandible. The sections were made with a water cooled rotating silicon carbide blade. After radiographic records, the sections were prepared for ultra-violet microscopic observation of tetracycline fluorescence. This process includes progressive alcohol dehydration, infiltration, and imbedding in a clear, hard setting plastic (8000 polylite). The sections are polished to approximately 20 μ and mounted unstained for ultraviolet fluorescent microscopy. The remaining segments were decalcified, dehydrated, and infiltrated and imbedded in paraffin. Serial sections were taken in a horizontal plane. They were mounted and stained alternately with hematoxyline and eosin and Masson stain and covered for histologic study.

### Experimental Design (Table I)

Eighteen animals were used in this phase of the study. Nine monkeys were in a mixed dentition stage at approximately 15 months of age (Group A). Nine animals were young adult monkeys with a full complement of permanent teeth. These animals were approximately 36 months of age (Group B). Healing characteristics were compared for these two major groups; the adolescent and the young adult monkeys. The animals in the mixed dentition stage were divided into two subgroups (1 and 2). Group 1 consisted of six animals and Group 2 consisted of three animals. The purpose of these two subgroups was to determine the effect of the presence of the unerupted bicuspid tooth bud in the line of fracture. All monkeys in Group A received the Risdon wire fixation appliance. The nine young adult monkeys in Group B were divided into two subgroups (1 and 2) in order to study healing under immobilized and mobilized conditions within this age group.

## EVALUATION TECHNIQUES

### Clinical Impression of Union

The firmness of fracture union was determined immediately after sacrifice by manual palpation and given a value of 1, 2, or 3, indicating: 1—good bridging, 2—slight bony bridge, and 3—no bony bridge.

TABLE I

| Monkey No. | Stage of Dentition | Bud in Fracture | Type of Immobilization | Weeks Healing | Evaluation | | |
|---|---|---|---|---|---|---|---|
| | | | | | Clinical | X-ray | Histological |
| Group A | | | | | | | |
| 37 | M | yes | Risdon | 6 | 1 | 1 | B |
| 38 | M | yes | Risdon | 6 | 1 | 1 | B |
| 39 | M | yes | Risdon | 6 | 1 | 1 | B |
| 40 | M | yes | Risdon | 6 | 1 | 1 | B |
| 41 | M | yes | Risdon | 6 | 1 | 1 | B |
| 42 | M | yes | Risdon | 6 | 1 | 1 | B |
| 43 | M | no | Risdon | 6 | 1 | 1 | B |
| 45 | M | no | Risdon | 6 | 1 | 1 | B |
| 48 | M | no | Risdon | 6 | 1 | 1 | B |
| Group B | | | | | | | |
| 47 | P | -- | Risdon | 6 | 1 | 1 | B |
| 52 | P | -- | Risdon | 6 | 2 | 2 | F |
| 53 | P | -- | Risdon | 6 | 2 | 2 | F |
| 54 | P | -- | Risdon | 6 | 2 | 2 | F |
| 55 | P | -- | none | 6 | 2 | 2 | F |
| 56 | P | -- | none | 6 | 3 | 3 | N |
| 57 | P | -- | none | 6 | 3 | 3 | N |
| 58 | P | -- | none | 6 | 1 | 1 | B |
| 59 | P | -- | none | 6 | 1 | 1 | B |

M - Mixed Dentition
P - Permanent Dentition

F - Fibrous Union
N - Nonunion
B - Bony Union

1 - Rigid
2 - Semirigid
3 - Nonrigid

## Radiographic Evaluation

The amount of bony bridge across the fracture site was evaluated from lateral jaw radiographs and given a value of 1, 2, or 3, indicating: 1—good bridging, 2—slight bony bridge, and 3—no bony bridge.

## Histologic Examination

The microscopic sections were evaluated as a bony union, fibrous union, or nonunion. The presence of osteoblastic activty and a calcium bond was designated as a bony union. A section showing active osteoblastic proliferation and new bond formation, absence of inflammatory elements in the presence of minimal fibrous tissue running perpendicular to the fracture site was termed a fibrous union. A diagnosis of nonunion was made on the basis of absence of osteoid or osteoblastic activity, osteoclasis, inflammation and dense fibrous tissue running parallel to the fracture line.

## Tetracycline

Routine sections were made on all animals for tetracycline fluorescence studies. We noted however that the information obtained from these studies was limited so that only representative tetracycline sections were studied and described. The tetracycline was incorporated into the mineralizing component of the osteogenic process at the fracture site. Under ultraviolet light, the characteristic tetracycline fluorescence was noted allowing for a subjective evaluation of degree of mineralization.

Although our evaluation methods were consistent, we still were unable to quantitate healing in order to statistically evaluate our results. Consequently a study of osseous enzymology was conducted as described in Part III of this report.

# RESULTS

The nine monkeys of Group A all demonstrated a rigid bony union. The presence or absence of a bicuspid tooth bud did not alter the result.

Of the nine monkeys in the young adult Group B, the four treated with immobilization showed various degrees of union from a bony to a definite fibrous union. Five monkeys in Group B, subgroup 2, received no immobilization. The results were varied: there were two bony unions, one fibrous union, and two definite nonunions.

Degree of Rigidity

This test proved to be critical when comparing the results of the two age groups of monkeys. Although it was quite gratifying to find the firm, rigid unions in the early parts of our study at six weeks, we would only expect to see this result in human in a particular age group corresponding to our young, adolescent monkeys. On the older young adult monkeys, the springy union after six weeks of immobilization matched our experiences with the typical appearance of a human fracture after six weeks of fixation. There was a firm but slight movable (rubbery) union of the fracture segments. Nonunions were obvious and were the same as experienced clinically in human nonunion situations and in our previous work with fracture Model I.

Radiographic Results (Figures 14-20)

One does not expect to see bony bridging of a fracture site following six weeks of fixation in humans. This result was observed also in our young adult monkeys. The lateral jaw radiograph showed the outline of the fracture site with only a slight opacity in certain bridging segments. There was absence of bony resorption. This union contrasted dramatically with the complete band of calcification observed in the younger adolescent monkeys. The presence or absence of the unerupted tooth did not alter the results of our 100% bony unions with the nine monkeys in the mixed dentition stage. There were instances when the bur actually perforated the tooth bud in this series without sequellae in fracture repair.

Nonunions were characterized by a complete radiolucency of the fracture site with widening of the area to indicate active bony resorption.

Radiographs of the horizontal sections produced predictable and interesting results in the case of nonunions and bony unions. The appearance of the fibrous union depended on the level of the section.

Histologic Results (Figures 21-25)

Sections of the mandibular fractures in Group A (mixed dentition) showed heavy osteoblastic activity with obvious subperiosteal anchoring callus. On some sections, the lingual cortex demonstrated heavy cartilage formation. All specimens in Group A consisting of adolescent monkeys demonstrated obvious bony unions regardless of the presence or absence of the tooth bud.

Adult bony unions demonstrated more subtle changes. Areas of bony bridging were present with very little fibrous barrier. Osteoblastic activity was present but the section lacked the massive build-up of new bone and obvious subperiosteal callus observed in the adolescent monkeys.

The only nonunions were in the young adult, nonimmobilized young adult monkeys bore the microscopic features of an eventual union. Although there was lack of a calcified bridge, heavy fibrous bands were running perpendicular to the fracture line with active osteoblast proliferation.

## DISCUSSION

The Macacca Rhesus monkey was ideal as the experimental animal for our study. Sernylan anesthetic in combination witha Model II fracture with the modified Risdon immobilization produced a predictable union. A most important step in our previous work was the realization that both buccal and lingual cortical defects offered to much challenge offered to much challenge to repair. By leaving the thin lingual cortex for manipulative fracture in Model II procedure, we were able to approximate the clinical situation of fractures with a potential to unite.

The modified Risdon appliance met all the necessary requirements for an immobilizing splint for the segments. It did not compound the fracture site.

Our observations were conducted under uniform conditions. A clinical judgment of a rigid, semirigid, or nonrigid union was verified by correlating lateral jaw radiographs. However, the most convincing correlation was upon histologic study. Characteristic semirigid or fibrous unions were observed. The amount and direction of fibrous tissue appeared significant. In nonunions, the heavy fibrous bands ran parallel to the fracture line, while in the semirigid union small short tenacious fibers ran perpendicular to the fracture line, joined periodically with a bridge of osteoid. This resembled the human fracture following six weeks of treatment. The adolescent monkeys developed a union demonstrating far more rapid calcification than humans. This rigid union in the adolescent group was not susceptible to variables. The young adult monkeys, however, produced various degrees of union. At six weeks, the fracture line was still quite evident with some calciffication and active osteoblastic activity. The semirigid or fibrous union observed in three out of four specimens was comparable to human clinical experience. The "springy" or "rubbery" consistency approximates that of the human mandibular fracture at this stage of repair. The lateral jaw radiograph also revealed a fracture line with only a slight degree of radiopacity. The histologic appearance suggested eventual complete bony union. This group's ability to respond to change revealed that the nonimmobilized segment produced a variety of results ranging from a solid bony union to a nonunion. Therefore, the experimental animal of choice for the conditions of Model II mandibular fracture study is the older young adult Macacca Rhesus monkey.

Development of a dependable experimental model has been a tedious and difficult task. We are now evaluating treatment modalities with a responsible

fracture model which is sensitive to the conditions of a human mandibular fracture.

## SUMMARY AND CONCLUSIONS

1. An experimental mandibular fracture has been developed to closely simulate a human fracture. It will follow a predictable course and is susceptible to induced variables to influence repair.

2. The adolescent monkey in the mixed dentition stage is not satisfactory because of the rapid rigidity obtained in the fracture and its unresponsibleness to experimental variation.

3. The older young adult Rhesus monkey presented a course of repair most closely simulating that which is seen in human clinical situations.

# P A R T I I

## INTRODUCTION

Pilot studies were intitiated in fracture wound healing with Isobutyl Cyanoacrylate Monomer, supplied by the Ethicon Corporation. The prototype "Eastman 910 adhesive," developed in 1960,[1] was methyl 2-cyanoacrylate monomer with various additives. The product was purified by the Ethicon Corporation and supplied to several qualified investigators. The methyl form of cyanoacrylate has been used extensively in animal surgery and in rare human trials. Applications included small vessel anastomoses, heart and aorta surgery, as well as procedures on the lungs, skin, eye, kidney, ureter, bladder, G. I. system, liver, and spleen.[2-10] Work on bone and tendon, with one exception,[11] has been limited to long bones. By 1965 further investigation revealed diminished toxicity achieved with an increase in the length of the alkyl chain.[12,13] The isobutyl form proved to be least irritating to tissues.

## MATERIAL AND METHODS

### PLASTIC ADHESIVE

Pilot investigation used the isobutyl cyanoacrylate monomer in repairing wounds of soft tissue and bone in the adult Macacca Rhesus monkey. The following tissue manipulation was performed:

### Soft Tissue

Under operating room conditions of anesthesia and preparation, a 1 cm transecting incision was made in the midline of the upper lip. The incision was opened and one drop of adhesive was applied immediately. The cut ends were placed together and held for one minute.

A mucoperiosteal flap was prepared in the anterior palate with a cuspid-to-cuspid incision. A single drop of material was placed on the bone and the palatal flap was returned under moderate pressure for one minute.

A mucoperiosteal flap was reflected on the mandible on the labial surface from midline to the first bicuspid region. The flap was returned to place and held in a similar manner with adhesive monomer.

Use in Osseous Tissue (Figure 26-31)

Four applications to bone defects were included. Routine "Model II" mandibular fracture was produced and two drops of adhesive were placed in the fracture site before reduction and closure of soft tissues. All standard fracture conditions were followed.

In another procedure, a rectangular segment was removed from the base of the left mandible. The defect was replaced with an autogenous graft taken from the right tibia. The graft was stabilized with two drops of adhesive. The periosteal envelope was returned around the graft and soft tissues were closed.

In two other animals, the right tibia was surgically exposed for drilling two holes in the cortex with a 558 carbide bur. The adhesive was drawn into a syringe and .3 cc was injected into the proximal hole. The distal hole was left as a control. Periosteum and other soft tissues were closed in layers.

Another tibial section, 1 x 2 cm was removed and replaced. This cortical bone block was returned to place and stabilized with adhesive monomer. Wound closure was similar to other wounds in the pilot study.

Radiographs

Radiographs were taken of all osseous structures impregnated with adhesive immediately after surgery and at time of the termination of the healing study.

Tetracycline Labeling

All animals received 100 mg of tetracycline IM one week before sacrifice.

Healing Period

Two healing intervals of 6 and 12 weeks were chosen. The sacrifice procedure and methods of evaluation were the same as developed on our fracture model.

## RESULTS (Table II)

Soft Tissue (Figures 32-37)

Adhesive application at time of surgery produced rapid bonding of margins with immediate hemostasis. The midline incision of the upper lip was ade-

TABLE II

| Monkey No. | Site of Adhesive Application | Weeks Healing | Results |
|---|---|---|---|
| 50 | (1) upper lip midline | 6 | slight foreign body |
| | (2) palatal flap | 6 | slight foreign body |
| | (3) mandibular flap | 6 | slight foreign body |
| | (4) Fx model II | 6 | nonunion, subcutaneous abscess |
| 51 | (1) upper lip midline | 12 | normal histologic pattern |
| | (2) palatal flap | 12 | normal histologic pattern |
| | (3) mandibular flap | 12 | normal histologic pattern |
| | (4) left mandibular bone graft | 6 | graft tolerated, moderate foreign body reaction |
| 59 | (1) right tibia marrow cavity | 6 | foreign body reaction |

quately closed and stable one minute after application of the adhesive. All soft tissue sites were clinically healed at six and twelve weeks postoperatively. Flap attachments were firm and the tissues were normal in color and tone.

Histologically, the lip midline incision showed little variation at 6 and 12 week intervals. The section at the end of 6 weeks showed some evidence of space occupied by adhesive crystals surrounded by foreign body giant cells. The 12 week section demonstrated only a slight disruption of the muscular layer.

The palatal mucoperiosteal flap also demonstrated, at 6 weeks, some isolated areas of adhesive with resultant foreign body reaction. The 12 week specimens represented normal healing with absence of inflammatory reaction. The mandibular flap yielded identical observations.

Bone (Figures 38-45)

Application of adhesive to the Model II fracture site resulted in rapid hemostasis. The immediate adhesive bond, however, was not adequate to stabilize a fracture under any degree of stress. Six weeks later the mandibular fracture was found in a state of definite nonunion with a subcutaneous abscess approximately 2 cm in diameter at the base of the jaw.

Lateral jaw radiographs were classical for nonunion with rounded resorption at the edges of the fracture site. Histologically there was a demonstration of heavy fibrous bands with no evidence of osteoid or calcified bridging. A large collection of spaces surrounded by foreign body giant cells was present in place of the resorbed buccal cortex. Lingually, a massive subperiosteal bony callus was present, but completely separated in the fracture line by a mass of fibrous tissue. The submandibular abscess showed lymphocytic and polymorphonuclear leucocyte infiltration, tissue necrosis, and foreign body reaction.

The autogenous graft at the base of the mandible appeared healed at the surface. The radiograph was unremarkable showing a possible bony bridge and the relative absence of resorption. Microscopically, the graph demonstrated minimal resorption with an adequate amount of viable bone. There was no bony bridge in our sections but inflammatory elements were minimal. An isolated prominent mass of ground substance representing adhesive material was present buccally, engulfed by many giant cells.

The injection of adhesive into the tibia marrow produced an intense foreign body reaction.

## DISCUSSION

The effectiveness of isobutyl cyanoacrylate adhesive in soft tissues has been documented by numerous and extensive studies.[14,15] Its use can be considered in the oral cavity to aid in retention of flaps. Although there was some foreign body reaction at 6 weeks, by 12 weeks most evidence of the monomer had been removed, substantiating its biodegradable characteristics.

This early trial found limitations to the use of the adhesive in bone. The technique of application to bleeding marrow is difficult. It was necessary to control hemorrhage before application. Although effective in soft tissue hemostasis, excessive marrow bleeding tended to carry the adhesive away from the desired site before coagulation occurred. The rapid setting property of the adhesive required that the bony segments be approximating before application. Capillary action distributed the adhesive. Thus adequate hemostasis was required.

For stabilization of a small fragment of bone which was not under a great deal of stress, such as a bone graft, the adhesive seemed adequate. The material did not possess structural strength to hold fragments subject to considerable distortion as in a mandibular fracture.

The single trial with the Model II fracture restricts major conclusions; however, one would doubt if a simple layer bond would stabilize a major fractured segment. Tissue reaction to the adhesive was not excessive even though the result was a nonunion. The effectiveness of the adhesive in bone grafting might prove to be helpful to several clinical situations.

### Addendum

On the basis of the pilot studies described, the action of the adhesive on bone surfaces subject to stress (mandibular fracture) and stabilized (tibial bone graft) are being studied at 3, 6, and 12 weeks intervals.

## SUMMARY AND CONCLUSIONS

1. A pilot study introduced isobutyl cyanoacrylate monomer adhesive application in soft tissue and bone injuries.

2. The results were assayed by clinical, radiographic, and histologic criteria.

3. A further limited study is now in progress to determine the effects of the adhesive in bone in a stable, versus nonstable experimental environment.

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# PART III

## PHASES OF BONE HEALING TRACED BY LACTATE DEHYDROGENASE ISOZYMES

Throughout our studies of mandibular fracture repair, we have felt the need for an objective quantitative method in evaluating degree of bony repair and union. Histologic, radiographic, and clinical examinations of the fracture site have allowed us to grossly and subjectively evaluate the fracture but a totally objective, quantitative examination and refined test for bone healing phases has not been possible thus far.

Work on enzymes, quantitated by computerized techniques, suggests promise as a method for evaluating healing in bone. If normal standards of enzyme activity could be related to the histologic degree of healing, a quantitative method of evaluation would result. This important objective has necessitated studies within the past year on a more basic level with the results easily applied to our studies of mandibular fracture repair in animals and humans.*

*This section has been accepted in a modified form for publication in the Journal of Oral Surgery. A reprint will be forwarded for inclusion when available.

## INTRODUCTION

The nature of the enzymatic mechanisms involved in bone repair and regeneration is a complex and unresolved biological phenomenon. Formation of bone during healing has been examined on a morphological basis which has afforded only a partial interpretation of osteogenesis. Enzyme histochemical techniques have been developed which allow new approaches to possible relations between morphological structures and chemical function. These techniques have been applied to the study of various tissues including bone[1,2] as well as fracture repair.[3] However, quantitative studies of enzymatic activity during bone repair have not been reported.

Many enzymes exist in multiple molecular forms called isozymes.[4] Specific patterns of isozymes (i.e., the relative amounts of one form to another) have been shown to change during development[5] and in response to changing cellular physiologic demands.[6] Lactate dehydrogenase (LDH) exists in five molecular forms in most vertebrate tissues.[7] The LDH isozymes of healing bone have not been investigated nor is quantitative data available on the amounts of LDH present in healing bone.

## CONDENSED REVIEW OF LITERATURE

Lactate dehydrogenase functions in the reversible reaction of pyruvate to lactate in which a pair of electrons are transferred to a coenzyme, nicotinamide adenine dinucleotide (NAD). The significance of the dehydrogenase enzymes is that they initiate energy-yielding reactions resulting in the eventual production of ATP.[8]

Walker, in a histochemical study of bone, failed to demonstrate isocitric dehydrogenase in bone and theorized that the lack of this enzyme allowed an increase in citrate by inhibition of the Krebs cycle.[9] He postulated that the local high concentration of citric acid fostered the decalcification of bone. However, subsequent studies by Balogh, et al.,[1] Fullmer,[2] and other authors[10-14] utilizing histochemical techniques have shown isocitric dehydrogenase as well as many other dehydrogenase enzymes including lactate dehydrogenase to be present in bone, demonstrating that bone has a functional citric acid cycle, pentose cycle, and the capacity to metabolize fatty acids and carbohydrates.[2]

In 1957, Vesell and Bearns[15] and Wieland and Pfleiderer[16] showed that LDH exists in five distinct forms and since then many studies have been concerned with the function and characteristics of LDH and other isozymes. The isozymes of LDH are referred to as $LD_1$, $LD_2$, $LD_3$, $LD_4$, and $LD_5$ with $LD_1$ carrying the highest negative charge and migrating farthest toward the anode during electo-

phoresis at alkaline pH.

