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An Organ Culture Technique for Maintaining the Pulp Tissue of Intact Human Teeth

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AN ORGAN CULTURE TECHNIQUE FOR MAINTAINING THE PULP TISSUE OF INTACT HUMAN TEETH

by

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I certify that I have read this thesis and recommend that it be accepted as fulfilling this part of the requirement for the degree of Master of Science in the field of Biochemistry

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INTRODUCTION

Tissue culture might be regarded as an extension of the biochemist's work with fresh tissue using a Krebs-Ringer type medium to maintain tissue activity in vitro. Two major divisions of tissue culture can be distinguished: monolayer outgrowth methods, in which individual and perhaps isolated cells are studied, and organ culture, in which attempts are made to maintain groups of cells arranged as seen histologically in the host tissue. Because of the high degree of interdependence of cells in a particular vertebrate organ the organotypic culture would give a clearer picture of the true organ as a whole. Hence, it was in the direction of organotypic culture that we did our studies. The use of organotypic culture gives the experimenter the great advantage of being able to pursue a continuous study of functioning tissue over a period of several days to a few weeks.

The first attempt at organ culture may be credited to Thomson in 1914, when he reported that individual parts of the young chick embryo enlarge en masse when cultured in vitro. Shortly thereafter, Fisher (1922) reported on the progressive differentiation of parts of young embryos, and the term "organotypic" was given to this particular type of tissue culture by Maximow (1935). One year later, Strangeways and Fell (1926 a, 1926 b) published two classic papers on differentiation of organ rudiments in vitro, and organotypic tissue culture became a new research tool.

Undoubtedly the most popular method of organ culture is the watch-

glass technique first reported by Fell and Robinson (1929). It was made up of a watch-glass placed in a petri dish containing cotton wool saturated with distilled water. The medium was placed in the watch-glass and the explants were grown on the surface of the medium. Since the first report of the technique many modifications have been incorporated into the procedure. Gaillard (1951) substituted an embryologic watch-glass. Martinovitch (1953) placed the explant on a series of glass rods to prevent its sinking into the cultivation medium. Trowell (1953) used fluid medium in organ culture. The explants were supported by cotton wool, and later he (Trowell, 1954) replaced the cotton wool support with a fine wire gauze. Further refinements of the culture chamber and a simplification of the medium were reported in a more recent excellent paper (Trowell, 1959). Chen (1954) modified Trowell's method by substituting lens paper for the wire gauze as a support for the explants. Grobstein (1956) introduced thin millipore filters to separate various tissues or medium components in a culture vessel. Shaffer (1956) substituted cellulose-acetate fabric for lens paper because of the difficulty in making histologic preparations from lens paper grown explants. Jensen, Gwatkin and Biggers (1964), using basically the method of Trowell (1959) except for adaptation to standard petri dishes, were able to isolate specific cell types simultaneously. Pinkel (1963) used a Teflon ring and lens paper to support the explant. The technique remains, however, basically the same.

Organ culture has been widely used in studies on calcified tissues,

including immature long bones and other skeletal rudiments. At first the studies primarily were concerned with morphology (Fell, 1956), but more recently attention has shifted to the biochemical and physiological properties of explanted cartilaginous rudiments. In recent years many reports have appeared on the action of numerous substances, such as parathyroid hormone (Gaillard, 1961), vitamin A (Lucy, Dingle and Fell, 1961), and others, on bone organotypic cultures.

Any tissue to be cultured must be removed from the host and made into a form suitable for culture, i.e., a form which will allow for nutrient intake, gaseous diffusion, and the elimination of waste products throughout its entire thickness. The optimal size of the tissue fragment varies with the type of tissue. Some tissues, e.g., most of the endocrine glands, must be cut into very small fragments to obtain an optimal result, while other tissues, e.g., chick-embryo heart or human fetal skin, can be cultured effectively in much larger fragments.

Tissues from adult organs, therefore, must be cut or dissected, and this results in regenerative changes. Franks (1959) has aptly shown a series of regenerative changes in organ culture of mouse prostate. "After 24 hours' incubation many cells show a peculiar degenerative change usually involving only the cells of acini at the edges of the culture. After three days, the epithelium shows great mitotic activity in areas similar to those that earlier showed degenerative changes. In some cultures the cellular regeneration is irregular and bizarre. After seven

days, mitoses are uncommon, but the irregular cells persist throughout the period of culture. This is almost certainly a regenerative reaction following nonlethal trauma during dissection. These regenerated cells are not normal and will not 'function' if treated with a substance that stimulated secretion in the normal cells" (Franks, 1963). Although it would be very desirable to eliminate these and other related, artificially induced abnormalities in the tissues cultured, it has not been possible because the culture is limited to such small size.

In practice the maximum thickness of a tissue in organ culture is about 2 mm., if internal necrosis (the result of poor gas and medium diffusion) is to be prevented. It is generally felt that the oxygen content is the single most important limitation to organ culture explant size. Because of this all organ culture techniques attempt to replace the interrupted blood supply of a tissue by positioning the tissue as close as possible to the atmosphere-medium interface to allow for a maximum supply of oxygen to the tissue.

Parker (1936) was the first investigator to draw attention to the importance of oxygen in the culture of adult tissues, although the advantage of culturing in oxygen was discovered by Loeb in 1897 (Loeb and Fleisher, 1919). Trowell (1959) using the model of Gerard (1931) concluded that, in theory, internal necrosis should occur with explants larger than 1.4 mm in diameter if oxygen is the limiting factor in explant size. This is close to the 2 mm diameter thickness which is generally regarded as the maximum size.

In the watch-glass technique of Fell and Robinson (1929) and in all the modifications of this technique, the fact still remains that small (2 mm D.) pieces of tissue must be cultured in order to prevent internal necrosis.

Organ culture could offer the biologist some interesting experiments if explants larger than 2 mm in diameter could be cultured. For example: 1) Organ cultures might be maintained for longer periods of time because as the cultures increased in size they would not tend to develop internal necrosis with its resulting toxic products. 2) A greater number of adult tissues, which are generally larger in size than their embryonic counterparts, might be cultured. 3) More human tissues, which are also larger in size, might be cultured. 4) The interaction of cell types, e.g., mesenchyme on epithelium, might be more clearly studied.