Cahn and others[17] and Kaplan and Goodfriend[18] have hypothesized that tissues which are well oxygenated and function under aerobic conditions such as the heart have a high concentration of $LD_1$ and those which function under an anerobic conditions such as skeletal muscle have a higher concentration of the $LD_5$ form. Markert and his colleagues[4,19] have demonstrated that during development of tissues there are changes in the LDH pattern.

Studies quantitating lactate dehydrogenase in bone and an evaluation of the LDH isozyme pattern during ostegenesis have not been carried out. The following investigation was designed to (1) quantitate LDH in healing bone at various time intervals and (2) to quantitate the LDH isozyme pattern during osteogenesis, in an effort to more fully understand the healing process.

## MATERIALS AND METHODS

Twenty New Zealand white rabbits were divided into four groups each containing five animals. Under pentobarbital general anesthesia, three standardized 3/8-in. defects were created at the inferior border of the body of the mandible bilaterally with trephine burs saline to cool the burs. (Three defects were placed on each side, a total of six defects per animal, Figure 46.) Animals in each group were sacrificed 6, 9, 12, and 15 days following surgery. Initial studies had shown that before 6 days of healing little evidence of osteogenesis was present and after 15 days of healing it was difficult to identify the healed defects.

To eliminate blood in the area of surgery which would influence the quantitive LDH activity, the animals were given 1,000 units of sodium Heparin and perfused with 0.9 per cent saline via the cartoid arteries. The animals were sacrificed by an overdose of pentobarbital and the mandible surgically reexposed. Healing tissue present in each of these defects was removed in the form of a plug by the use of hand rotated trephine burs. The tissue samples from the same position on each side of the mandible were combined to obtain an adequate weight for analysis. Histologic sections were prepared of the healing mandibular defects from one animal in each group and stained with Masson's stain to examine healing morphologically. Normal perfused rabbit bone was also prepared as a control.

The tissue samples were frozen, thawed, and the wet weight obtained. The samples were placed in 3 cc of 0.2 M Tris BCl buffer at pH 8.9 and homogenized in a Kontes type tissue homogenizer cooled in ice water. The homogenate was centrifuged for five minutes and 1 cc of the supernatant removed and diluted with 0.2 M Tris BCl buffer to a concentration of 1 mg of tissue/0.1 cc to be used for spectrophotometric analysis. The remaining 2 cc of supernatant was diluted with 40% sucrose in 0.05 M Tris glycine buffer pH 8.3 to a final concentration of 8 mg/0.1 cc. This solution was used in the acrylamide gel

electrophoresis technique.

The total lactate dehydrogenase activity was measured using 0.72 M sodium lactate as the substrate, 0.05 M NAD in 0.05 M Tris HCl (pH 8.9) as the coenzyme. The rate of reduction of NAD at 340 μ was measured for six minutes on a Beckman DU spectrophotometer at 38°C.

The techniques and reagents used for the acrylamide disk electrophoresis were essentially those described by Davis[20] except no sample gel was used since it was possible by careful technique to add the sample which was in a dense sucrose solution directly to the electrophoresis columns. Staining of the gels for LDH activity was carried out by a method similar to that described by Dewey and Conklin.[21]

Quantitation of the stained gels was accomplished by spectrophotometric scanning of the gels at 440 μ. The spectrophotometer was coupled to a digital voltmeter and the output relayed to an IBM key punch. A program was written to graphically present the data, compute the area of each of the LDH isozyme bands, and determine the per cent of the total area for each band. By the use of the Student Fisher T-test the significant differences between means of the grouped data was tested.

## RESULTS

### Histologic

Six days after surgery organization of the blood clot in the defects were evident with angioblastic and fibroblastic proliferation. The buccal callus was well formed approximately 5 mm from the edge of the defects but the defect was not bridged by callus. After 9 days of healing, the granulation tissue was more mature and there was evidence of intramembranous type of bone formation in the medullary portion of the defects. At 12 days newly formed woven bone bridged the defect except near the lingual cortex where connective tissue was evident. The bone which had formed the buccal callus had become dense lamellar bone with noticeable Haversian systems. After 15 days of healing, the defect was completely filled with organizing fibrous bone.

The results of the spectrophotometric analysis of lactate dehydrogenase activity in healing bone are presented in Figure 47. There was a generalized decrease in enzymatic activity as the bone healed. The activity decreased from a high at 6 days to about one half of this activity at 15 days. There was a significant difference between the activity at 9 days and that at 12 days. There was no significant difference between the 6 and 9 day samples but a significant difference was noted between the 12 and 15 day samples. No significant differences were noted between the defects at different positions through the mandible at any given day.

An analysis of the isozyme pattern of tissue homogenates from the healing bone showed various changes as the bone healed. Although in some cases all five isozymes were noted, in general isozymes $LD_1$, $LD_2$, and $LD_3$ exhibited the greatest activity throughout healing with minimal activity noted in the slower migrating isozymes $LD_4$ and $LD_5$. The results of the isozyme patterns at each of the healing periods are presented in Figures 48-52.

The changing isozyme pattern is presented in Figure 53. In general, there was a shift toward a more cathodal isozyme pattern between the 6th, 9th, and 12th days and another shift to an anodal pattern as healing progressed as in the 15 day samples and in mature bone. It was found that there was a significant decrease in the total area of the isozymes at each of the healing days which corresponded to the decrease in the total LDH activity as measured by the quantitative assay.

## DISCUSSION

Histologically, healing bone is composed of a heterogenous mixture of cell types including fibroblasts, angioblasts, osteoclasts, osteoblasts, and osteocytes. These various cells exhibit LDH activity and all contribute to the total activity in healing bone. Studies by Walker,[9] Fullmer,[2] and Balogh, et al.,[1] showed that LDH activity decreased from a high in osteoclasts through osteoblasts to a low in osteocytes. We can only speculate on the relationship between the presence of osteoclasts in the early healing samples and high LDH activity and decreasing LDH activity as the dominant cell type changed.

Balogh and Jajeck's[3] histochemical study of repairing rat tibial fractures indicated that the greatest enzymatic activity occurred as the osteoprogenator cells approached maturity and decreased as repair progressed.

Van Reen's[22,23] studies on the quantitation of aconitase and isocitric dehydrogenase in mature bone showed the highest activity of these oxidative enzymes in marrow, less in proliferating epiphyseal areas, and little activity in bone cortex. Roberts and Strachan[24] also showed a decrease in lactate dehydrogenase activity in tooth buds as calcification increased.

Our results tend to substantiate these findings. If LDH activity can be considered to be an indicator of cellular energy requirements, it would seem that these needs are greatest during the initial stages of osteogenesis and decrease as bone becomes more mature.

If the hypothesis of Kaplan and Goodfriend[18] and Cahn[6] concerning the functional role of isozymes are valid, there appears to be a change from aerobic metabolism in healing rabbit bone at 6 days to a tendency toward an anaerobic type of metabolism at 12 days and a return to aerobic metabolism after 15 days of healing.

There is other experimental evidence that changes in metabolism in healing bone occur. Ham,[25] Girgis and Pritchard,[26] and Urist[27] in studies of bone and fracture repair theorized that tissue ischemia due to local vascular insufficiency resulted in areas of cellular hypoxia. Mesenchymal cells in the area respond by the production of various mature cell types including fibroblasts, chondroblasts, or osteoblasts. Bassett and Herrmann[28] in tissue culture experiments modified after those of Fell[29] were able to produce various cell types, fibroblasts, chrondroblasts, and osteoblasts, by varying the environmental oxygen and carbon dioxide concentrations. It is possible that during bone repair there is a change from an aerobic to an anaerobic type of metabolism as osteogenesis occurs within a relative ischemic area of the defect. As the area becomes more vascularized, a shift to an aerobic type of metabolism could occur.

Markert and Ursprung's[19] studies of changes in isozyme patterns may occur with maturity. For example, in the mouse embryo the heart contains high $LD_5$ but in the adult there is a pronounced shift to the $LD_1$ and $LD_2$ forms. In rabbit healing bone as shown by this study the isozyme pattern also changes with tissue maturation. The pattern found in mature bone may be due to tissue development beyond initial maturity to senescence in which the tissue may be considered to be in a resting or less dynamic state.

## SUMMARY AND CONCLUSIONS

Tissue samples from healing mandibular rabbit bone defects were analyzed for LDH activity after 6, 9, 12, and 15 days of healing by means of qunatitative spectophotometric methods and by acrylamide gel electrophoresis techniques.

Fibrous bone filled the defects after 15 days of healing. The highest LDH activity occurred in the 6 day samples with a continual decrease in activity through 15 days.

Healing rabbit bone was highest in the fastest migrating isozymes $LD_1$, $LD_2$, and $LD_3$. As healing progressed, the isozyme pattern shifted to a pattern in which $LD_3$ and $LD_4$ increased. The later stages of healing the pattern again shifted to a pattern in which $LD_1$ and $LD_2$ predominated.

The decrease in LDH activity may be related to a decrease in cellular metabolic activity as healing progresses. The changing patterns of LDH isozmes during bone healing suggest that the metabolism in healing bone changes from an aerobic to an anerobic type and the pattern may be related to tissue maturity.

## Addendum

From this study, it is obvious that the quantitative isozyme pattern relates

closely to the degree of healing. We are now able to sample the healing bone from the fracture site and quantitate the degree of healing by the isozyme pattern. This will be a most valuable tool in our future studies of fracture repair.

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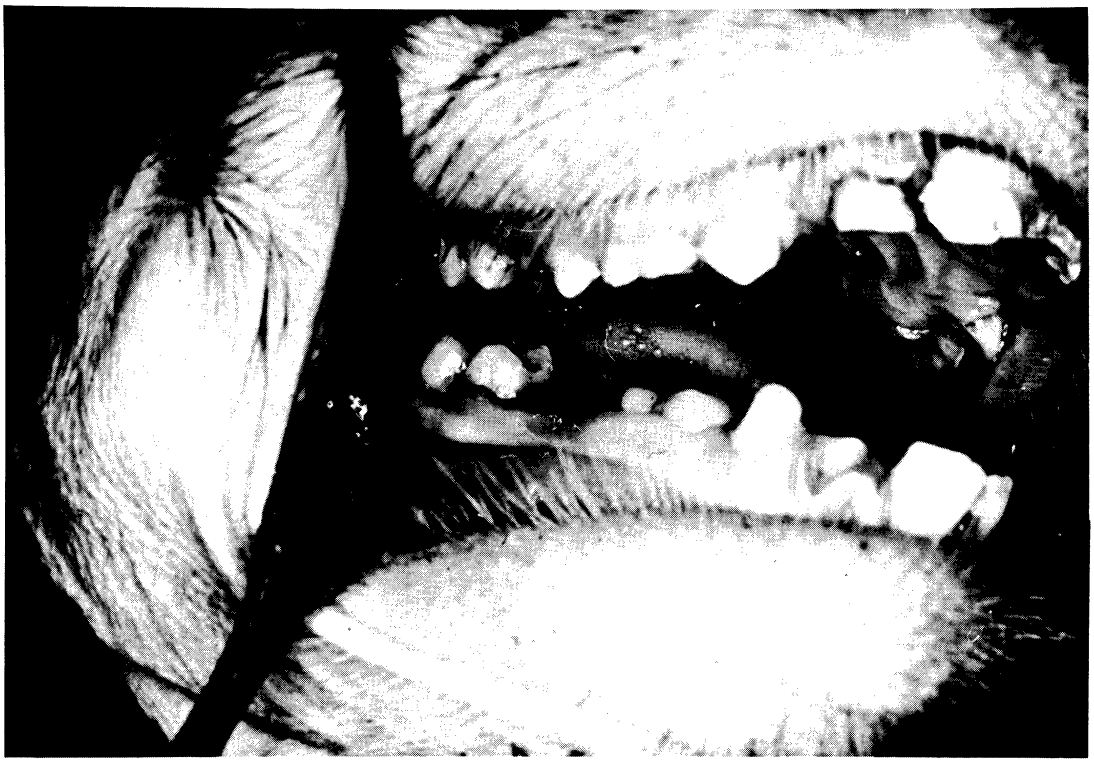

Figure 1. Extraction of second primary molar prior to surgery.

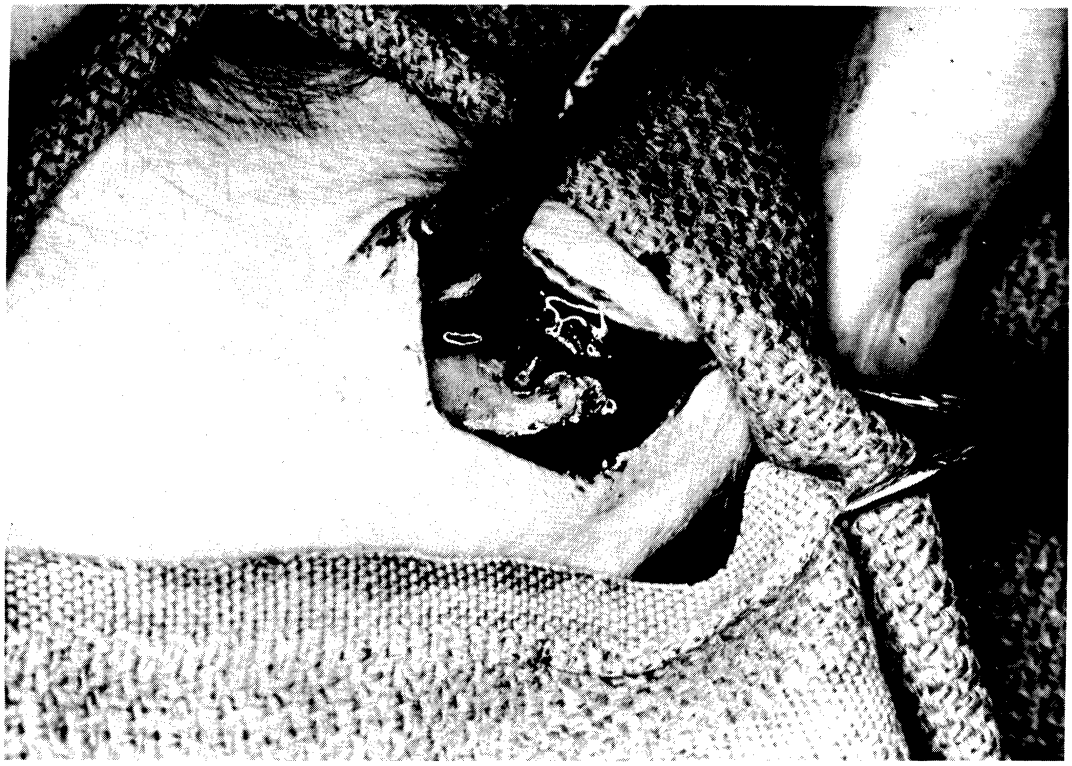

Figure 2. Exposure of the right mandibular body. The mental neurovascular bundle is visible.

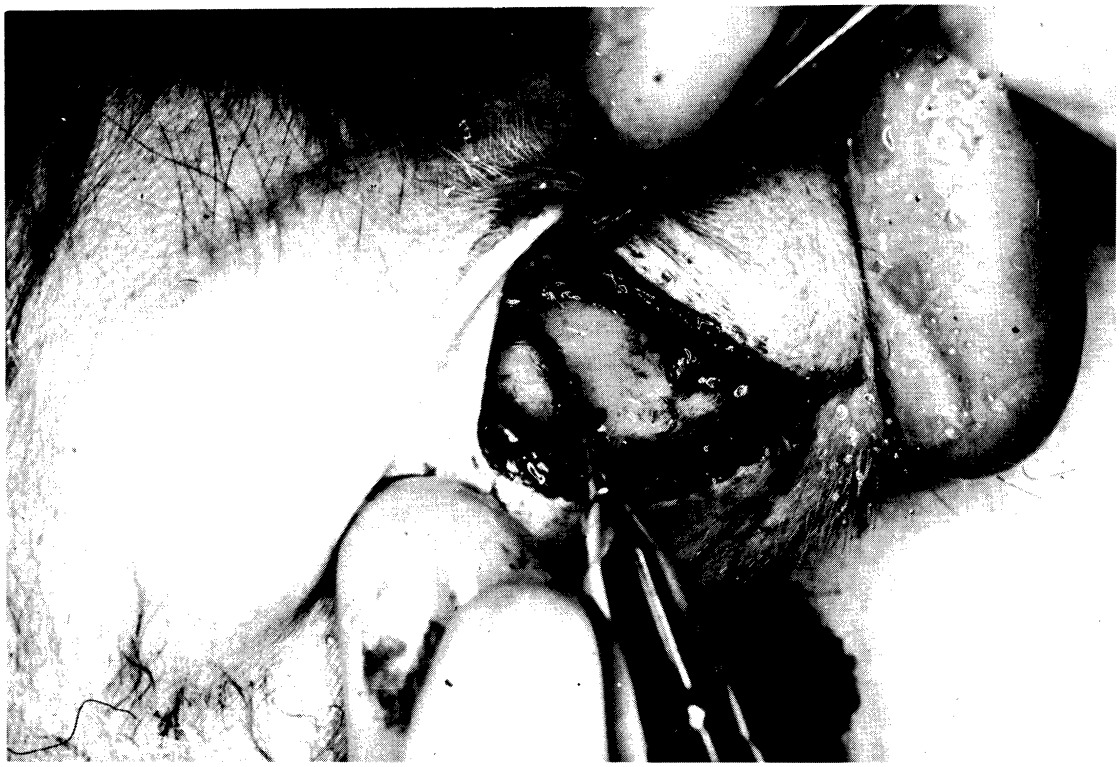

Figure 3. A cut with a No. 6 dental bur in the buccal cortex.

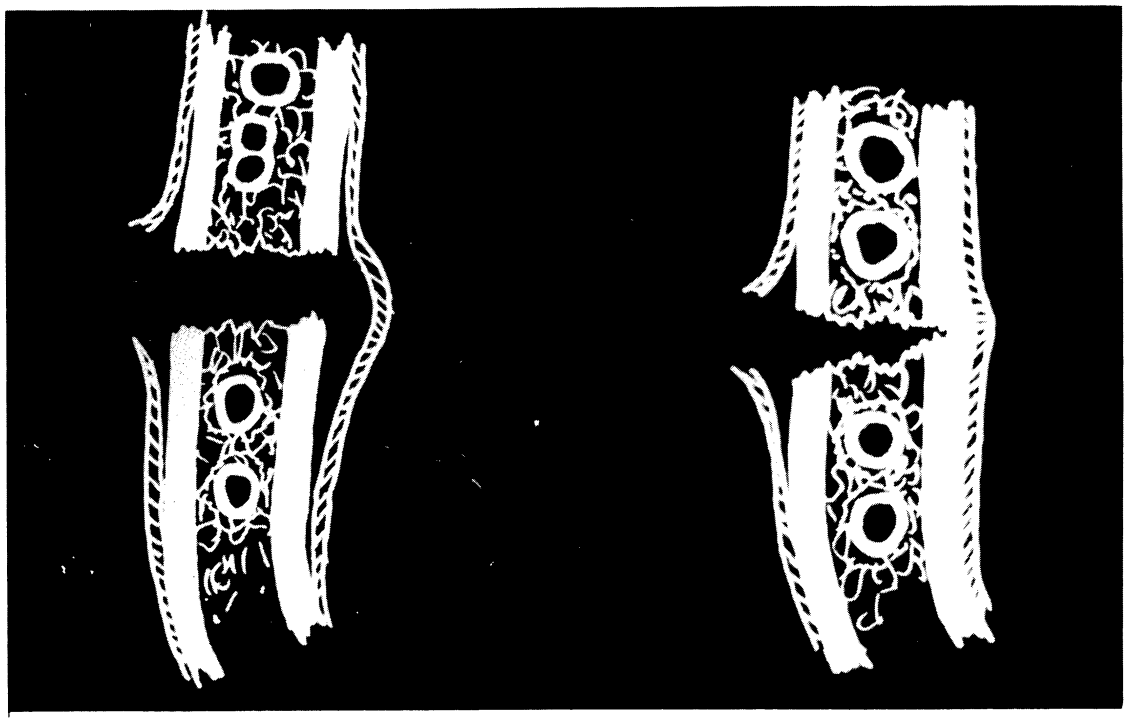

Figure 4. Comparison of Model I and Model II fracture techniques. Model I at the left shows complete penetration of both cortices. In the Model II fracture on the right, fracture of the lingual cortex would be produced by the twisting of an elevator.