A possible solution to the limit of the size of an organotypic explant might be to place small needles into the tissue and perfuse tissue culture medium through these needles. This could have the effect of increasing gaseous exchange (from the perfused medium), and it could also better bathe the tissues in medium. It would appear that, theoretically, there might be no limit to the size of an organ explant so long as the perfusing needles were placed every 2 mm.

As a tissue to test the feasibility of this idea, the adult human tooth appeared to offer the following advantages over any other: 1) The pulp tissue of a human bicuspid tooth contains only around 10-20mg

wet weight of tissue. 2) The somewhat ribbon shape of the pulp in bicuspids should facilitate medium and gaseous exchange. 3) Pulp tissue is considered to be a type of embryonic connective tissue even in the young adult, and embryonic tissues have always been easier to culture in vitro than have mature tissues. 4) The QO_2 for adult tooth pulp is low when compared to that of other tissues (Pincus, 1951; Fisher et al., 1959; Bolke et al., 1958), indicating that pulp tissue oxygen requirements are lower. 5) The hexose monophosphate shunt (pentose shunt), which is a direct oxidative pathway for carbohydrate metabolism, may play a significant role in pulp carbohydrate metabolism (de Shazer, 1961). This could imply that the oxygen content could markedly affect the type of carbohydrate metabolism which occurs in the tooth pulp, so that the type of carbohydrate metabolism, as measured by an end product, might indicate the quantity of O_2 available to the tissue. 6) Since the human is a relatively large animal the oxygen requirement of a constituent tissue (Krebs, 1950) would be lower than that of tissue from most vertebrates. 7) The pulp tissue is encased in a calcified structure which would inhibit direct gaseous exchange of the pulp tissue with the atmosphere of the incubator. This would mean that the sole source of oxygen would be delivered by the medium and would be more easily regulated. 8) The calcified case would serve to stabilize the needles inserted into the pulp tissue. 9) The calcified case would permit internal hydrostatic pressures of greater variation than in a soft tissue.

Greater pressures would ensure more complete bathing of the tissue by the perfusing medium. Apparently intrapulpal pressures are normally quite high *in vivo* (Brown and Yankowitz, 1964; Beveridge, 1965).

10) The pulp may be considered a complete organ with specialized cells (odontoblasts), mesenchymal cells (fibroblasts), etc., which can be removed from the host with a minimum of trauma. The trauma should be localized at or near the small apical end(s) of the tissue and should be only that resulting from severed vessels.

Organ culture has not been without its representatives in the dental field. Glasstone (1936) first reported the successful growth *in vitro* of tooth germs. She continued work in this field for several years and published an interesting paper on the morphogenesis of developing molars (Glasstone, 1939). The work on tooth organ culture then lay dormant for almost 15 years until the simultaneous reports of Szabo (1954) and Lefkowitz, Bodecker, and Mardfin (1954) were published. Since then a number of investigations, including that of Hay (1961), Holloway and Mellanby (1961), and Lefkowitz and Swayne (1958), have been reported. Glasstone (1964) accomplished *in vitro* growth of tooth germs in a chemically defined medium. Recently Main (1966) succeeded in culturing tooth germs of 14-day mouse embryos on gelatin sponges in Leighton tubes. All the techniques for tooth germ culture employed in the above reports were those already in use in other types of organ culture. In almost all instances fetal tissues were used and the size of the organ explant was kept as small as possible. Human tissue was not used.

Comparison of a tissue in an organotypic culture with the in vivo counterpart is extremely important in evaluating the success of the culture. But the first stumbling block to comparison with the normal is that the normal must be accurately recognized. In the past, by far the most important criterion for success of an organotypic culture technique was the state of the cells seen in the morphological and histological examination. However, the histological and morphological examinations may not be as accurate in determining the viability of a tissue as most investigators have assumed.

Gillette (1963) pointed out a great need in organ culture for a test system which could differentiate between "structural integrity" and "functional viability." Organ culture may maintain cellular integrity and general architecture while the original organ function may be greatly altered. Recently Gillette, Goulian, and Conway (1965) have shown that the application of certain cortisone derivatives gives a more favorable organ culture viability depending upon what criteria are used. For example, skin treated with cortisone acetate functioned as autographs after 3 to 4 weeks in vitro but failed to maintain the histologic structural integrity of skin treated with deoxycorticosterone acetate. However, with the latter compound the skin lost its ability to function as an autograph after one week in organ culture.

Stanley (1965) reported that a tooth placed in balanced salt solution for 24 hours after extraction had a normal histologic pulp. However,

after three days in the balanced salt solution the odontoblasts were merely ghost cells, though the tissue at the apical end was still rather normal in appearance. Nerve and collagen degeneration could be detected after three days in the balanced salt solution. This implies that at best a histologic examination could not reveal anything until after three days of perfusion, since the pulp, which was greatly altered biochemically, still appeared normal histologically until about the third day.

Eranko and Kahri (1965) used histologic structure and catecholamine content as criteria for the successful organ culture of adult and new-born rat adrenal medullae. In the classic paper of Fell and Robinson (1929) a number of criteria were used to evaluate the success of their organ culture procedure. They used phosphatase activity, dry weight, increase in length, anatomical and histological development.

It would seem that the level of a measurable, sensitive indicator of metabolism would be a good criterion for the success of an organ culture procedure, since continuous energy production is essential for cell viability. Since adenosine triphosphate (ATP) is a common medium for energy exchange, the level of this compound should serve effectively as an indicator of viability. Steinman (1961) demonstrated by an autophotographic technique that the human pulp contains ATP. Thus it might be possible to measure quantitatively the concentration of pulp tissue ATP and to use this measurement as an indication of viability.

The object of the work reported here was to develop an organ

culture technique which would not be limited by the size of the cultured explant. The success of the technique was judged by pulp tissue ATP content, histologic appearance, and monolayer outgrowth explants from perfused pulps after a time in organ culture.

METHODS AND MATERIALS

A. The Perfusion Procedure

The pulp tissue of an intact, freshly extracted human tooth was perfused with a standard tissue culture medium. The medium was delivered to the pulp tissue through two 30 gauge, stainless steel needles (seamless, stainless steel cartridge, Mizzy, Clifton Forge, Va.) threaded from the tooth apices to the pulp horns. This was accomplished in the following manner. A pair of needles was autoclaved within a cotton roll. Two lenghts of polyethylene tubing (Intramedic polyethylene, Clay Adams, New York) each consisting of connected sections of approximately 6 mm of P.E. 90, 100 mm of P.E. 50, and 50 mm of P.E. 10 tubing were sterilized by soaking them one hour in a solution containing 1,250 units penicillin G, 250 units streptomycin sulfate and 5 mg Fungisone (amphotericin B) per 100 ml of sterile water. Rinsing was accomplished by forcing three changes of sterile distilled water through the tubing with a sterile syringe. The tubing and needles were connected and stored in a sterile petri dish until ready for use. The needles were attached to the size P.E. 10 tubing.

Just prior to insertion of the two needle-tubing complexes into a freshly extracted tooth, they were filled with the tissue culture medium with the aid of a syringe. Then each needle was inserted into the tooth from the apex to the pulp horn. All handling of tooth, needles, and tubing was done using sterile gloves. With needles in place, the syringe

filled with medium was once again applied to the P.E. 90 end of the tubing and medium was forced through the needle-tubing complex by gentle pressure on the syringe plunger. Medium seen to accumulate at the apical end of the tooth indicated that all passageways were clear. If no visible accumulation of medium was produced at the apex the inserted needle was withdrawn a few millimeters and reinserted. This usually allowed the medium to flow freely. The perfusion was not begun until the medium did flow freely with slight pressure applied to the syringe. Thus the medium was continuously delivered to the pulp horns and was perfused through the pulp tissue. After perfusing the pulp tissue the perfusate was drained from the tooth apices and was absorbed by the cotton gauze surrounding each tooth.

To simulate the metabolic function of the periodontal membrane the perfused tooth was placed in a small sterile petri dish containing 2 x 2 in. cotton gauzes saturated with perfusate on the upper and lower lids. The glass rims of the upper and lower petri dish lids were depressed at one point so as to allow free passage of needles and tubing. A 2 mm hole was made in the lower lid at the base to allow drainage of the perfusate.

The supply of tissue culture medium was contained in a sterile 300 ml Erlenmeyer flask located above an incubator. The flask was connected by a short piece of glass tubing of 10 mm internal diameter and by sterile glass and Tygon tubing to a sterile blown glass delivery

manifold housed in the incubator. A 100 mm length of sterile P. E. 50 tubing was passed into each spigot of the manifold and stoppered with 0. 030 orthodontic stainless steel sterilized wire.

A Precision Thelco Incubator was used; the temperature was kept at or near 100% humidity by pans of water placed inside the incubator.

A gas mixture of 0_2 and $C0_2$ was continuously delivered to the atmosphere of the incubator. A sample of incubator air was passed into a 100 ml bottle of the same medium as that being perfused into the pulp tissue. The ratio of 0_2 and $C0_2$ in the incubator was determined by the color of the phenol red indicator. The pH was kept near 7. 4 as determined by the color of the indicator. As a double check, the color of the indicator in the medium contained in the petri dish housing the tooth also was observed.

The medium was delivered to the pulp chamber of each tooth at a constant pressure of 20 mm Hg. throughout the perfusion period. However, in the early part of the investigation, before we developed the procedure using hand pressure on the syringe to clear the inserted needles before perfusion was begun, much higher perfusion pressures were used before the medium could be seen leaving at the apices. Some pressures had been as high as 64 mm Hg. In the early experiments a fast perfusion rate (12 ml per tooth per hour) was used. However, a rate of about 4 ml per tooth per hour was settled on for the later experiments.

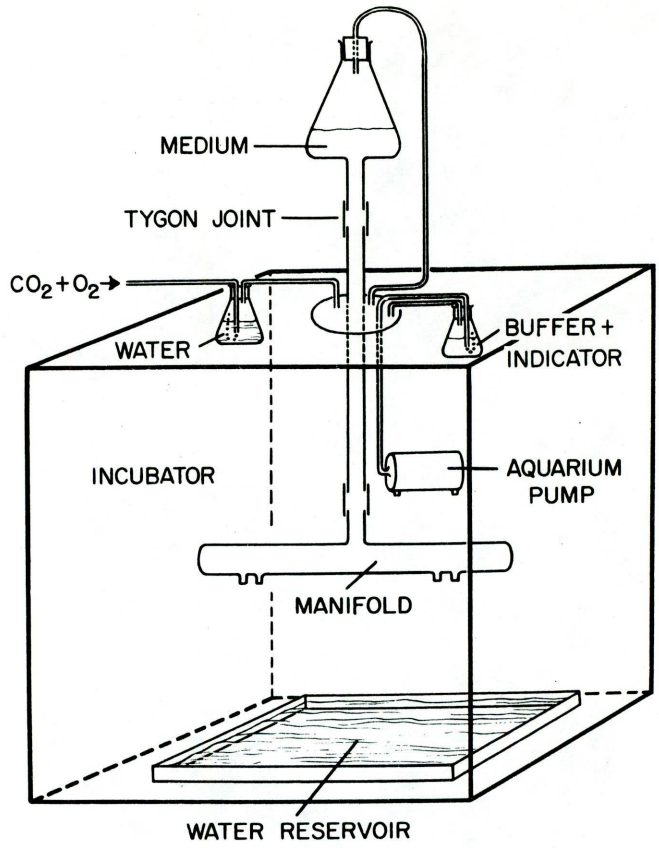


Figure 1. A diagramatic representation of the perfusion apparatus. The distance from the medium to the manifold was about 25 cm. The petri dishes which contained the perfused teeth and the acquarium pump were stabilized in the incubator by racks which were not shown in the drawing.