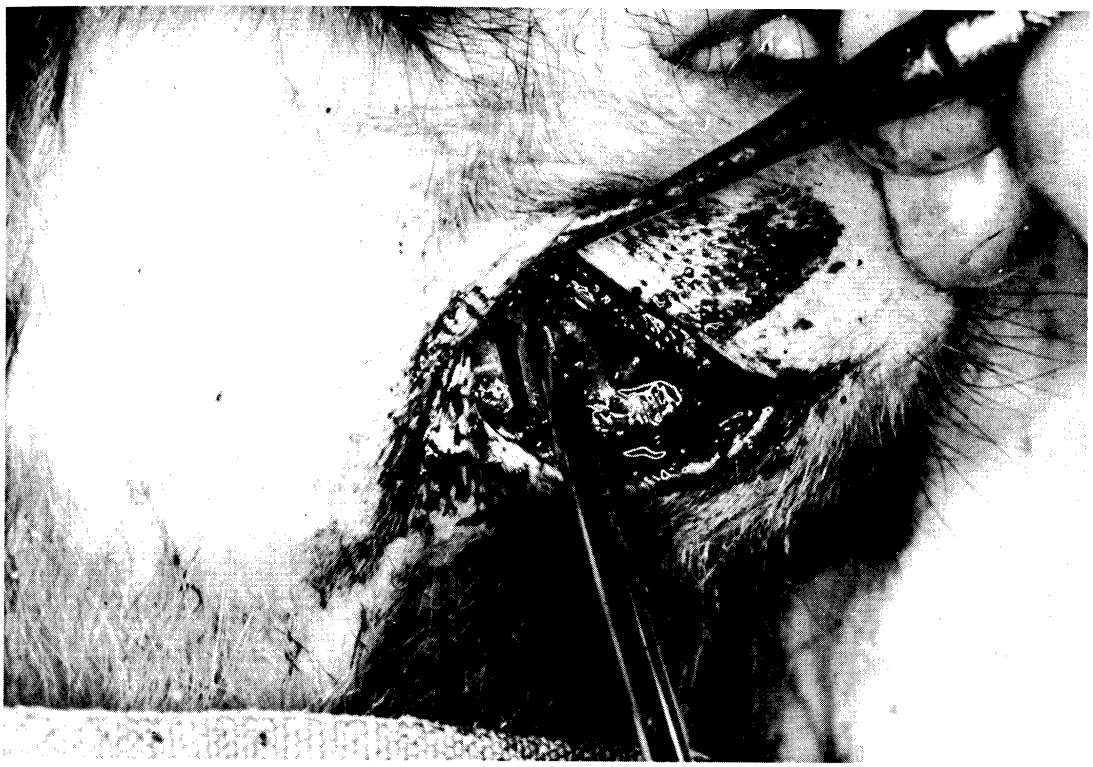

Figure 5. Completed mandibular fracture.

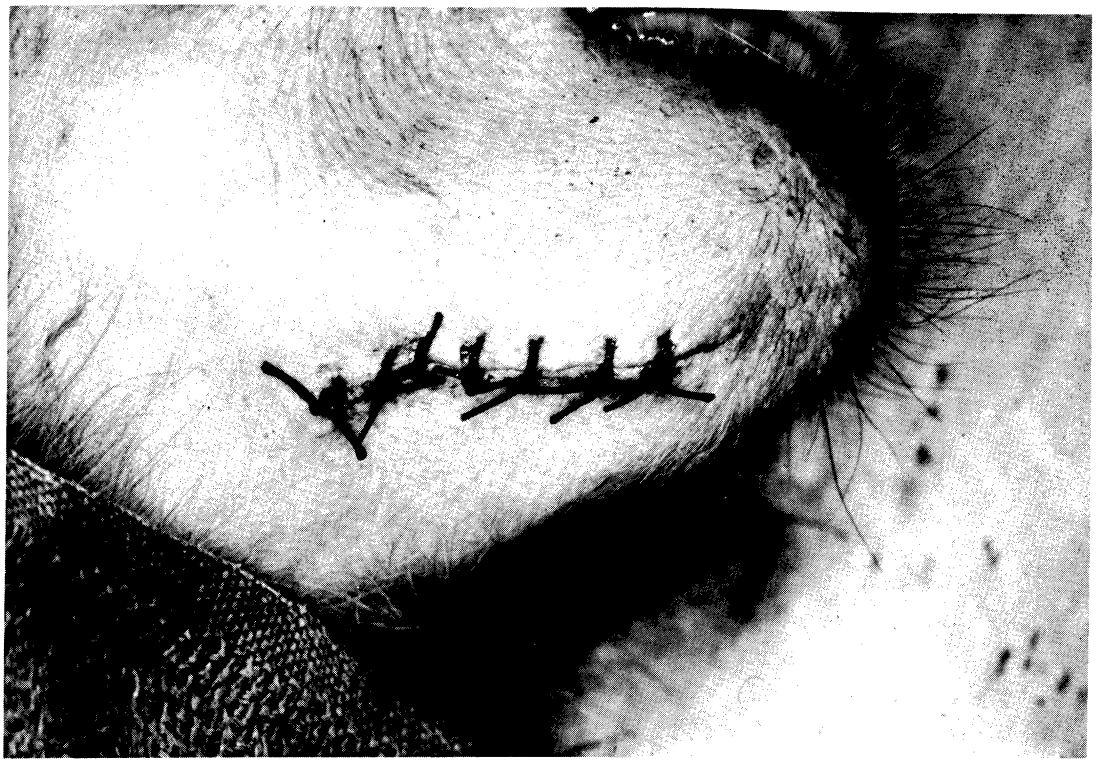

Figure 6. Sutures placed at the completion of the first stage of the Model II operation.

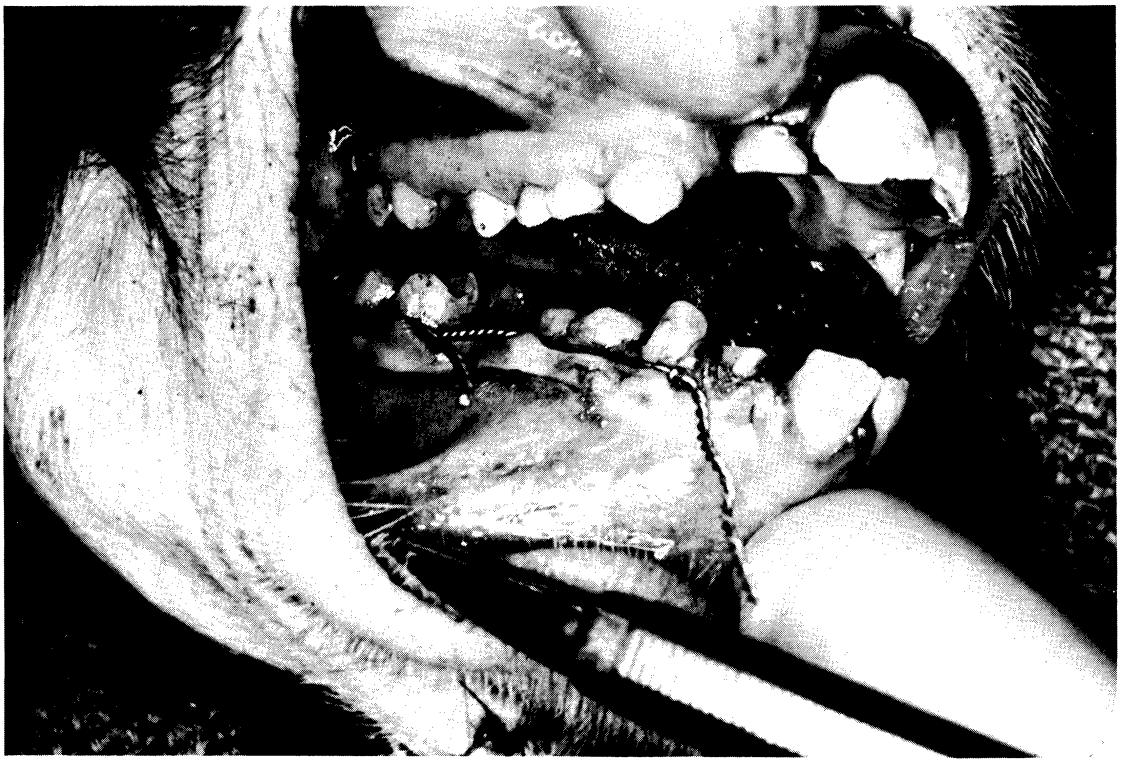

Figure 7. The 0.020 stainless steel wire applied to teeth adjacent to the fracture.

Figure 8. Completion of interdental Risdon wiring.


Figure 9. Materials used in the modified Risdon appliance.

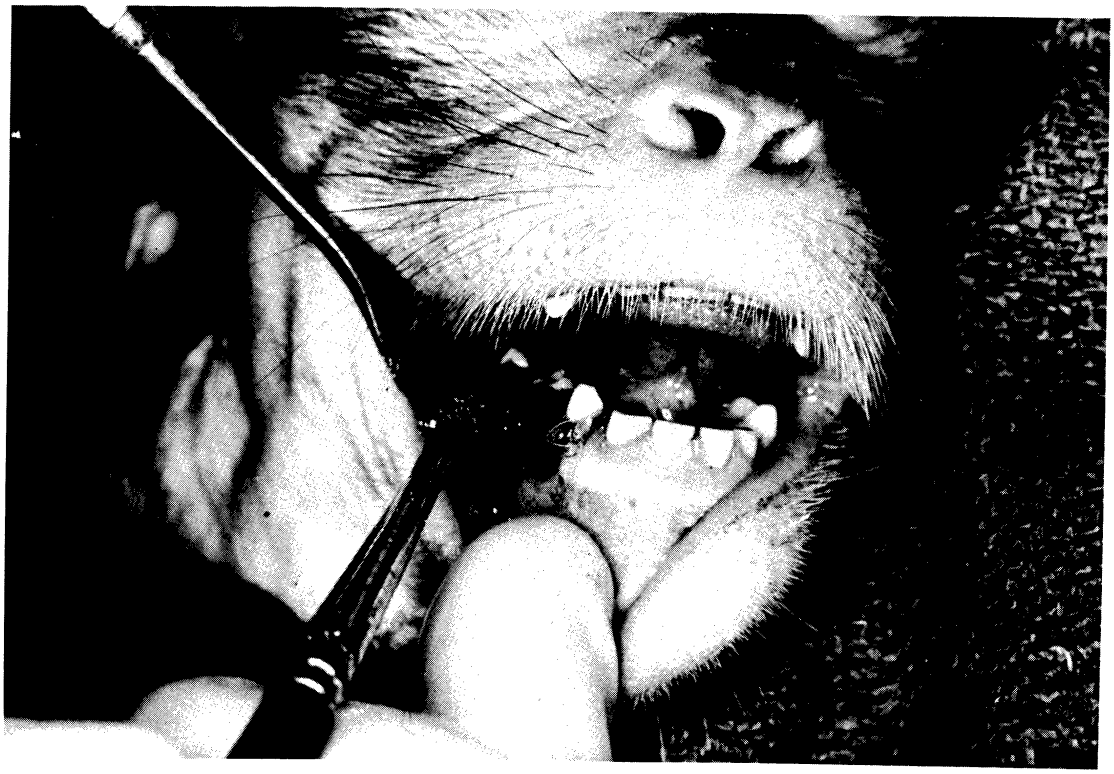

Figure 10. Incorporation of the self-curing acrylic in the Risdon wire.

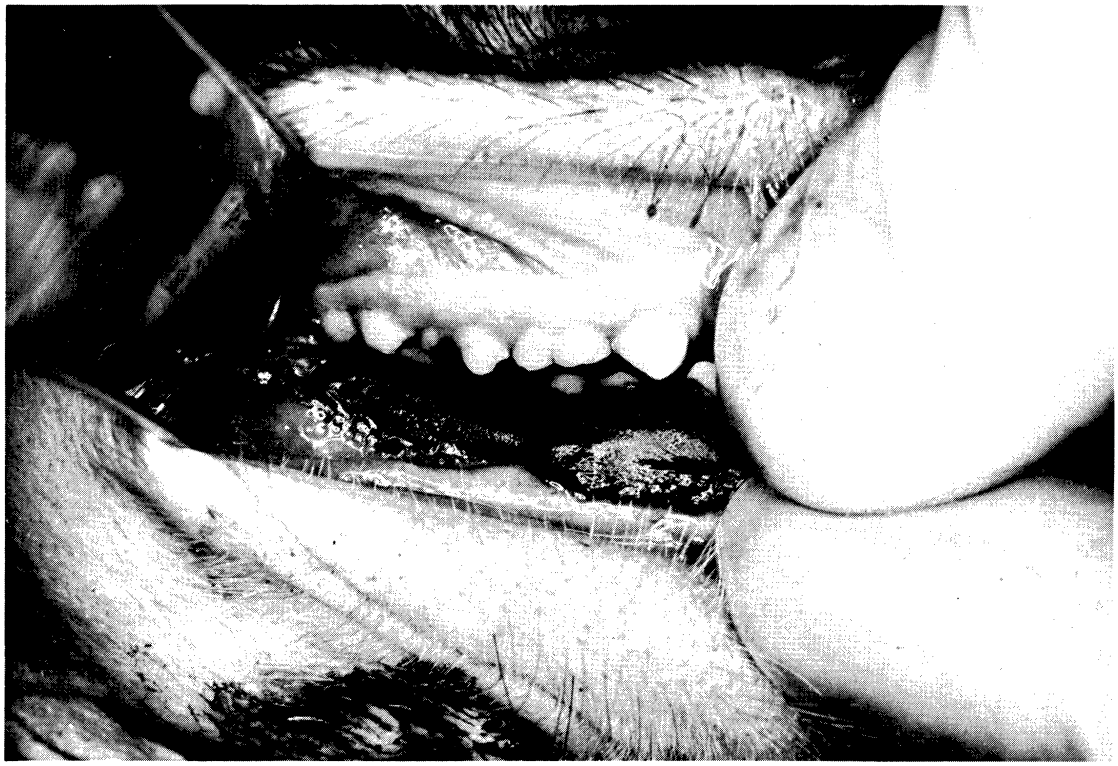

Figure 11. Completed and modified Risdon appliance.

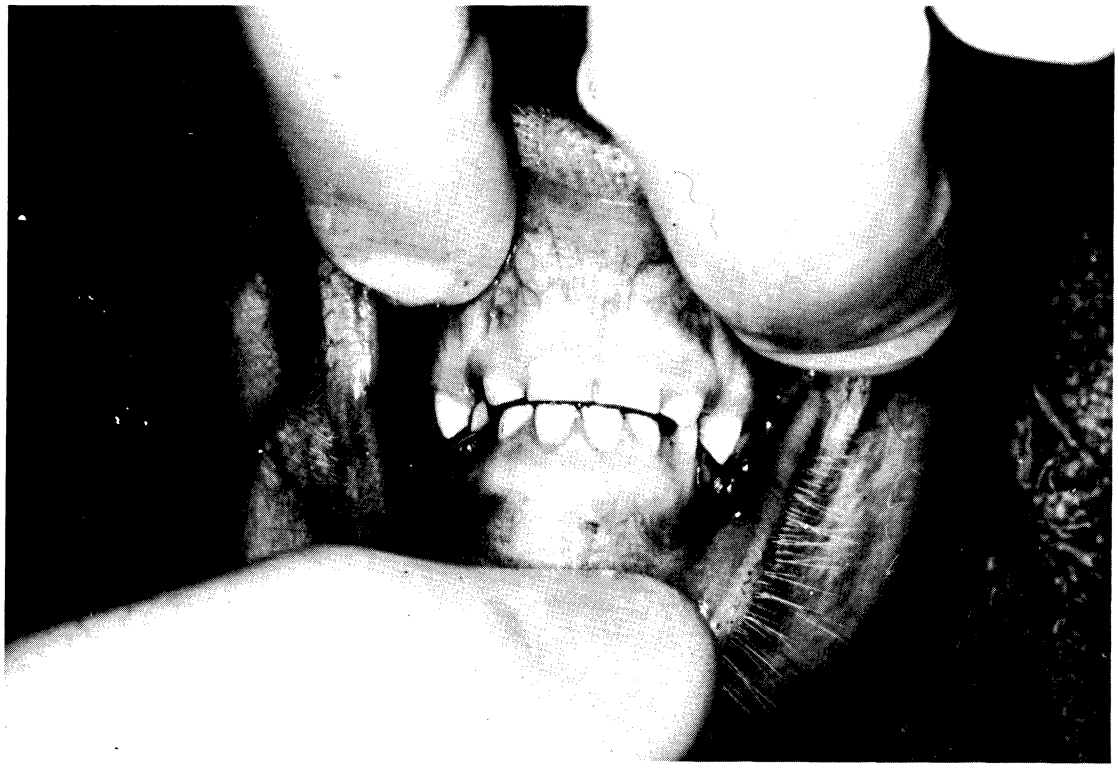

Figure 12. Check for fracture alignment and clearance of the Risdon appliance.

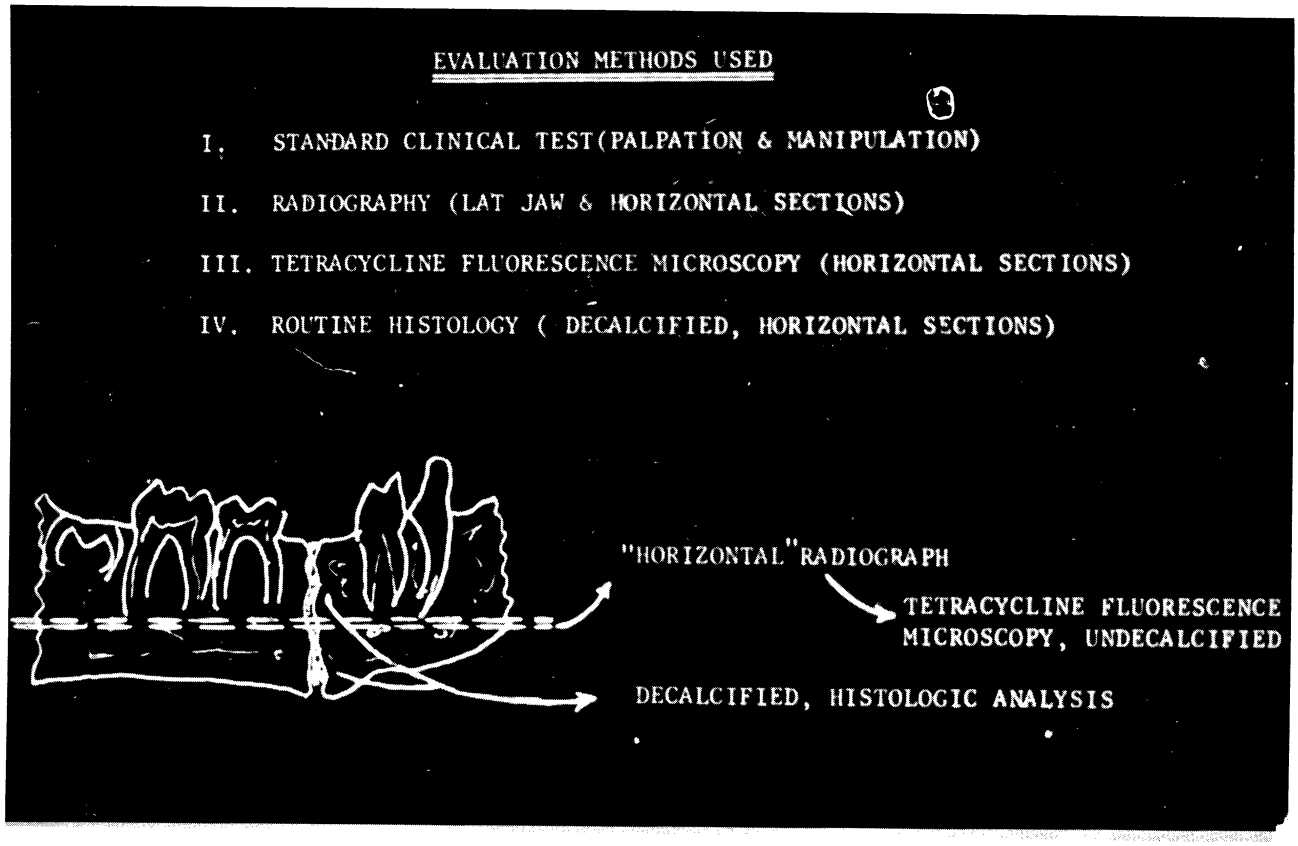


Figure 13. Evaluation methods used.