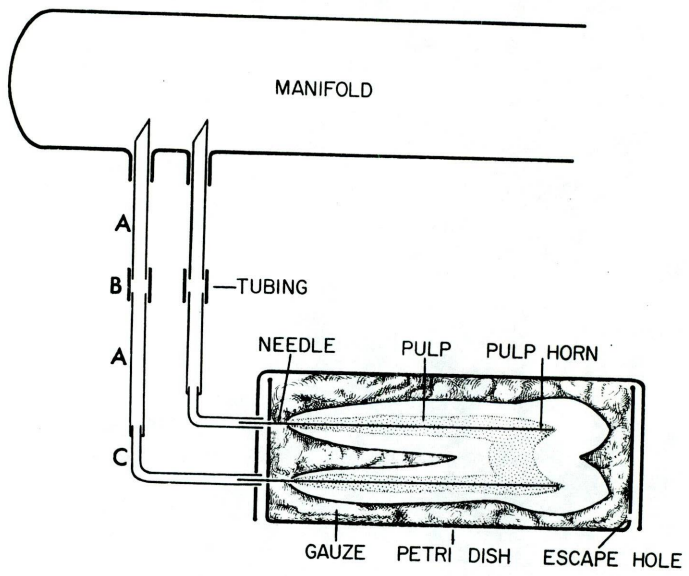


Figure 2. A schematic representation of the manifold to pulp horn connections and the housing of a perfused tooth in the petri dish of 6 mm diameter. The letters A, B, and C represent various sizes of polyethylene tubing. A was P.E. 10 (0.28 mm I.D.); B was P.E. 90 (0.86 mm I.D.); C was P.E. 50 (0.58 mm I.D.). The needles were 30 gauge stainless steel.

The type of medium used in the perfusion procedure was changed several times during the course of development of the procedure. At first NCTC 109 (Microbiological Associates) supplemented with 10% horse serum (obtained from Dr. Robert Nutter) and 5% embryo extract was used. The embryo extract was prepared from 10 day chick embryos obtained from a local hatchery. After the embryos had been removed from the eggs under sterile conditions they were placed in a sterile 50 ml syringe without a needle and squeezed through the opening. This material was immediately centrifuged at 2° C in a Harvester Model PR-2 centrifuge for 30 minutes at 5,000 RPM. The supernatant fluid was drawn off with sterile volumetric pipets and frozen in sterile prescription bottles until ready for use.

Because of the time involved in making the embryo extract and horse serum and the cost of NCTC 109, CMRL-1066 (Microbiological Associates) was used without the addition of any growth factors. Finally, Waymouths 752/1 (Microbiological Associates) plus 10% fetal bovine serum (Microbiological Associates) was used. All three types of media contained 100 units penicillin G and 100 micrograms streptomycin sulfate per ml. Whenever a perfusion was planned to last longer than three days, 5 micrograms per ml of Fungisone was also added.

All teeth used in the perfusion experiments were permanent bicuspids extracted exclusively for orthodontic reasons from human subjects ranging in age from nine to eighteen years.

For extraction, local carbocaine anesthesia was given as either infiltration or block, and the epithelial attachment was loosened. The time required from placement of the extraction forceps on the tooth until the tooth was removed from the alveolus was noted in each instance. In the early experiments, if the extraction time exceeded 30 seconds the tooth was not perfused but was used as a saline control. Later, it was found that the extraction time was not that critical, and no such restriction was set. However, seldom did an extraction time run over one minute. (The author did about 90% of all the extractions).

The freshly extracted tooth was wrapped in a sterile gauze pre-warmed to 38° C, was immersed in a petri dish of tissue culture medium at 38° C containing 100 mg of penicillin G and 250 mg of streptomycin sulfate per 100 ml, and was rushed to the perfusion apparatus. The needles with attached tubing were threaded from the apices to the pulp horns. This tubing was then connected to the manifold tubing at the P.E. 90 joint after checking for clear flow as described earlier with syringe pressure. From the instant the tooth was removed from the alveolus until it was attached to the manifold tubing required a maximum of 120 seconds. If more than 120 seconds were required, the tooth was used as a saline control. Sixty seconds was the limit set in the early experiments, but this did not include checking the flow with the syringe before connection to the manifold.

Frequently in teeth from the older orthodontic patients the apices

were too small to allow passage of the needles and exit of the perfusing medium. In these instances, the lower quarter of the root was removed with side cutting pliers.

B. Procedures Related to Adenosine Triphosphate Determination

The general steps involved in all the ATP extraction and quantitation procedures here described consisted of: a) preparation of the donor in such a way that the tissue could be removed, b) removal of the tissue from the donor, c) treatment of the tissue immediately after removal from the donor to prevent loss of ATP, d) in the case of pulp tissue, the removal of the tissue from the calcified tooth, e) extraction of the ATP-containing portion of the tissue in boiling water, f) freeze-storage of the extracted samples, g) quantitative ATP determinations, h) measurment of the wet weight of the tissue. Numerous experiments were conducted to determine how each of the above steps should be carried out to give optimum levels of ATP.

The donors were either humans between the ages of eight and nineteen or young dogs. In the case of dogs, pentobarbital sodium (Nembutal), 35 mg per kilogram body weight, was used as the anesthetic. All animals were sacrificed at the end of the experiment. All the human donors were anesthetized with Carbocaine local anesthetic by either infiltration or block anesthesia. No premedication or general anesthesia was administered.

The method of removal of the tissue from the donor depended,

to some extent, on the type of tissue under study. Dog peripheral nerve tissue was obtained by gently exposing the femoral nerve (by blunt dissection) for a distance of approximately 5 to 8 cm. Mammalian Ringers solution was used to prevent dehydration. When the nerve was completely exposed successive lengths of it were quickly cut with scissors and the ATP extracted. Teeth were removed as rapidly as possible using a No. 150 Universal upper forceps. On human subjects the epithelial attachment was first loosened and an average time of 15 seconds was required to remove a tooth. In dogs the duration of the extraction procedure varied greatly; for the bicuspids 10 seconds was quite adequate; for the molars--30 seconds; for the incisors--10 seconds; for the canines--several minutes if at all.

As soon as the tissue was removed from the donor it was plunged in toto into a test tube containing 2 ml of distilled water at 100°C., in a boiling water bath, and was boiled 10 minutes. When the tooth was removed from the donor, the pulp was not immediately accessible. Thus the tooth was split, with a pair of side cutting pliers, and the pulp subsequently removed in toto with the aid of a small cleoid-discoid (a dental spoon). This procedure required 60-120 seconds. The pulp then was either plunged into a boiling water bath or it was divided and cut into regions and then placed in the boiling water bath.

After the 10 minute boiling period all samples were stored at -15°C if ATP determinations could not be made within a few hours. All ATP

determinations were made within 1 week after boiling, and the majority were completed within 72 hours after the boiling period. The tissue samples were weighed at the time of the quantitation of ATP. The tissue was blotted dry, rapidly weighed on an analytical balance, and discarded after weighing.

The luciferin-luciferase complex of the fire fly tail, as first reported by McElroy (1947) and by McElroy and Strehler (1949), was used for the quantitative determination of ATP. Lyophilized fire fly lantern extract (FLE-50, Sigma Chemical Co.) was obtained in 5 ml vials, and when reconstituted with 5 ml of water each vial contained 0.05 M Potassium Arsenate and 0.02 M Magnesium Sulfate at pH 7.4. The reconstituted enzyme preparation was kept in an ice bath or in a 2°C, constant temperature water bath while the ATP determinations were made.

A standard light response curve was determined for each vial of luciferin-luciferase complex and for each series of ATP determinations. Ten mg of crystalline ATP (Disodium $3H_2O$, Sigma) were weighed on an analytical balance and placed in a test tube containing 10 ml of distilled water. Serial dilutions were made to 0.1 microgram per ml and the light production of 0.1, 0.2, 0.5, 1.0, and 2.0 micrograms ATP were recorded. A 1.0 microgram standard determination was done for every 10 unknown samples to check the stability of the enzyme preparation.

All light responses were measured at room temperature by placing the unknown or standard ATP solution plus distilled water to make 3.5 ml in the glass tube placed in the holding rack on the door of a Model 110

Turner fluorometer. Five tenths of a milliliter of the fire-fly extract was added rapidly by pipet to the glass tube in the holding rack. The solution was mixed 10 seconds with a glass stirring rod; the door was shut; the light response was read on the dial at exactly 30, 60 and 120 seconds after addition of the enzyme. In determinations in which the activity of the luciferin-luciferase complex was maximal the ATP measurements were accurate to within ± 0.02 micrograms when the phosphorescence produced at 30 seconds was measured. The luminescence was read by blocking out all light coming from the activating lamp and using a filter with a sharp cut at 415 millimicrons (secondary filter 2A).

An internal standard was used whenever the tissue sample was large enough to contain a quantity of ATP which could be divided into two aliquots and still produce a light response of 20 (scale 100) on the fluorometer after 30 seconds. The internal standard, consisting of a known amount of crystalline ATP, was used with an aliquot of each unknown sample just prior to light production as a check to determine if some elements in the unknown solution were interfering with light production of the luciferin-luciferase complex.

C. Histologic Procedures to Determine Structural Integrity

A total of eight perfused human teeth plus a number of freshly extracted and non-perfused teeth were used in this study. All eight teeth were perfused from eight to fourteen days using Waymouths 752/1 base

medium plus fetal bovine serum and pen-strep antibiotics with added Fungisone. The percentages of constituents are described in section A of Methods and Materials.

After the period of perfusion, the teeth were immediately frozen in a deep freeze. After fifteen hours at -15°C the teeth were thawed and a dental air rotor and water jet were used to drill a 2 mm hole at the dentino-enamel junction through the calcified structure of the tooth into the pulp chamber.

The method used for the processing of the teeth prior to sectioning was basically the same as that used by Harold R. Stanley at the National Institutes of Health. The only modification was in the type of fixative used. A fixative of 90 parts ethanol, 10 parts formalin and 5 parts acetic acid as routinely used by Sol Bernick (University of Southern California, Department of Anatomy) was substituted.

If the apices were completely formed the root tips were clipped off. The teeth were then placed in the above described fixative for forty-eight hours. The volume of the fixative was approximately twenty times the volume of the teeth. The teeth were then placed in 5% formic acid and gently rotated. The acid was changed daily. After ten days in acid, 2 mm thick portions of the mesial and distal surfaces were cut off with a razor blade. The teeth were returned to the acid for three more days. Then the teeth were washed in running tap water for twelve hours, placed in 80% ethyl alcohol for twenty-four hours, placed in 95% ethyl alcohol for

twenty-four hours, and finally placed in 99.9% ethyl alcohol for twenty-four hours. All alcohol solutions were changed every twelve hours. The final dehydration solution was a 50-50 mixture of absolute alcohol and ether in which the teeth were placed for two hours. Finally, this solution was renewed and the teeth left another two hours. The teeth were then ready for routine histologic procedures. The Hematoxylin-Eosin stain was used.

D. The Monolayer Outgrowth Procedure to Assess Viability

The four first bicuspids were removed from a twelve year old girl. One upper and one lower bicuspid were treated as saline controls; the other two were perfused for three days. After the three day period, the pulps of both the perfused and saline control teeth were removed under sterile conditions, cut into approximately 2 mm fragments, and placed in a freshly prepared plasma clot located on one of the cover glasses of the Rose miltipurpose chamber (Rose, 1954). The chambers were filled, as described by Rose (1954), with the medium used to perfuse the teeth (Waymouths 752/1 plus 10% fetal bovine serum plus antibiotics) and were incubated for 48 hours at 37°C in the perfusing incubator.

The plasma clot was made by putting 10 drops of cockerel plasma on the cover glass and then adding 3 drops of 50% embryo extract (in balanced salt solution). This was gently agitated with a glass rod until the medium began to clot (approximately 60 seconds). The plasma clot method for the outgrowth of pulpal cells was found to be superior to the method of Pomerat and Contino (1964).

After three days of incubation the explants were photographed under phase contrast microscopy at approximately a magnification of 150X.

RESULTS

A. The ATP Determinations

The temperature of the 2 ml of water used for the extraction of ATP contained in the test tube in the boiling water bath at the moment the pulp tissue was placed therein, was critical. As can be seen from the data of Table 1, the pulp tissue of each of 12 human teeth placed in 2 ml of water at room temperature yielded virtually no ATP on analysis, whereas, the pulps of 10 human teeth each placed in 2 ml of boiling water yielded a mean of 0.19 micrograms of ATP per mg of wet weight of tissue.

Since in the ATP determination the term "wet weight" referred to the wet weight after boiling and freezing, it was necessary to determine the magnitude of the change in weight which was caused by these procedures. The data of Table 2 showed that about a 50% reduction in the wet weight occurred due to the boiling and freezing. Except for samples 2 and 9 the reduction in weight was fairly uniform.