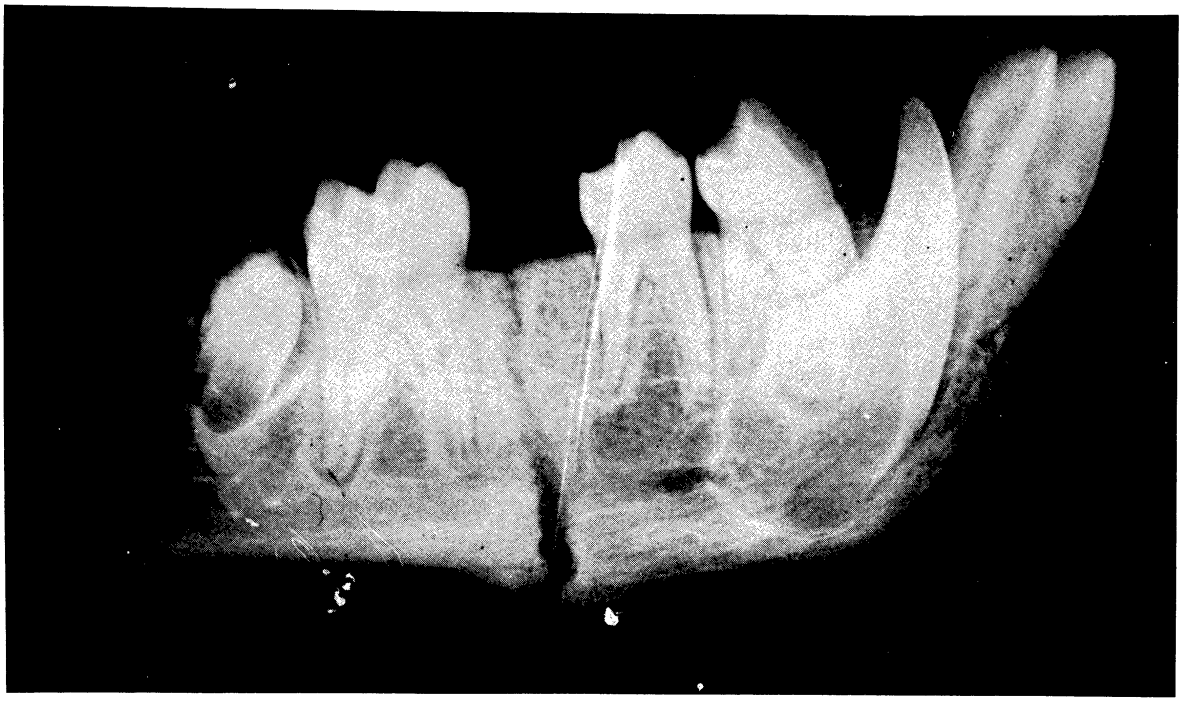

Figure 14. Radiograph of a fracture union on a young adult monkey. Note slight bridging of fracture line and absence of resorptive process.

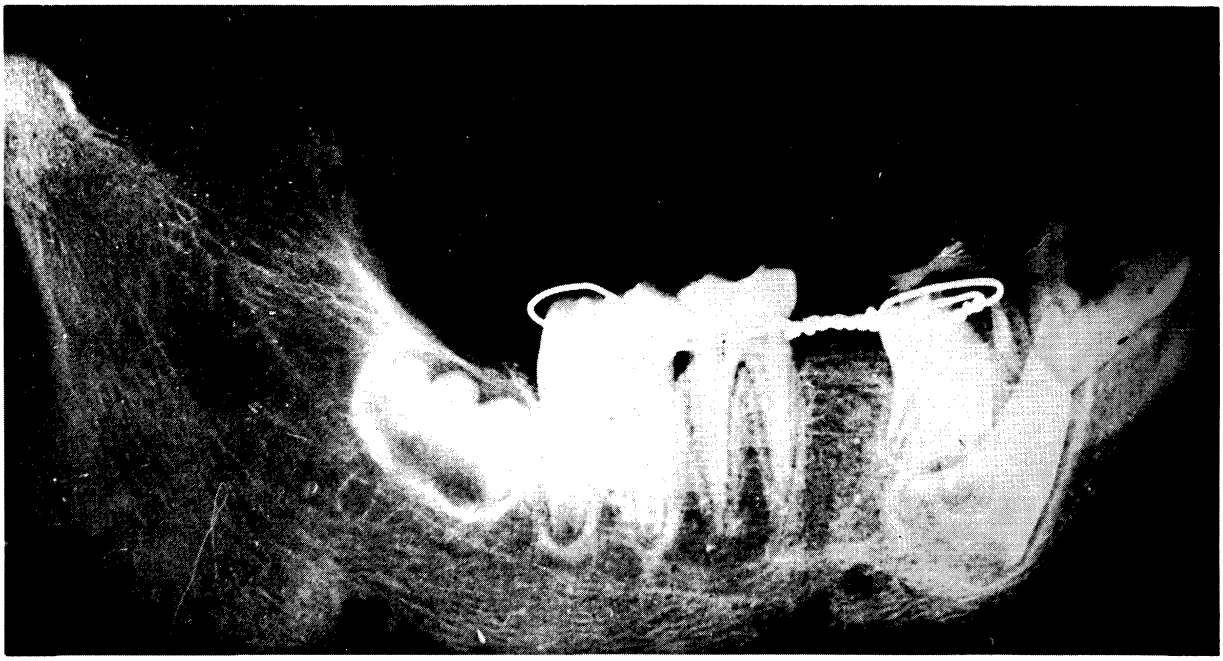

Figure 15. Union observed in adolescent monkey. Note complete obliteration of fracture site with calcified material.

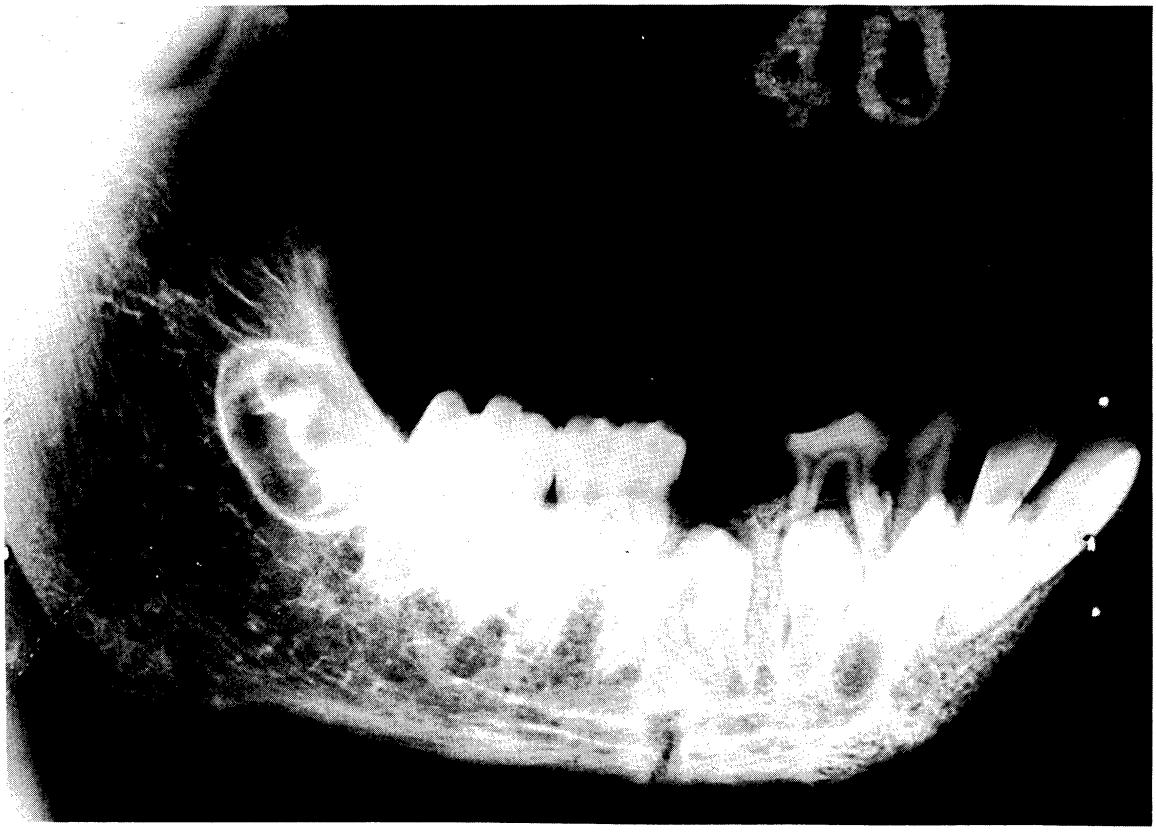

Figure 16. Complete union in adolescent monkey. Note bur cut through the tooth bud.

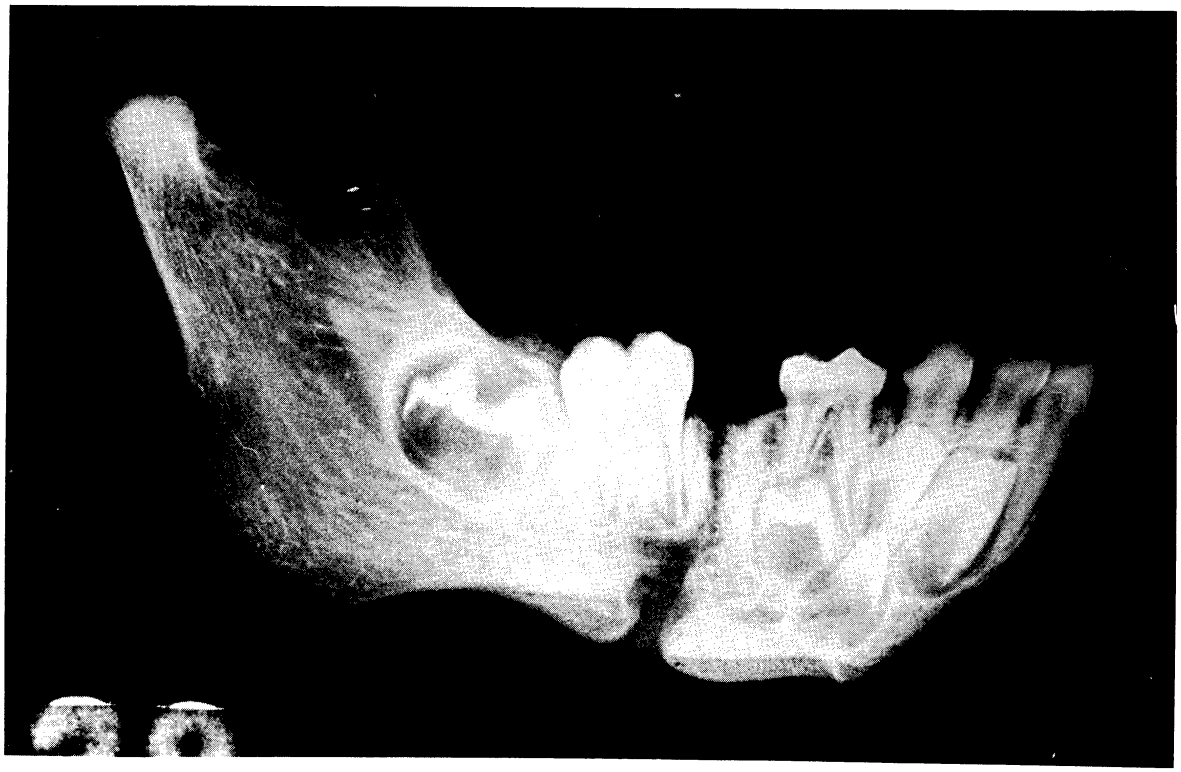

Figure 17. Nonunion demonstrating bony resorption in fracture site.

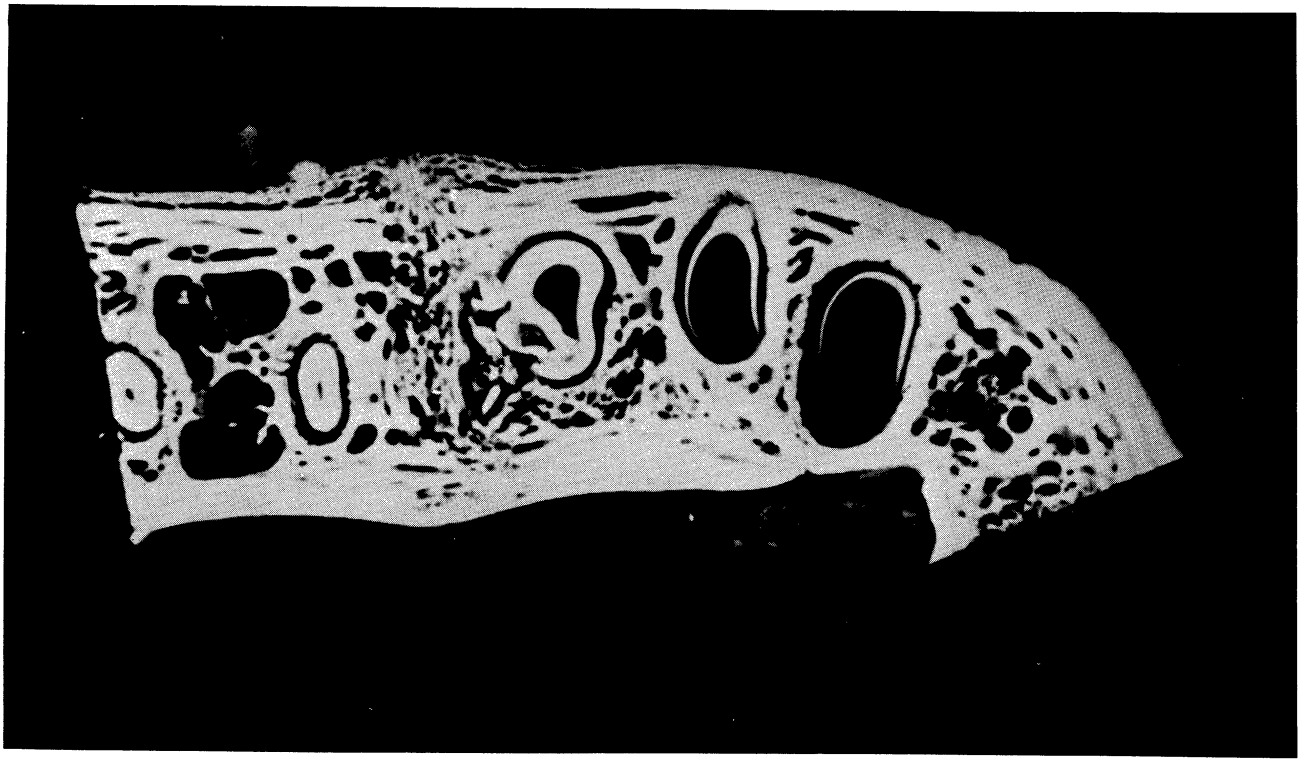

Figure 18. Rigid bony union demonstrated by horizontal section radiograph.

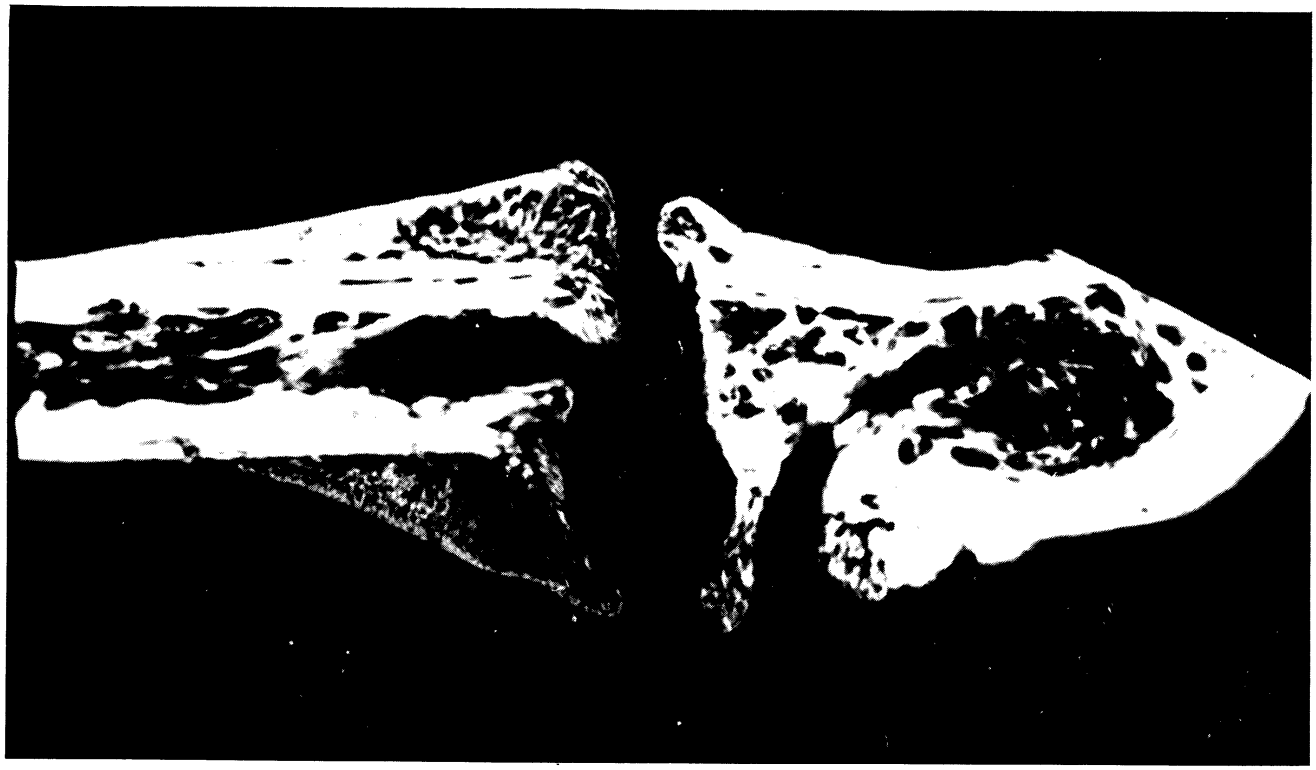

Figure 19. Horizontal section radiograph demonstrating obvious nonunion.

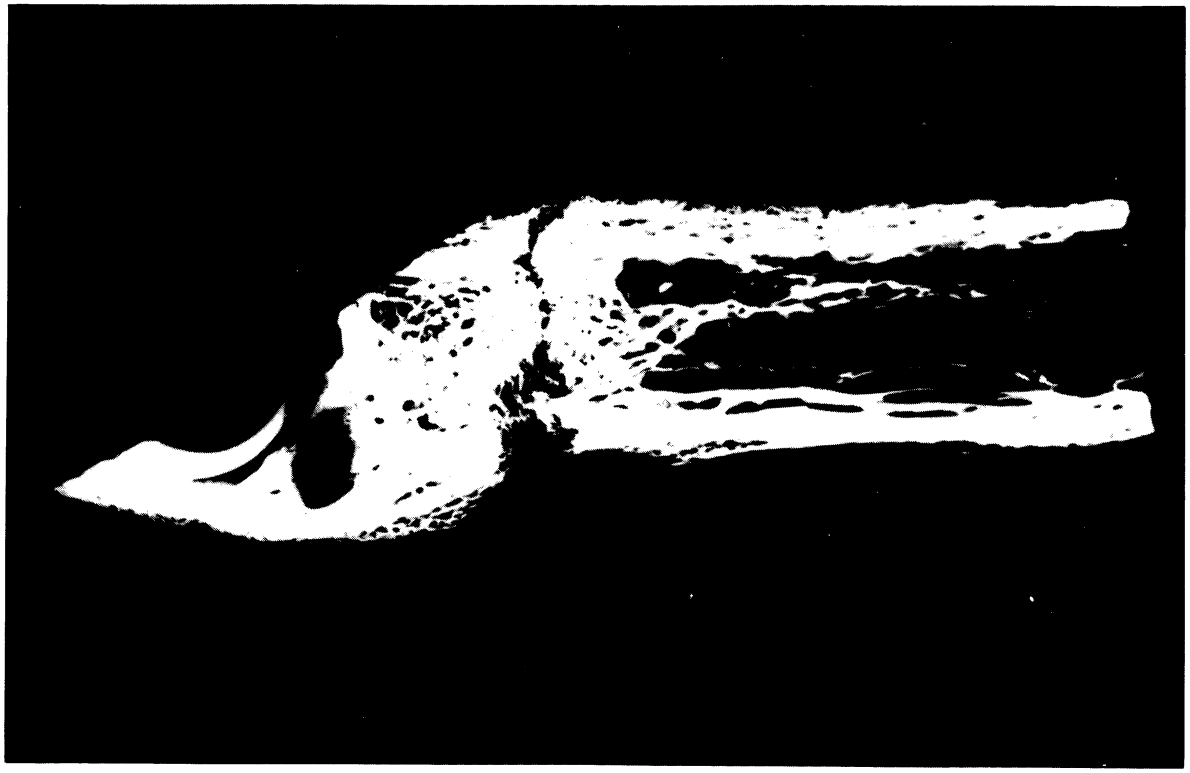

Figure 20. Union in young adult monkey.