The concentration of ATP in some representative tissues of the dog are shown in Table 3. The femoral nerve of dog A contained a mean ATP value of 0.14 micrograms of ATP per milligram wet weight, while the value for the same tissue from dog B was 0.22 micrograms ATP per milligram. There was little variation in the ATP levels of successive samples of the same tissue. This was clearly seen in the samples of femoral nerve from dog B where a standard deviation of ± 0.02 or a percent standard deviation of ± 7.3 was obtained.

Table 4 contains data on the localization of pulp tissue ATP. In dog A, when the pulp was divided into three areas, it was found that the mean and standard deviation in the coronal pulp ATP level was 0.42 ± 0.15, that in the body pulp ATP level was 0.25 ± 0.12, and that in the apex pulp ATP level was 0.16 ±0.09 micrograms of ATP per mg wet weight. In dog B, a similar localization of pulp ATP occurred with the coronal and apical areas showing a mean and standard deviation of 0.54 ± 0.25 and 0.21 ± 0.09 micrograms ATP/mg tissue respectively. It is interesting to note that in both dog A and dog B there occurred a 61% decrease in the ATP level of apical pulp tissue as compared to coronal pulp tissue.

Table 5 presents data obtained in the same manner as those in Table 4 except that human teeth were used. The mean values and standard deviations for crown, body and apical pulp tissue ATP were 0.37 ± 0.29, 0.15 ± 0.13, and 0.15 ± 0.12 respectively. There was a 59% decrease in apical pulp ATP compared to the coronal counterpart. The body pulp also contained the same amount of ATP as the apical pulp; this does not agree with the dog samples. However, the human sample contained only 5 complete crown-body-apex divided pulps.

In Table 7 is seen the normal loss of ATP in unperfused human pulp tissue. The pulp tissue ATP level was almost negligible 15 minutes after removal from the patient and showed no sign of increasing for 5 hours thereafter, which therefore indicated that the tissue ATP level might be a sensitive indicator of viability.

In Table 8 the pulp tissue ATP levels of perfused and non-perfused control teeth were compared. A tooth was considered to be successfully perfused if its pulp tissue contained more that 0.03 micrograms ATP/mg of tissue. Using this figure, 4 of the 29 perfused teeth or 14% were considered unsuccessfully perfused.

As can be seen from the summary of Table 8 the saline and gauze control teeth contained mean ATP pulp levels of 0.01 and 0.02 respectively. The gauze controls had a pulp tissue ATP level of twice that of the saline controls however, In contrast to these low values for pulp ATP in the controls, approximately 10 times that amount of ATP was found in the pulps of perfused teeth.

The data indicate that the pulp ATP level of the perfused teeth decreases as the perfusion period lengthens. The pulps of all teeth perfused 24 hours or less contained 0.17 micrograms of ATP/mg, whereas in teeth perfused 24-72 hours, 0.10 micrograms of ATP/mg of tissue was found. Perfusion periods longer than three days reduced the pulp tissue level to 0.07 micrograms of ATP/mg.

All perfused teeth had a mean and standard deviation ATP level of 0.11 ± 0.08 micrograms ATP/ mg tissue. All successfully perfused teeth had a mean and standard deviation ATP level of 0.14 ± 0.07 micrograms ATP/mg tissue. The ATP level in successfully perfused teeth closely approximated that of the immediately extracted teeth of Table 6. The mean value for pulp tissue ATP in all teeth of Table 6 was 0.15 ± 0.10 micrograms of ATP/mg.

TABLE 1

THE EFFECT OF THE TEMPERATURE OF THE 2 ML OF WATER CONTAINED IN THE TEST TUBE ON THE ATP CONTENT OF PULP TISSUE

A. The 2 ml of water contained in the test tube were at room temperature when the pulp tissue was added, and then the test tube was put in the boiling water bath for 10 minutes.

Based on 12 teeth from three patients:

The light production readings on the Turner were so small (a maximum of 7 on a scale of 100, and 5 of the 12 teeth gave no detectable light production at all) that the weights were not recorded, and thus ATP/mg tissue could not be calculated.

B. Same as A. above except the 2 ml of H_2O contained in the test tubes were pre-heated in the boiling water bath for 5 minutes before the pulps were added.

Subject[1]	Reading[2]	Wet[3] Weight	Micrograms ATP	Micrograms ATP/mg
1a	89	5.5	1.93	0.35
1b	62	3.5	0.78	0.22
1c	79	3.8	1.44	0.38
1d	65	4.6	1.58	0.34
2a	18	4.7	0.48	0.10
2b	14	6.0	0.36	0.06
3a	77	17.8	2.18	0.12
3b	72	24.5	2.04	0.08
3c	33	6.1	0.91	0.15
3d	69	23.6	1.85	0.08

1. Numbers refer to subjects; letters refer to teeth
2. Thirty second fluorometer reading (scale 100)
3. Weight after boiling and freezing in mg.

TABLE 2

THE EFFECT OF BOILING AND -15°C STORAGE ON THE WET WEIGHT OF HUMAN PULP

Sample[1]	Wet weight before boiling[2] (mg)	Wet weight after boiling[3] (mg)	Percent reduction in wet weight[4]
1	5.6	2.7	52
2	3.9	1.6	59
3	7.5	3.6	52
4	8.0	3.8	53
5	3.7	1.9	49
6	3.2	1.6	50
7	3.5	1.8	49
8	4.6	2.4	49
9	6.7	3.8	43
10	7.1	3.8	47
Summary	53.8	27.0	50

1. Taken from 4 unerupted wisdom teeth from a 19 year old patient.

2. The weight immediately after exposure of the pulp tissue. The pulp was blotted dry with a tissue prior to weighing.

3. The weight after 10 minutes in 2 ml of 100°C distilled water in a boiling water bath and 24 hours of storage at -15°C. The pulp was blotted dry with a tissue prior to weighing.