Figure 21. Union in adolescent monkey. Note obvious subperiosteal callus with heavy calcified bond.

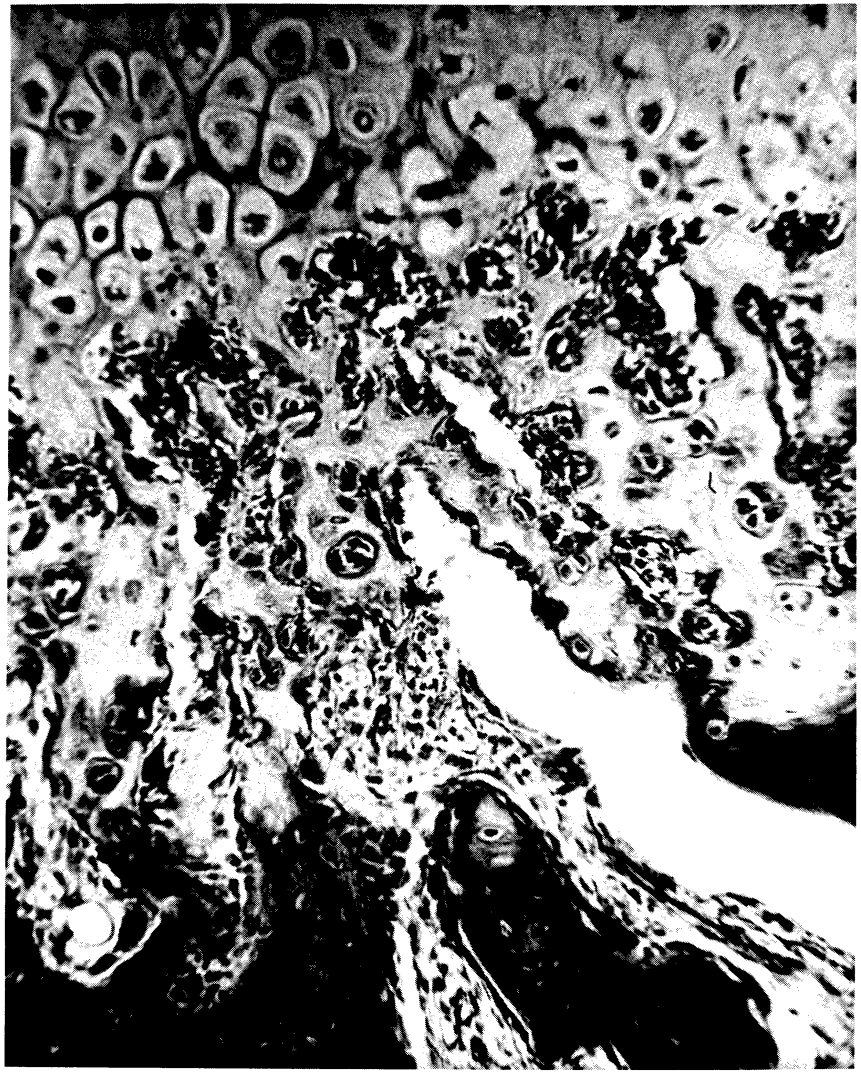

Figure 22. Transition from fibrous callus through fibrocartilage, hyalinized cartilage and final formation of bone.

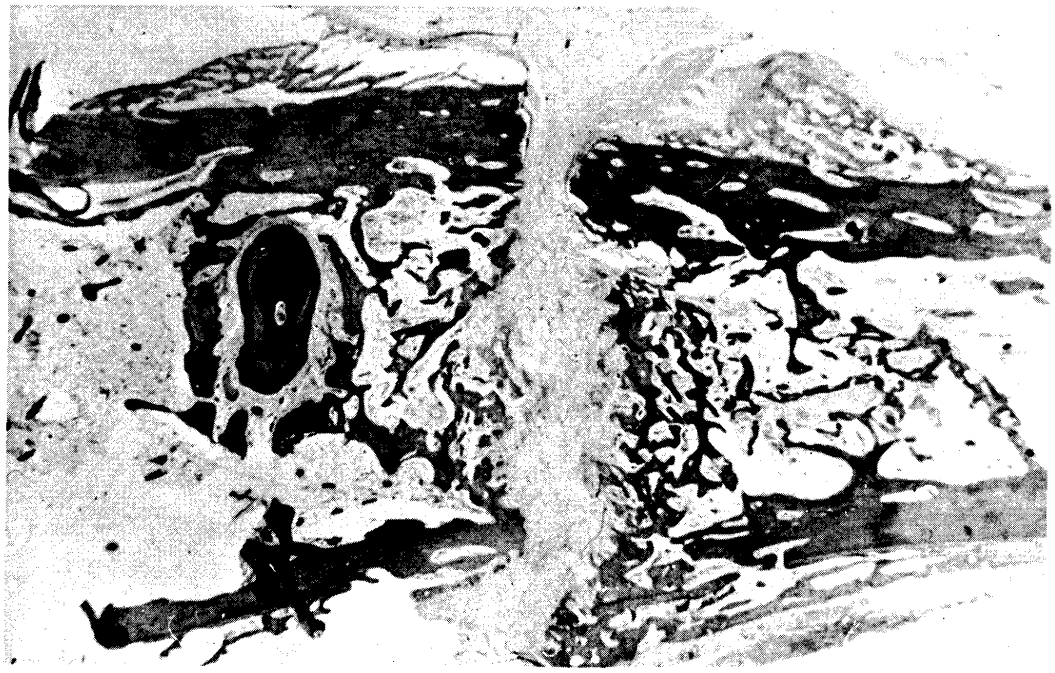

Figure 23. Histologic nonunion.

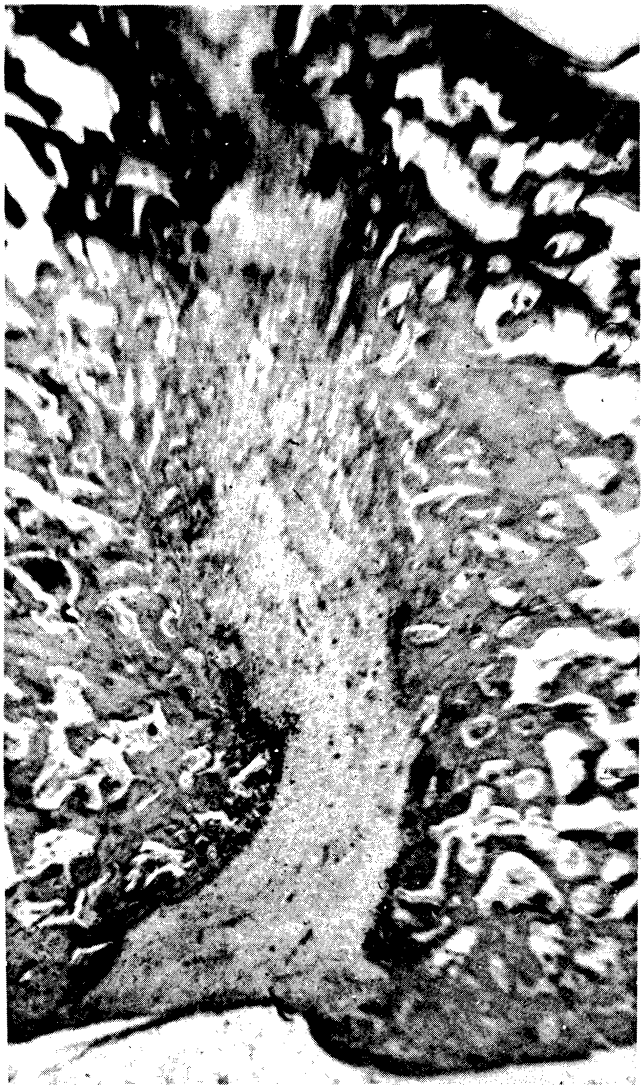

Figure 24. Histologic nonunion, medium power.

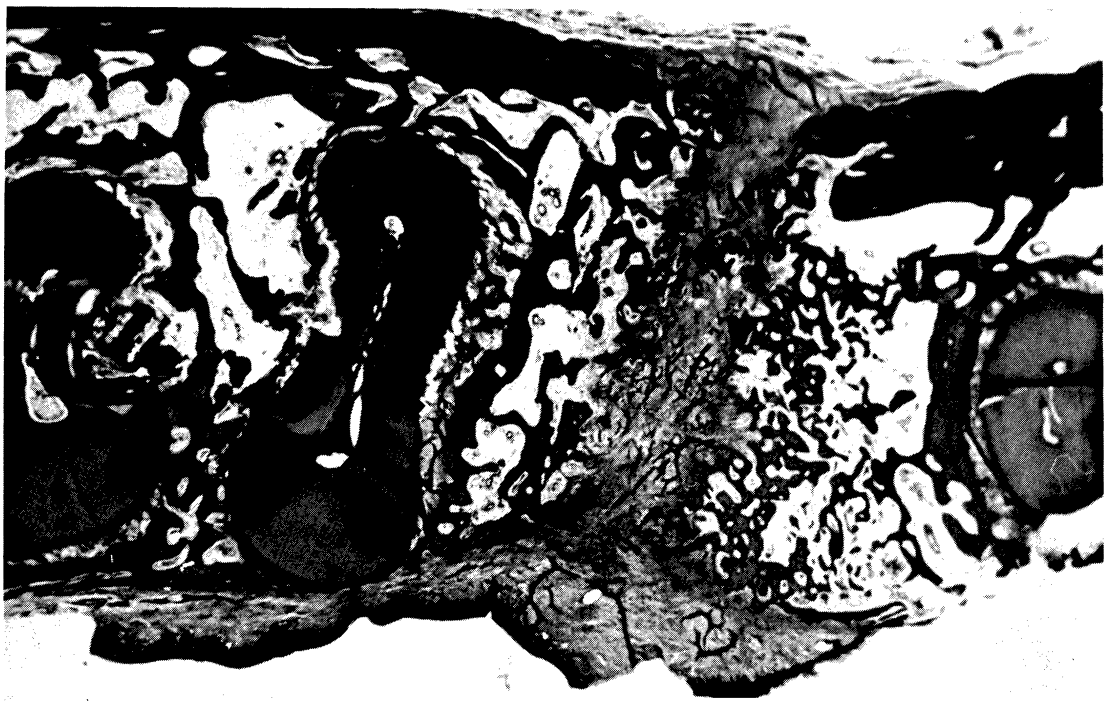

Figure 25. Semi-rigid union observed in young adult monkey. Note bridge of osteoid tissue.


Figure 26. Application of isobutyl cyanoacrylate adhesive to the Model II mandibular fracture.

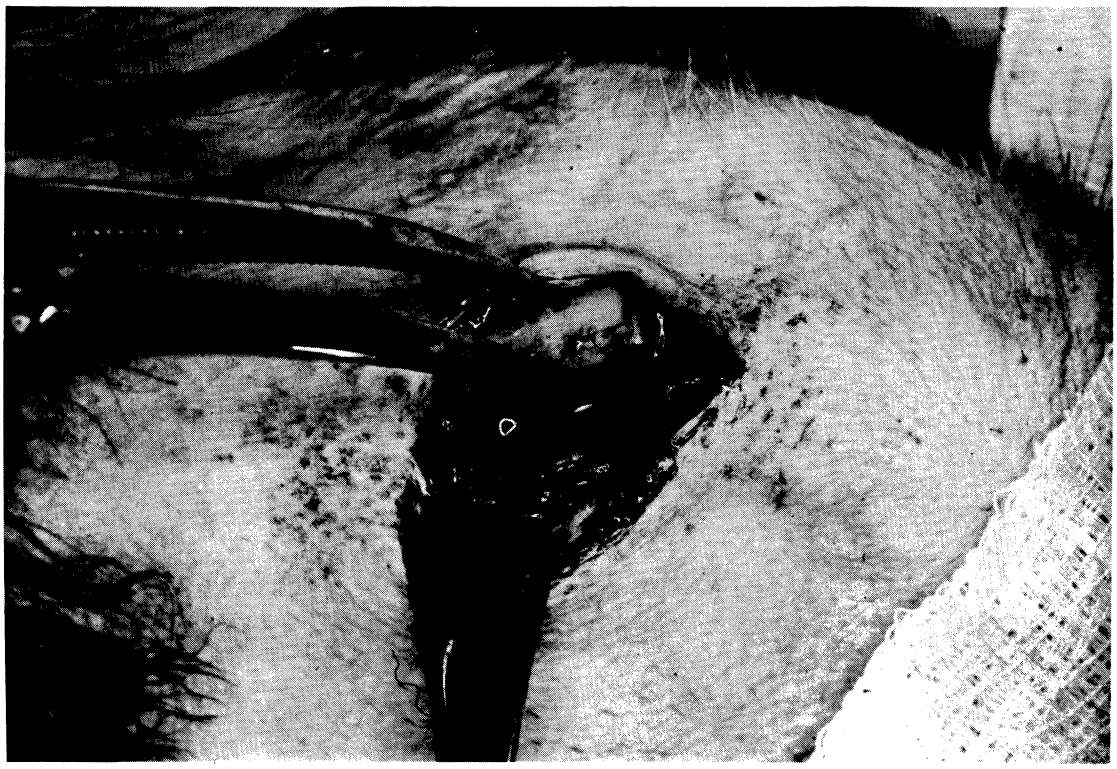

Figure 27. Placement of a tibia autogenous bone graft to the freshly prepared site at the base of the left mandible.

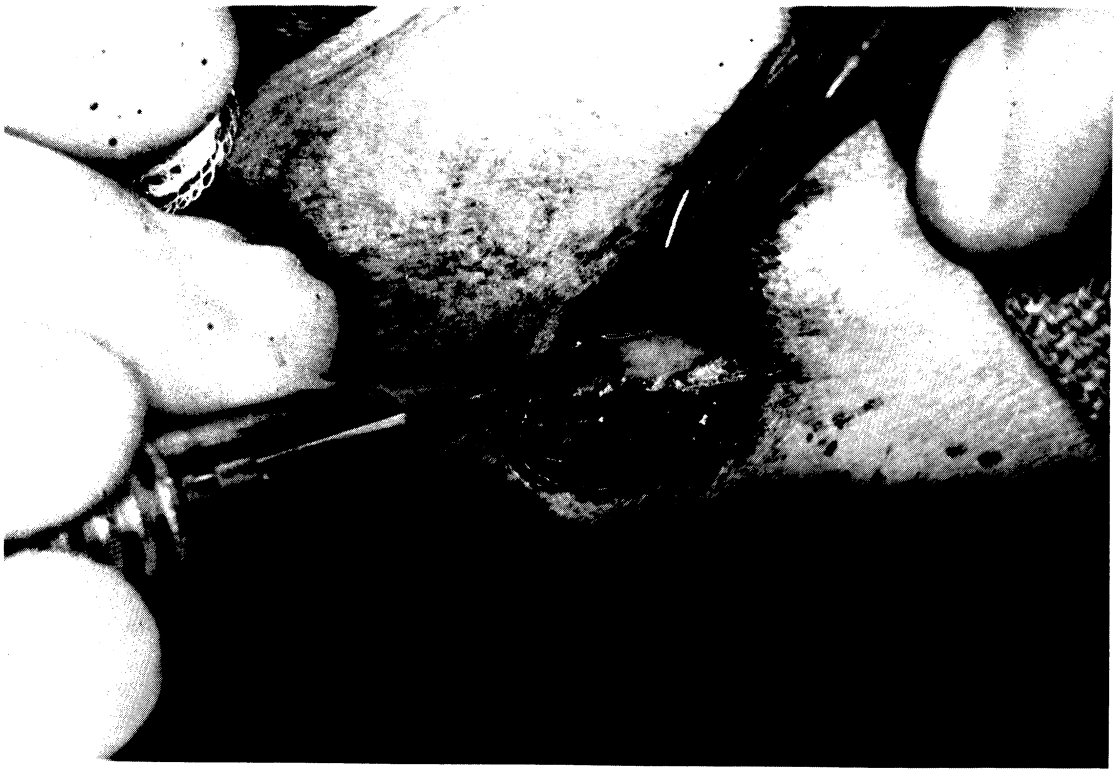

Figure 28. Drilling of cortical holes in the right tibia.

Figure 29. Right tibia, the proximal hole (right) prepared to receive the adhesive, the distal to act as a control.

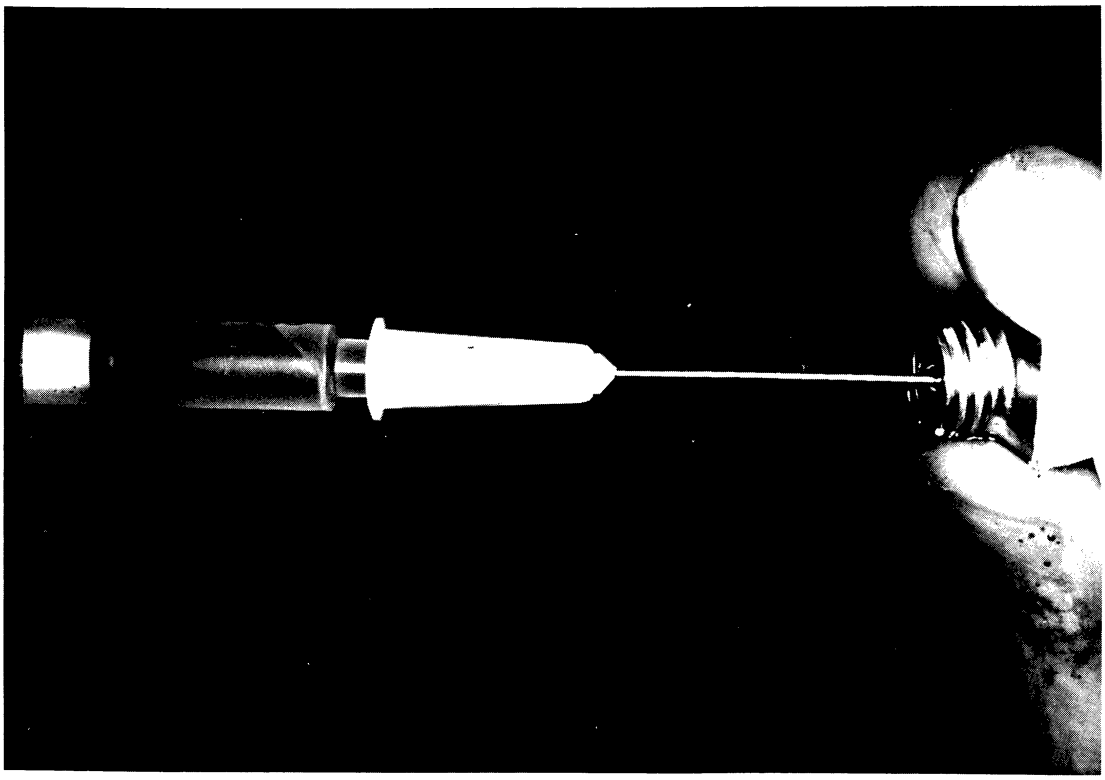

Figure 30. Method used to place adhesive in 2 cc syringe.

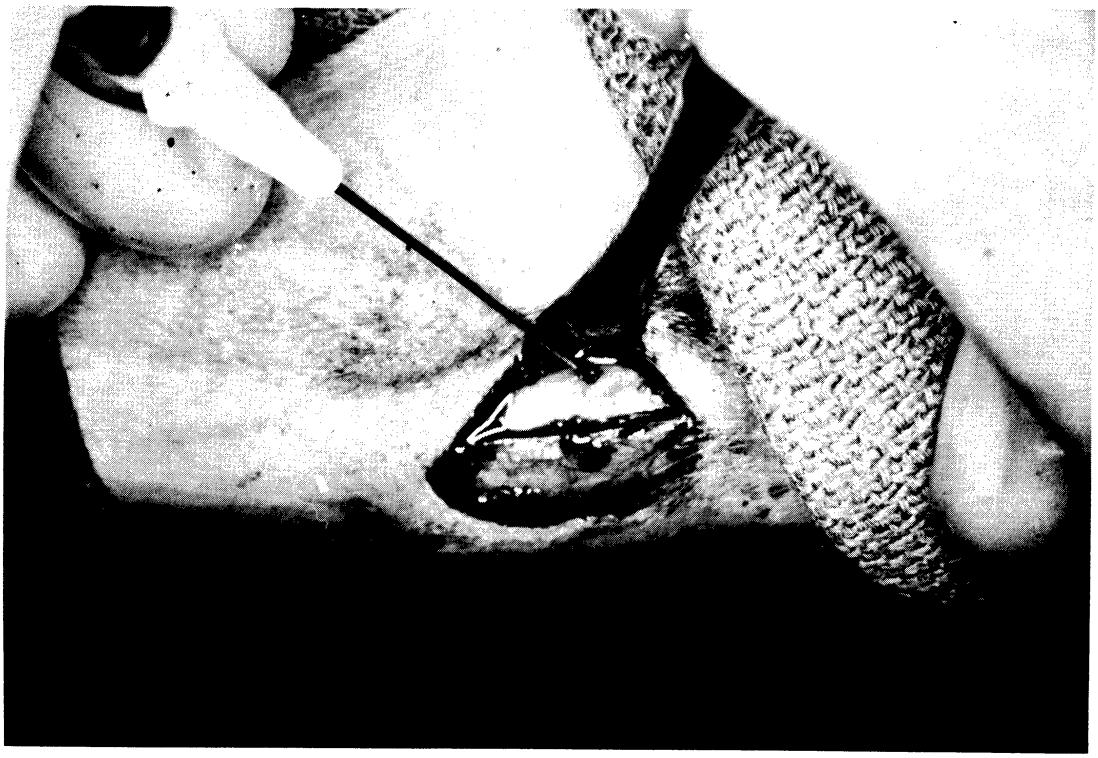

Figure 31. Injection of 0.3 cc of adhesive into medullary space.

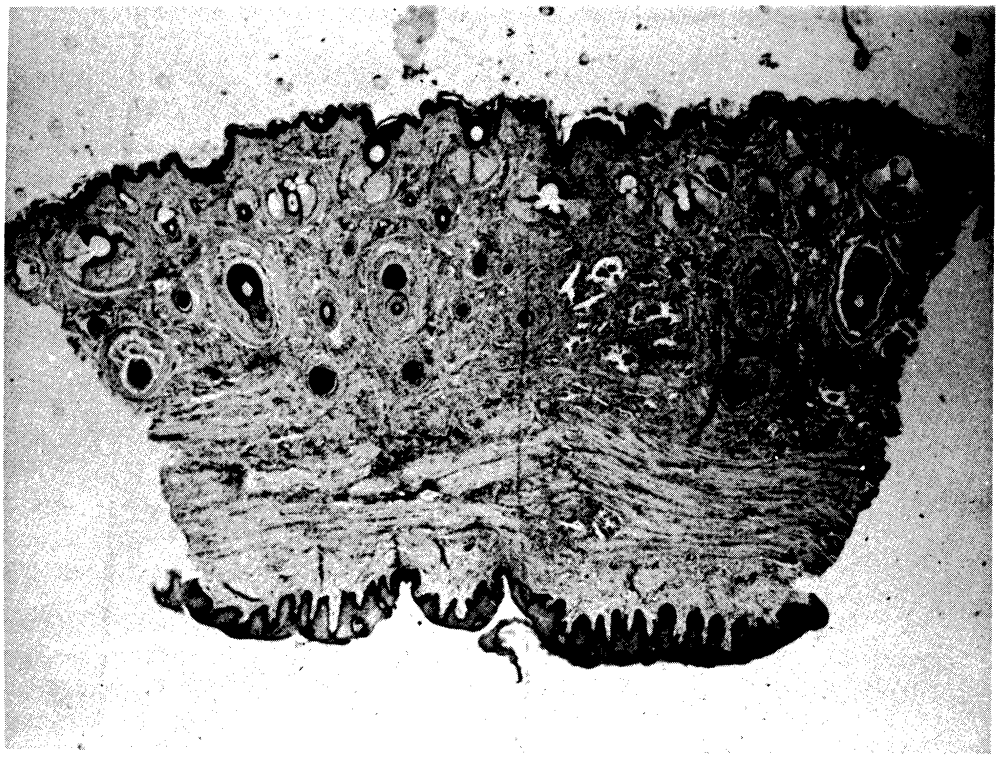

Figure 32. Midline of upper lip six weeks following application of adhesive. Note minimal foreign body response in muscular layer.

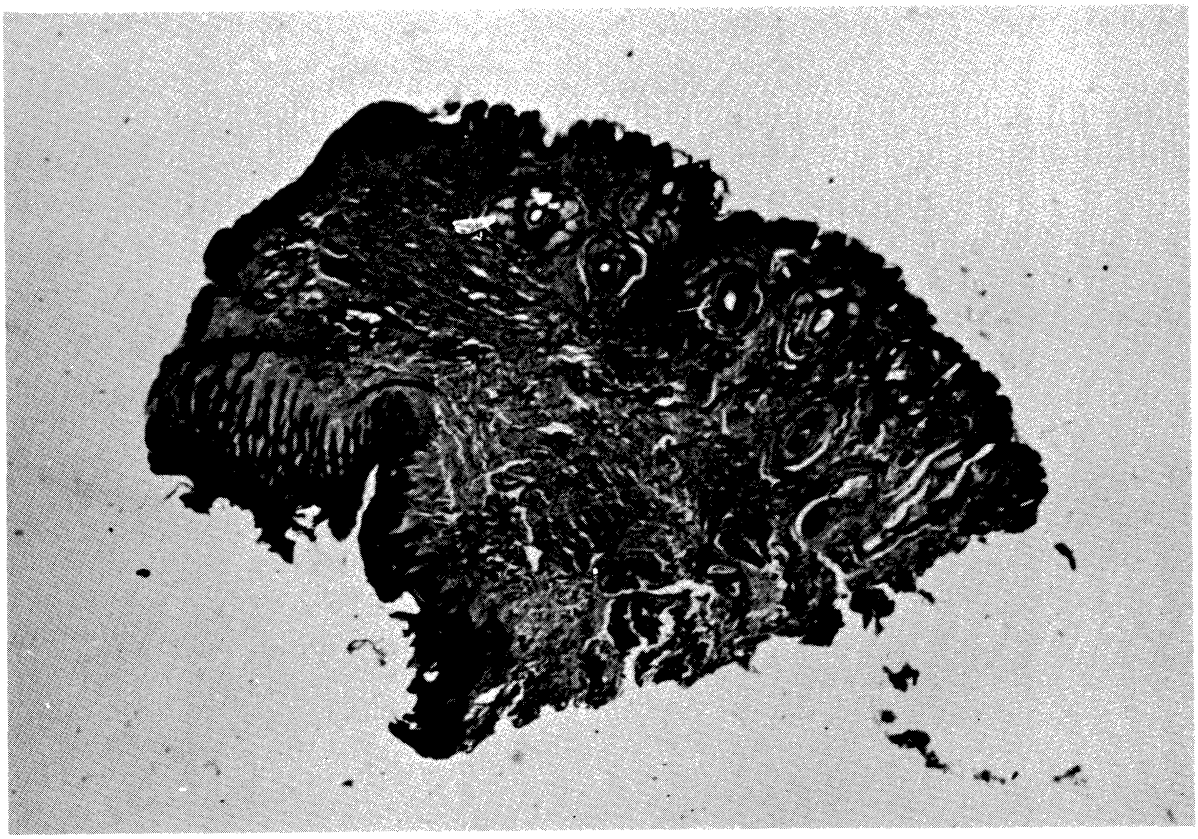

Figure 33. Midline of upper lip twelve weeks following application of adhesive.

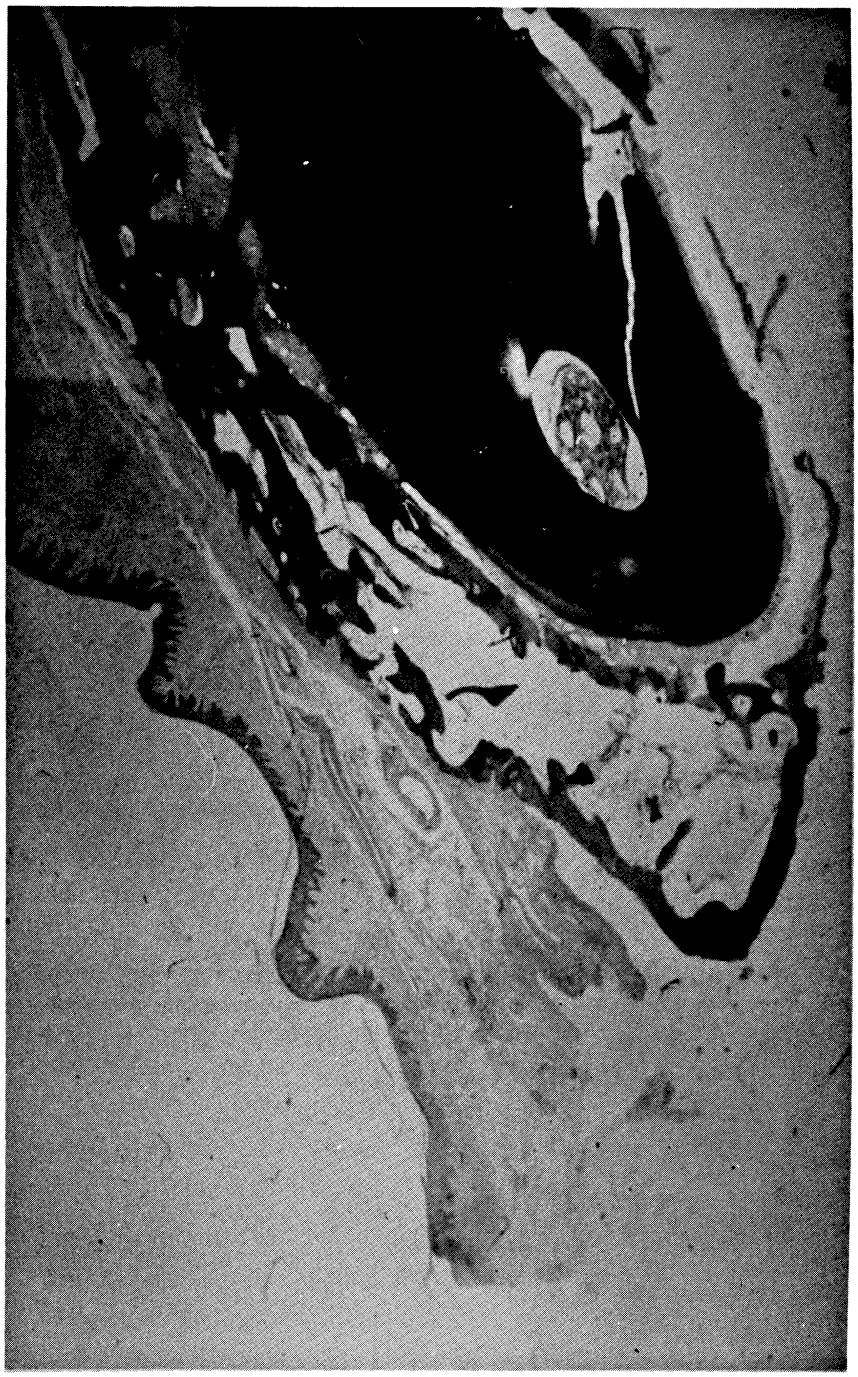

Figure 34. Saggital section of palate six weeks after application of adhesive. Note minimal foreign body response between mucosal layer and palatal cortical bone.

Figure 35. Palatal area twelve weeks after application of adhesive.

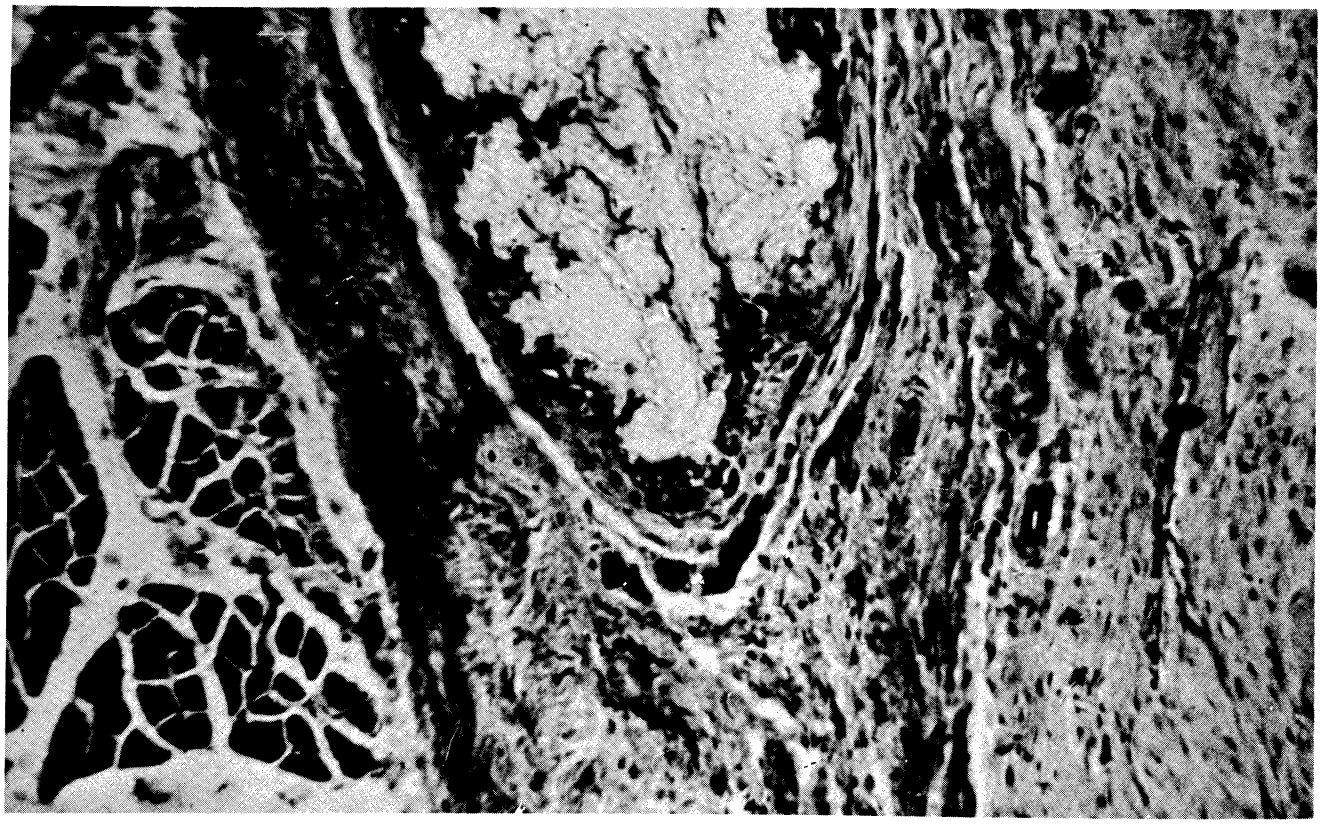

Figure 36. Medium power view of space created by adhesive crystals with surrounding foreign body cells.

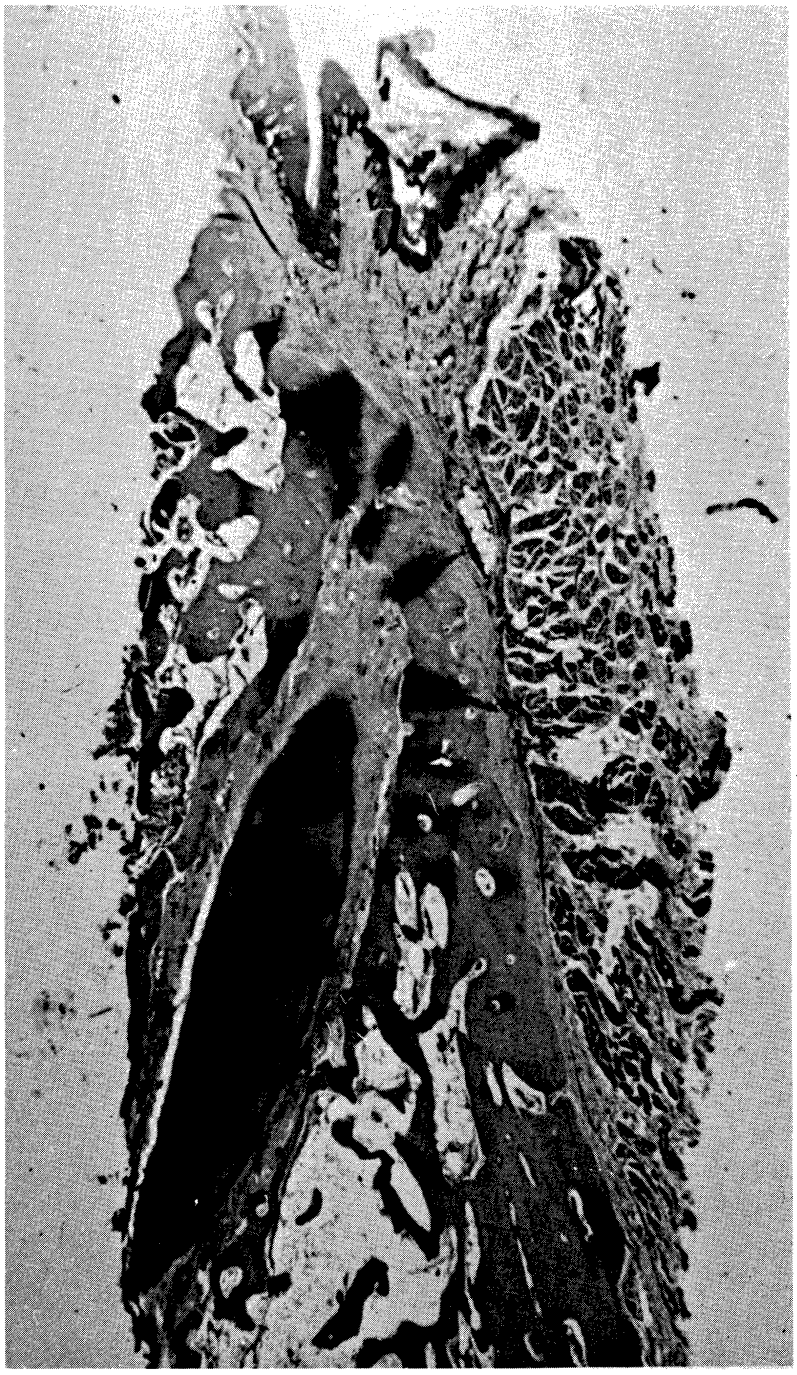

Figure 37. Mandibular flap 12 weeks after application of adhesive.