4. $\frac{\text{Post-boiled}}{\text{Pre-boiled}} \times 100 - 100.$

TABLE 3

THE AMOUNT OF ATP IN VARIOUS TISSUES OF THE DOG

Tissue Type[1]	Reading (30 sec)	Wet Weight (mg)	Total Micrograms ATP	Micrograms ATP/ mg tissue
A. Femoral N. 1	20	7.9	1.04	0.13
A. Femoral N. 2	24	7.9	1.07	0.14
A. Femoral N. 3	35	9.7	1.55	0.16
A. Femoral N. 4	50	15.1	2.32	0.15
A. Femoral N. 5	47	17.8	2.35	0.13
B. Femoral N. 1	23	6.7	1.40	0.21
B. Femoral N. 2	36	15.3	3.06	0.20
B. Femoral N. 3	13	8.4	1.72	0.25
B. Femoral N. 4	36	12.4	2.70	0.22
B. Femoral N. 5	34	10.2	2.55	0.25
B. Femoral N. 6	50	17.2	3.70	0.22
B. Femoral N. 7	38	17.6	3.70	0.21
B. Femoral N. 8	32	13.4	2.62	0.20
A. Liver 1	17	4.0	0.71	0.18
A. Liver 2	54	25.6	5.66	0.22
A. Liver 3	35	82.1	16.00	0.20
A. Skeletal M. 1	18	3.7	12.6	3.4
A. " Muscle 2	34	6.2	19.2	3.1
A. " " 3	23	4.8	14.4	3.0

1. A & B refer to different dogs.

TABLE 4

THE LOCALIZATION OF ATP IN DOG PULP

Dog	Pulp Area	Reading (30 sec)	Tooth Type	Wet Weight	Micrograms ATP	Micrograms ATP/mg
1A	c	12	Upper	0.7	0.33	0.47
	b	43	Incisor	3.8	1.25	0.33
	a	12		1.5	0.33	0.22
2A	c	14	Lower	0.5	0.39	0.78
	b	13	Incisor	0.7	0.36	0.52
	a	9		0.9	0.25	0.28
3A	c	18	Lower	1.0	0.50	0.50
	b	15	Incisor	2.3	0.42	0.18
	a	2		1.1	0.04	0.06
4A	c	8	Lower	1.0	0.22	0.22
	b	10	Bicuspid	1.8	0.28	0.16
	a	5		1.0	0.14	0.14
5A	c	10	Lower	0.8	0.28	0.35
	b	9	Bicuspid	2.4	0.25	0.10
	a	2		1.3	0.04	0.03
6A	c	11	Lower	0.8	0.31	0.39
	b	18	Bicuspid	2.8	0.50	0.18
	a	19		2.4	0.25	0.10
7A	c	22	Lower	1.9	0.61	0.32
	b	20	Bicuspid	2.5	0.56	0.22
	a	9		2.0	0.25	0.16
8A	c	18	Upper	1.5	0.50	0.33
	b	19	Bicuspid	2.3	0.53	0.23
	a	13		1.7	0.36	0.21
9A	c	24	Upper	1.6	0.67	0.42
	b	26	Bicuspid	3.6	0.72	0.20
	a	5		1.3	0.35	0.11
10A	c	6	Lower	0.4	0.17	0.43
	b	14	Incisor	1.2	0.39	0.33
	a	8		0.7	0.22	0.31

TABLE 4 (CONTINUED)

Dogs	Pulp Area	Reading	Tooth Type	Wet Weight	Micrograms ATP	Micrograms ATP/mg
11B	c	1	Lower	0.70	0.05	0.07
	a	5	Lateral	1.80	0.35	0.20
12B	c	14	Lower	1.50	1.15	0.79
	a	8	Bicuspid	2.91	0.58	0.20
13B	c	18	Lower	2.21	1.55	0.72
	a	12	Bicuspid	2.61	0.93	0.36
14B	c	8	Lower	1.40	0.58	0.41
	a	9	Molar	4.00	0.68	0.17
15B	c	12	Lower	1.70	0.93	0.56
	a	6	Molar	1.20	0.43	0.28
16B	c	13	Upper	1.8	1.00	0.55
	a	10	Lateral	4.7	0.75	0.16
17B	c	5	Upper	0.6	0.35	0.45
	a	6	Bicuspid	1.9	0.43	0.23
18B	c	12	Upper	1.2	0.93	0.77
	a	5	Bicuspid	4.3	0.35	0.08

Explanation of Table 4

Letters refer to dogs; numbers refer to teeth. Small letters c, b, and a refer to coronal, body and apical areas of the pulp. These areas represent arbitrary regions. The coronal area included all pulp tissue above the dentino-enamel junction. The apical portion was that part contained in about the apical 1/4 of the root.

TABLE 5

THE LOCALIZATION OF ATP IN HUMAN PULP TISSUE
(see table 4 for explanation)

Subject		Reading (30 sec)	Wet Weight (mg)	Micrograms ATP	Micrograms ATP/mg
1A	c	35	1.0	0.91	0.96
	b	29	2.4	0.61	0.27
	a	25	2.1	0.41	0.20
2A	c	25	0.7	0.41	0.59
	a	24	1.0	0.37	0.37
3A	c	31	1.3	0.71	0.55
	b	29	1.4	0.61	0.44
	a	19	1.1	0.12	0.11
4A	c	42	1.2	1.26	0.57
	b	23	2.4	0.32	0.13
5B	c	10	4.0	0.10	0.0.03
	b	4	2.0	0.26	0.13
6B	c	5	0.7	0.13	0.19
	b	13	4.0	0.35	0.09
7C	c	31	4.4	0.85	0.19
	b	44	10.4	1.28	0.12
	a	2	3.0	0.05	0.02
8C	c	16	2.6	0.44	0.15
	b	13	10.9	0.35	0.03
	a	43	11.3	1.25	0.11
9C	c	16	4.6	0.44	0.10
	b	28	12.8	0.74	0.06
	a	25	6.2	0.67	0.11
10C	c	18	1.4	0.50	0.36
	b	15	4.7	0.41	0.09

TABLE 6

ATP LEVELS IN IMMEDIATELY EXTRACTED HUMAN TEETH USING WHOLE PULP TISSUE

Subject[1]	Reading (30 sec)	Weight (mg)	Micrograms ATP	ATP/mg
1a	20	16.3	0.80	0.05
b	40	20.0	1.60	0.08
2a	17	12.7	1.02	0.08
b	24	9.1	1.64	0.18
c	33	7.5	2.02	0.27
d	19	14.4	1.16	0.08
3a	77	17.8	2.18	0.12
b	72	24.8	2.04	0.08
c	33	6.1	0.91	0.15
d	69	23.6	1.85	0.08
4a	18	4.7	0.48	0.10
b	14	6.0	0.36	0.06
5a	17	1.8	0.58	0.32
b	13	1.6	0.45	0.28
6a	1	1.0	0.04	0.04
b	8	3.1	0.28	0.09
7a	61	5.6	2.10	0.32
b	21	2.8	1.05	0.37
8a	72	10.4	1.38	0.13
b	53	5.5	1.02	0.19
c	90	10.6	1.74	0.16
d	74	8.1	1.42	0.17
9a	8	1.6	0.21	0.13
b	0	1.9	0.00	0.00
c	10	3.4	0.28	0.08