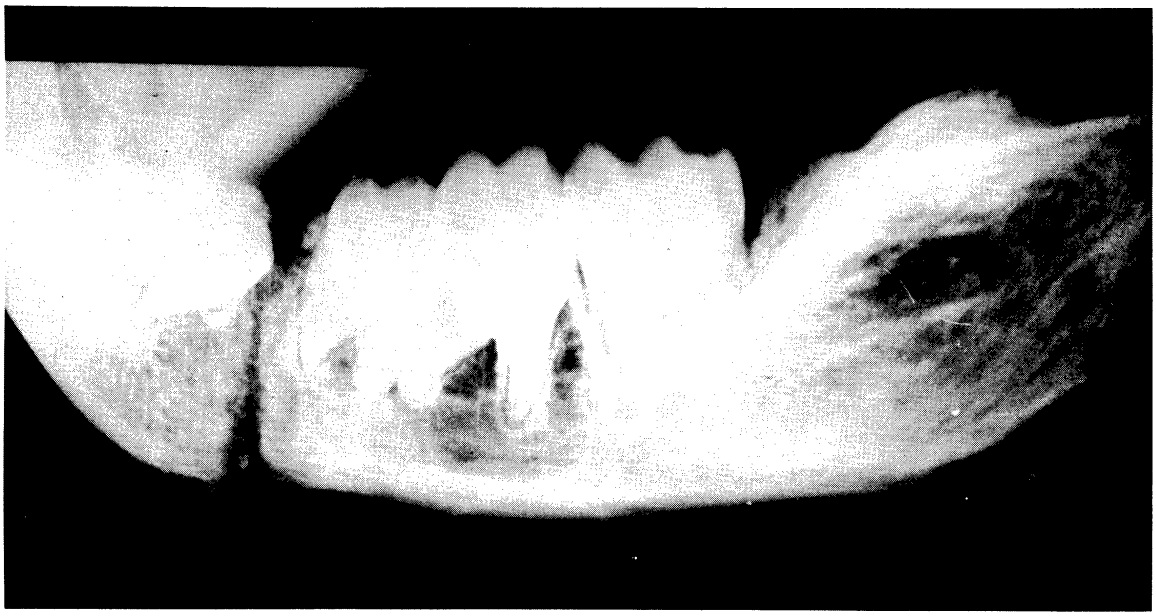

Figure 38. Radiographic evidence of nonunion six weeks after incorporation of adhesive to the fracture site.

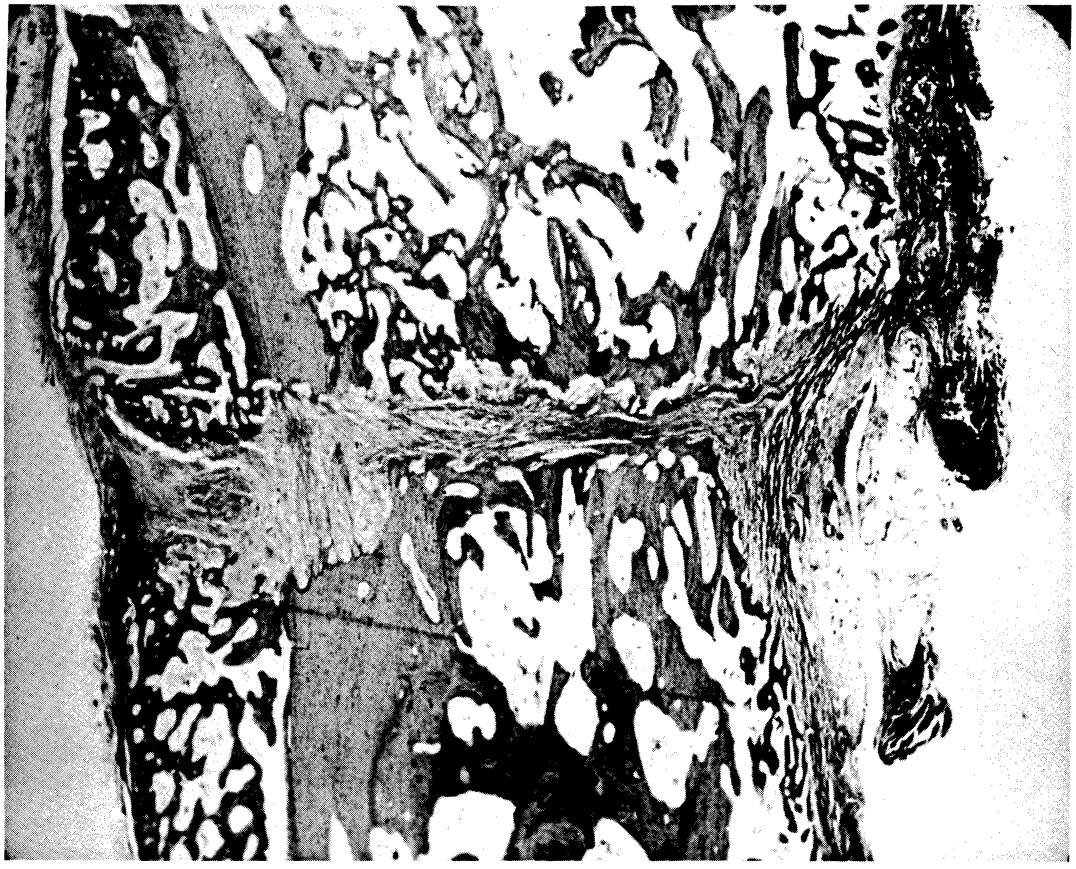

Figure 39. Histologic section of the mandibular fracture six weeks after inclusion of adhesive. Concentration of adhesive is seen at left (buccal cortex).

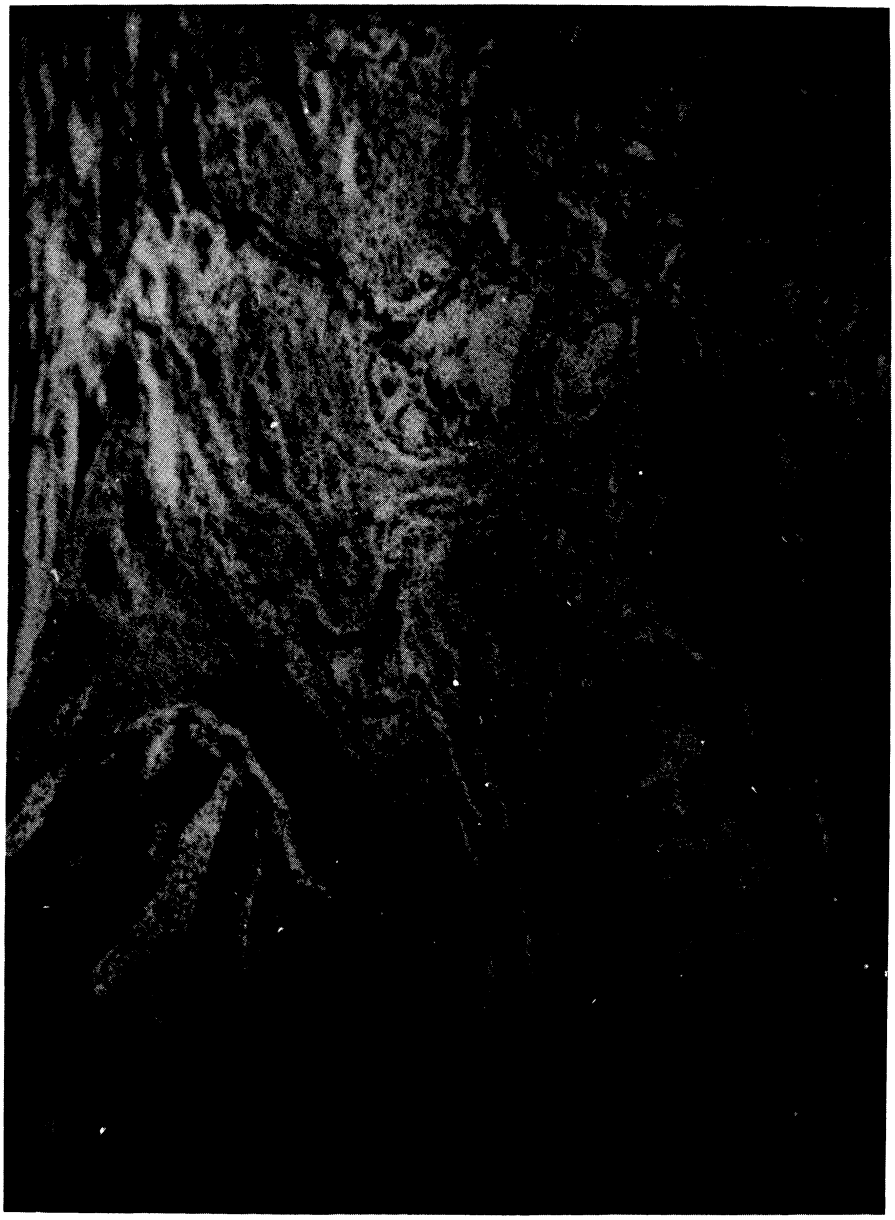

Figure 40. Medium power view of adhesive concentration at buccal cortex.

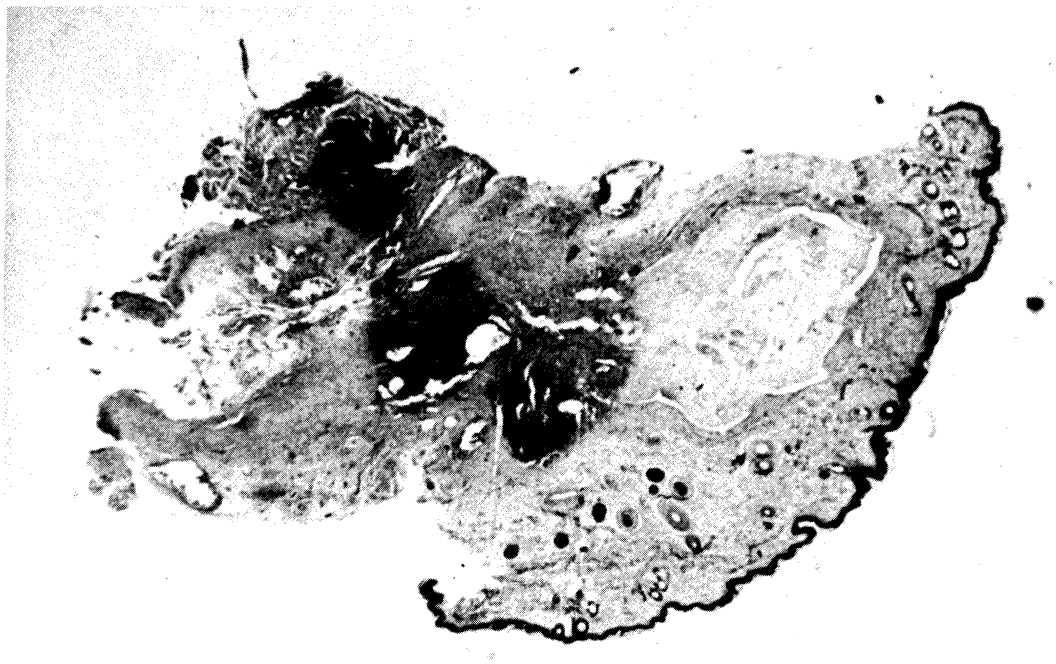

Figure 41. Subcutaneous abscess associated with mandibular fractures. Intense lymphocytic and polymorphonuclear leukocytic infiltration was noted.

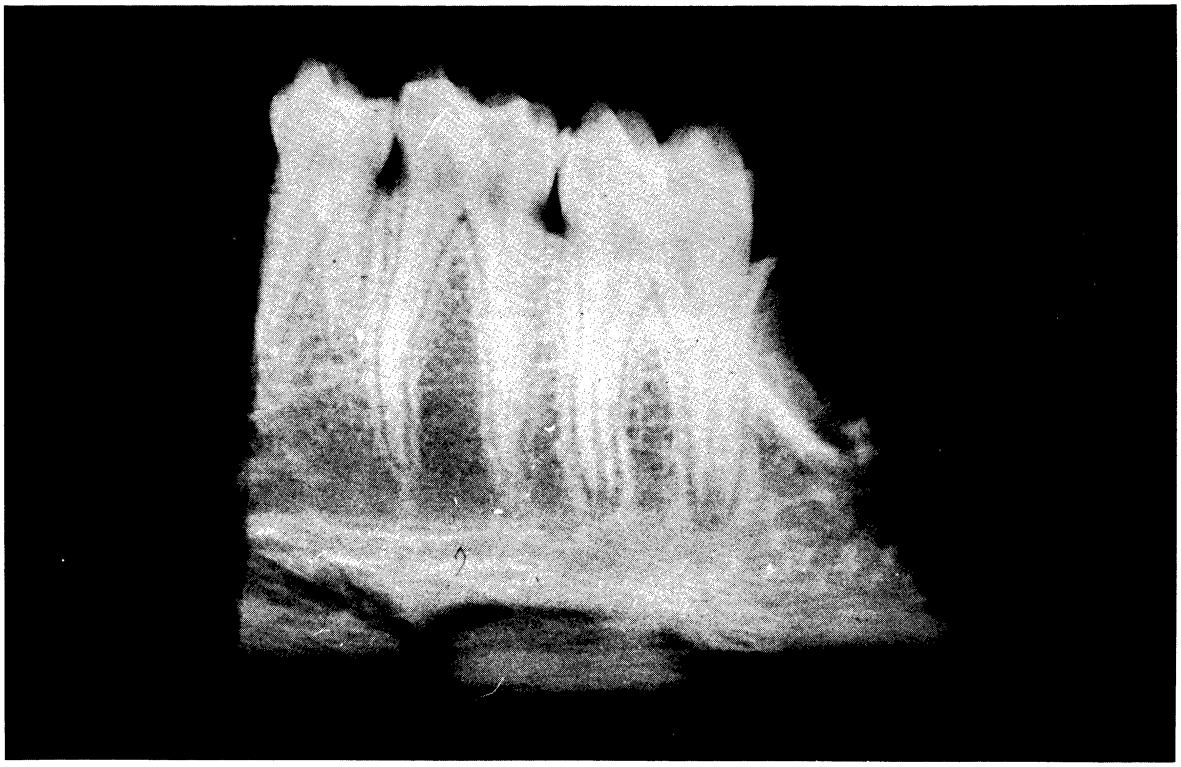

Figure 42. Radiograph taken six weeks after stabilization of mandibular bone graft with adhesive. Questionable bony bridging can be noted.

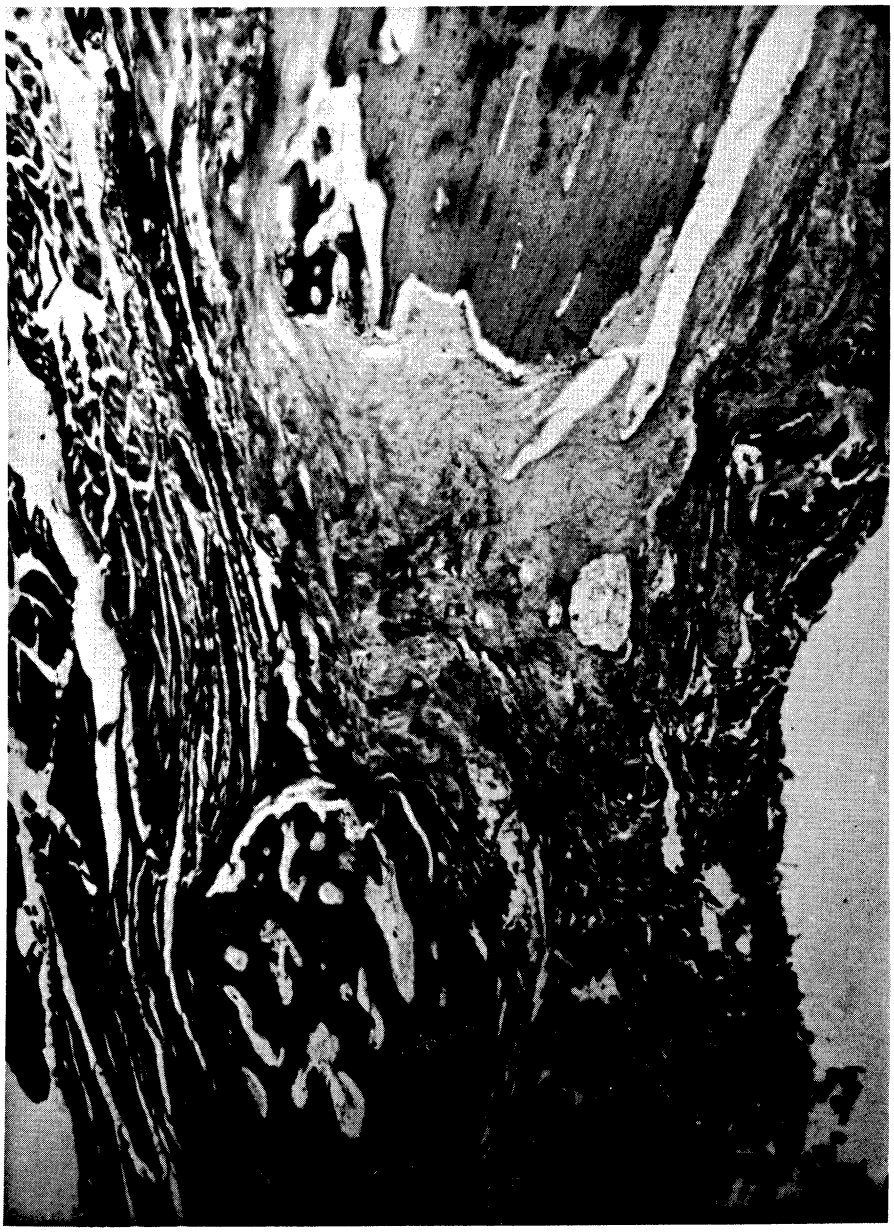

Figure 43. Microscopic section of bone graft. There is evidence of acceptable toleration, presence of viable bone. Graft is at inferior margin of section.

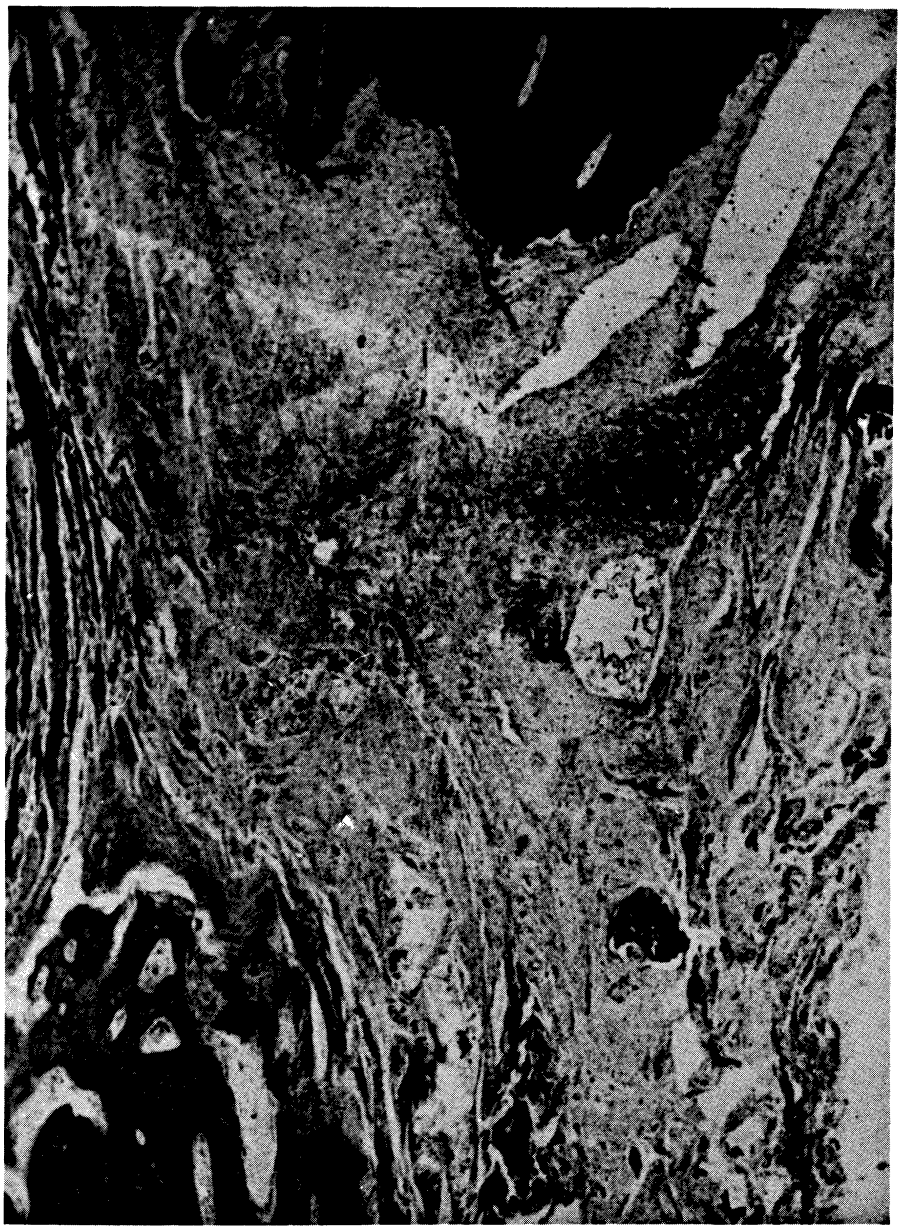

Figure 44. Medium power of junction between mandible and bone graft. Isolated areas of adhesive concentration can be seen with foreign body cells.

Figure 45. Tibia medulla six weeks after injection of adhesive. Reaction to adhesive can be seen on the right.

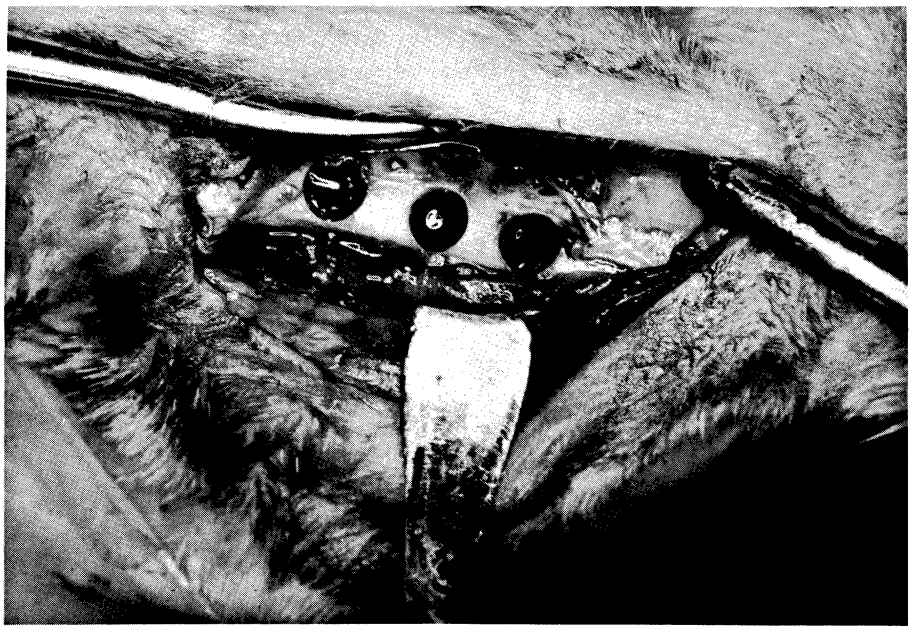

Figure 46. Rabbit mandible exposed with three defects through the buccal and lingual cortices.

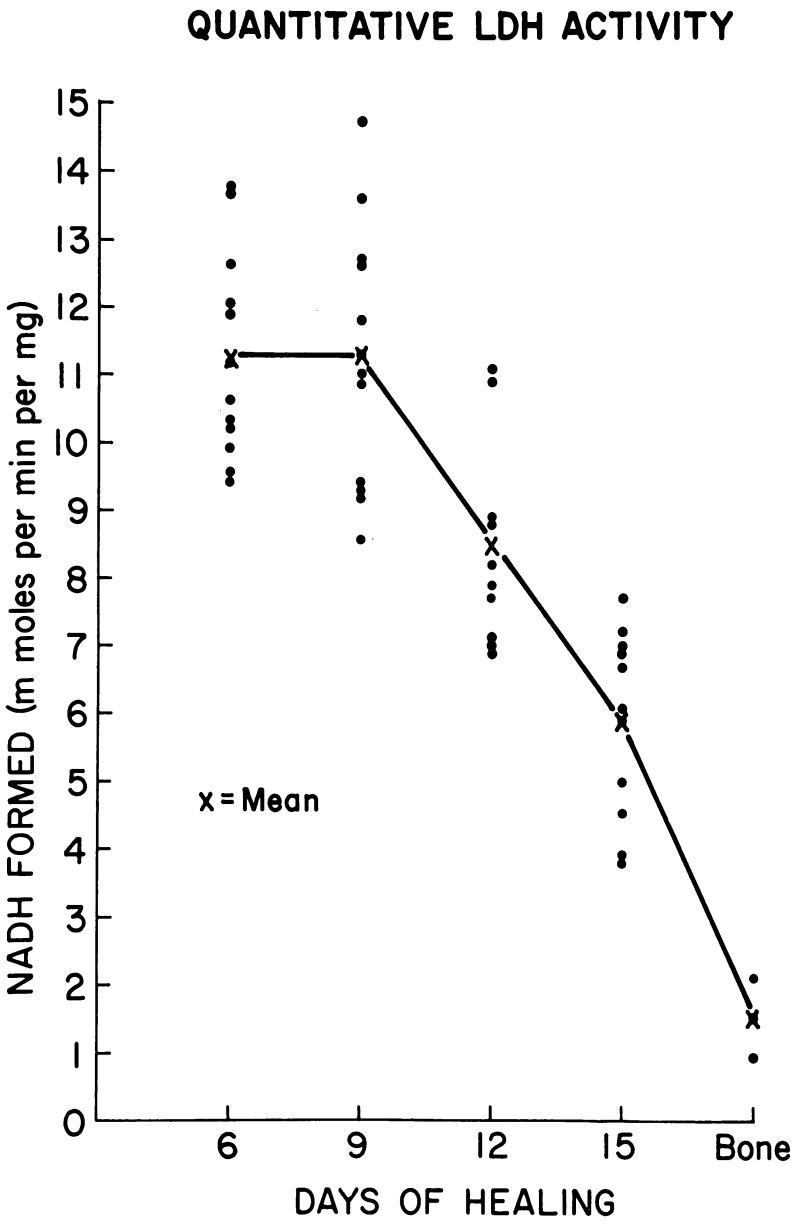


Figure 47. Graphic presentation of the total quantitative LDH activity at various times. The dots represent the numerical value of NADH formed in m mole/minute/mg of tissue as measured by the spectrophotometric analysis. The line is drawn through the mean values at each healing period.

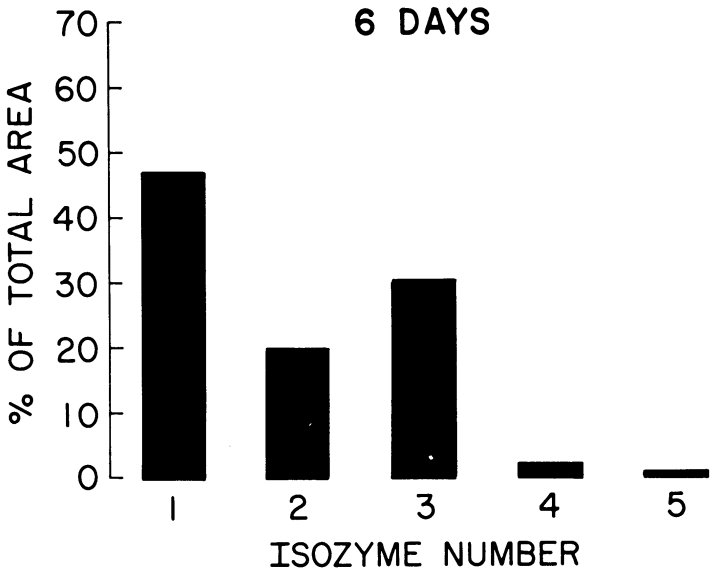


Figure 48. Graphic representation of the isozyme pattern after six days of healing.

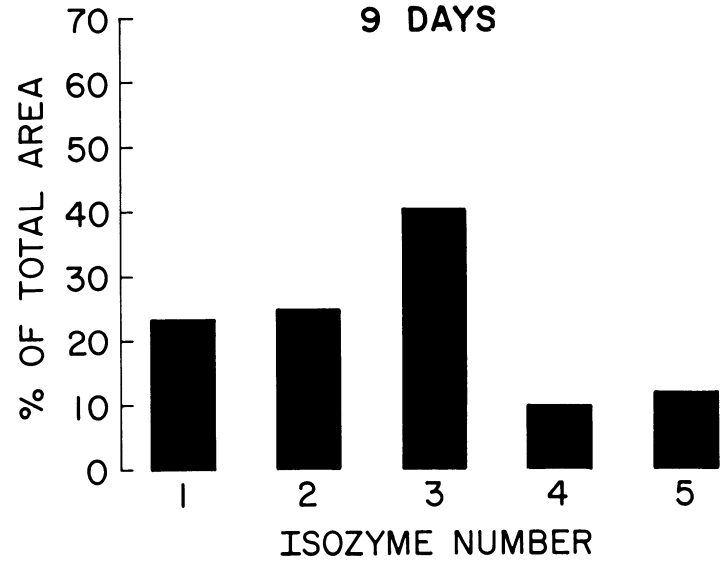


Figure 49. Graphic representation of the isozyme pattern after nine days of healing.

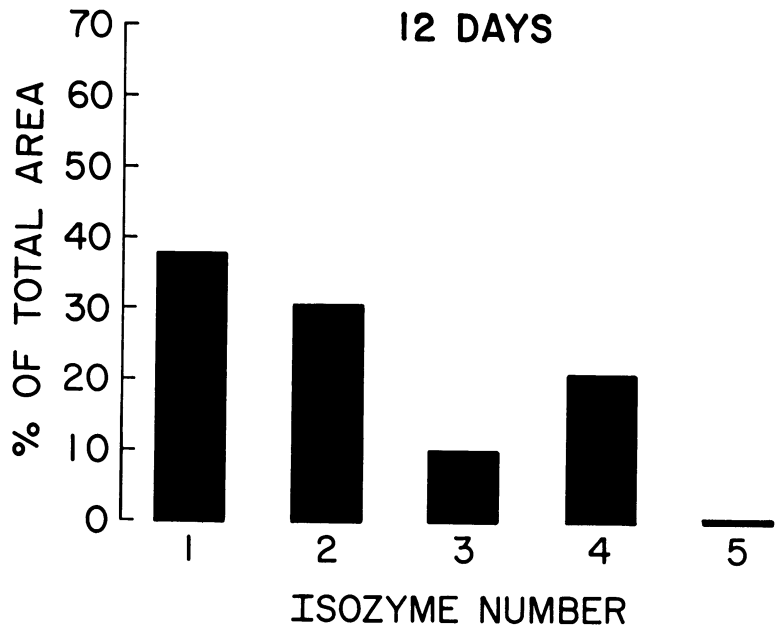


Figure 50. Graphic representation of the isozyme pattern after twelve days of healing.

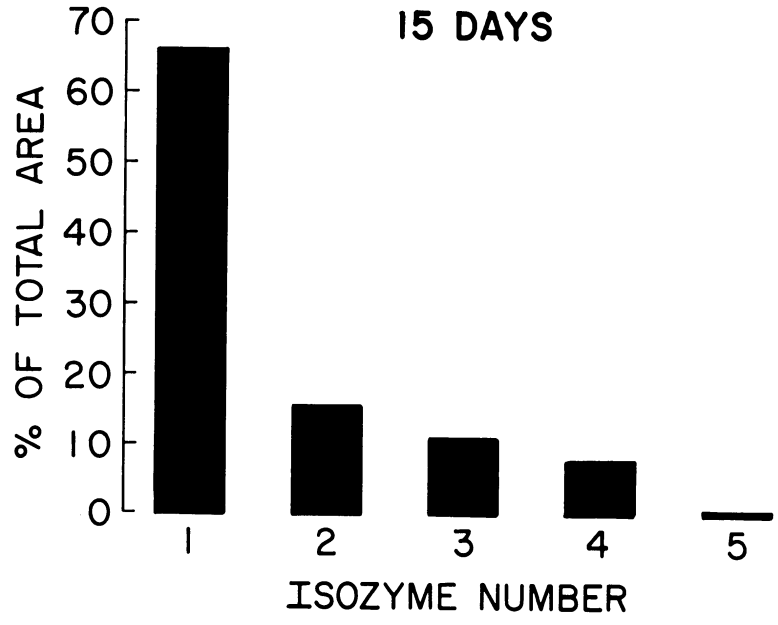


Figure 51. Graphic representation of the isozyme pattern after fifteen days of healing.

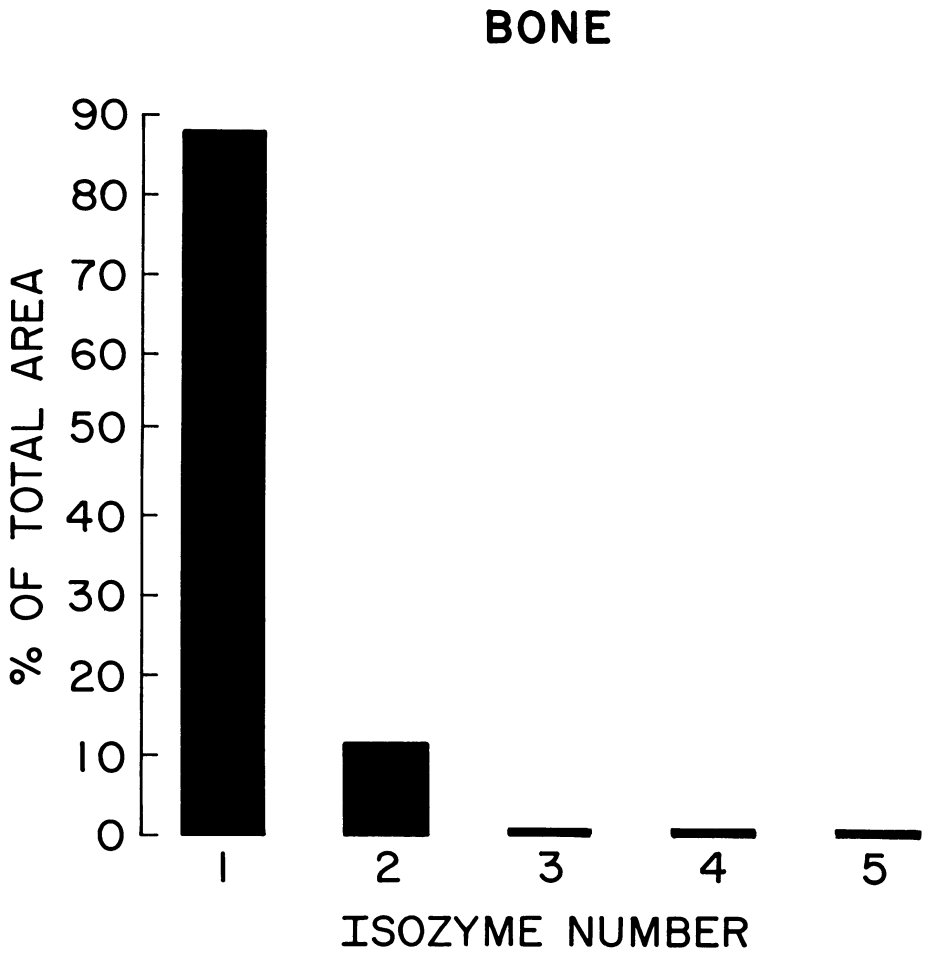


Figure 52. Graphic representation of the isozyme pattern in the control bone sample.

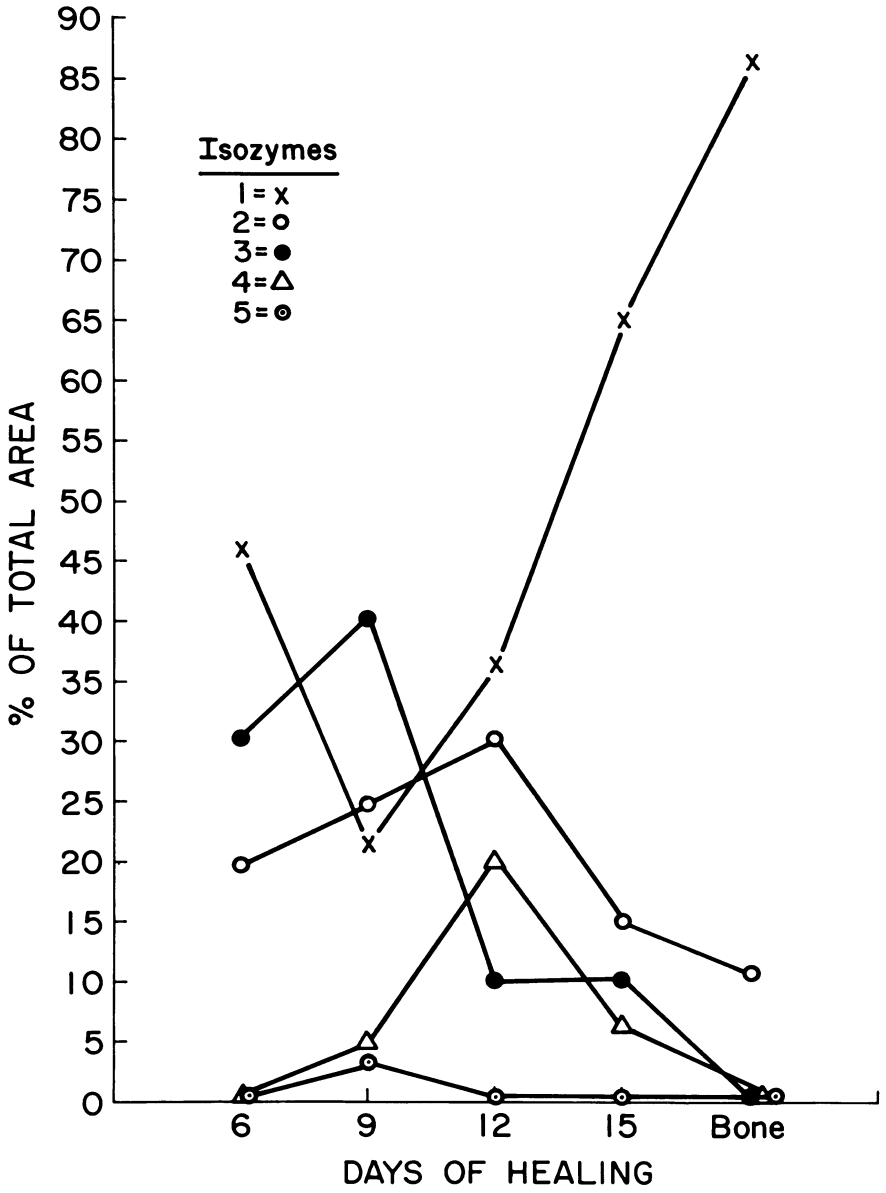


Figure 53. Composite graph of five LDH isozymes showing the interrelationship among the five forms.

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13. ABSTRACT

A method was developed to produce mandibular fracture in an experimental animal which closely simulates the human injury. The fracture model was followed by predictable healing and was labile to induced variables.

Eighteen Macacca Mulatta Rhesus monkeys were studied in two groups: An adolescent group with mixed dentition and a young adult group with full permanent dentition. It was found that fracture healing in these two groups differed. In young adults, the fracture healing proved to be influenced by alterations in therapy; in the younger adolescent group, very few differences could be observed from these same variables upon fracture healing.

The use of isobutyl cyanoacrylate monomer in soft tissue flaps was clinically effective and adequately tolerated. The use of adhesive with stable bone fragments such as onlay bone grafts found clinical control of the parts and minimal foreign body response. In a mandibular fracture with bone segment distortion, the application of the adhesive was technically difficult and did not stabilize the fragments. In this setting, foreign body response was more evident.

Tissue samples from healing mandibular defects in the rabbit were analyzed for LDH activity after 6, 9, 12, and 15 days of healing by means of quantitative spectrophotometric methods and by acrylamide gel electrophoresis.

The changing patterns of LDH isozymes during bone healing suggest that the metabolism in healing bone changes from an aerobic to an anaerobic type and the pattern may be related to tissue maturity.

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