1. Numbers refer to subjects; letters refer to teeth.

TABLE 7

THE RATE OF LOSS OF ATP FROM PULP TISSUE OF UNPERFUSED EXTRACTED TEETH WRAPPED IN COTTON GAUZE AND KEPT MOIST WITH MEDIUM

Subject	Treatment	Reading (30 sec)	Wet Weight (mg)	Micrograms ATP	Micrograms ATP/mg
1a	Imm. extraction	12	6.1	0.58	0.10
b	15 min. at 37°C	5	6.3	0.21	0.03
c	30 min. at 37°C	4	8.7	0.20	0.02
d	60 min. at 37°C	4	7.4	0.20	0.02
2a	Imm. extraction	26	6.2	1.18	0.19
b	2 1/2 hrs. at 37°C	0	2.8	0.00	0.00
c	5 hrs. at 37°C	0	3.7	0.00	0.00

TABLE 8

ATP LEVELS IN PERFUSED AND NON-PERFUSED TEETH

Subject[1]	Treatment[2]	Medium	Duration	Reading (30 sec)	Wet Weight (mg)	Micro grams ATP	Micro grams ATP/mg Tissue
1a	Saline	Saline	.25 hr	16	5.4	0.80	0.15
b	Saline	Saline	1 hr	0	2.9	0.00	0.00
c	Perfused	1066	.25 hr	14	4.7	0.70	0.15
d	Perfused	1066	1 hr	10	4.4	0.50	0.11
2a	Saline	Saline	1.5 hr	1	3.9	0.05	0.01
b	Perfused	1066	1.5 hr	8	2.4	0.40	0.17
3a	Saline	Saline	3 hr	2	4.2	0.10	0.02
b	Saline	Saline	5 hr	0	4.0	0.00	0.00
c	Perfused	1066	3 hr	14	3.6	0.70	0.20
d	Perfused	1066	5 hr	17	4.8	0.85	0.18
4a	Saline	Saline	12 hr	4	11.7	0.13	0.01
b	Perfused	1066	12 hr	47	5.5	1.58	0.29
c	Perfused	1066	24 hr	43	5.4	1.45	0.26
5a	Perfused	752/1	.75 hr	17	8.5	1.02	0.12
b	Perfused	752/1	2 hr	8	5.6	0.78	0.14
c	Perfused	752/1	5 hr	8	6.1	0.49	0.08
d	Immediate			43	9.0	2.70	0.30
6a	Immediate			11	3.2	0.92	0.29
b	Perfused	752/1	7 hr	11	4.9	0.92	0.19
c	Perfused	752/1	60 hr	9	4.3	0.75	0.17
d	Perfused	752/1	72 hr	13	4.9	1.08	0.22
7a	Perfused	752/1	24 hr	12	2.3	0.22	0.10
b	Perfused	752/1	56 hr	9	6.2	0.17	0.03
c	Gauze	752/1	56 hr	9	3.9	0.17	0.04
8a	Perfused	752/1	4 days	15	1.5	0.28	0.19
b	Gauze	752/1	4 days	6	3.6	0.11	0.03

TABLE 8 (CONTINUED)

Subject[1]	Treatment[2]	Medium	Duration	Reading (30 sec)	Wet Weight (mg)	Micro grams ATP	Micro grams ATP/mg Tissue
9a	Perfused	752/1	2 days	6	3.6	0.11	0.03
b	Gauze	752/1	2 days	3	3.1	0.06	0.02
10a	Immediate			0	1.3	0.00	0.00
b	Perfused	752/1	3 1/2 days	6	2.4	0.11	0.05
c	Perfused	752/1	3 1/2 days	15	2.4	0.28	0.12
d	Gauze	752/1	3 1/2 days	0		0.00	0.00
11a	Gauze	752/1	30 hrs	0	2.3	0.00	0.00
b	Perfused	752/1	30 hrs	0	6.9	0.00	0.00
c	Perfused	752/1	30 hrs	15	2.3	0.28	0.12
d	Perfused	752/1	11 days	9	5.1	0.17	0.03
12a	Gauze	752/1	10 days	27	12.9	0.50	0.02
b	Perfused	752/1	10 days	0	6.8	0.00	0.00
c	Perfused	752/1	10 days	24	8.8	0.46	0.05
d	Perfused	752/1	10 days	34	7.0	0.69	0.10
13a	Perfused	752/1	5 days	32	17.4	1.28	0.07
b	Perfused	752/1	5 days	30	20.6	1.20	0.06
14a	Perfused	752/1	4 days	13	18.0	0.70	0.04
b	Perfused	752/1	8 days	25	18.9	2.02	0.11

Explanation of Table 8

1. Numbers refer to subjects; letters refer to teeth.

2. "Saline" refers to saline control teeth which were threaded with needles and placed in a beaker of physiologic saline. These teeth were not perfused with saline. "Immediate" refers to teeth which, immediately upon removal from the mouth, were extracted with boiling water. "Gauze" refers to gauze control teeth which were not threaded with needles nor perfused but were placed between sterile cotton gauzes kept moist with medium.

TABLE 8

SUMMARY

Number of teeth	Treatment	Microgrms ATP/mg
5	saline controls	0. 01
6	gauze controls	0. 02
29	all perfused teeth	0. 11 ± 0. 08
12	perfused 24 hours or less	0. 17 ± 0. 05
6	perfused more than 24 hours but less than 73 hours	0. 10
11	perfused more than 3 days	0. 07 ± 0. 04
25	all perfused teeth with more than 0. 03 microgrms ATP/mg	0. 14 ± 0. 07

* Subject la was not included.

B. The Histologic Study

1. General

In successfully perfused teeth the collagen fibers of the pulp appeared intact, discrete, and fairly abundant. In some areas possible collagen deposition was occuring with many fibroblasts present. In other areas the connective tissue was composed primarily of undifferentiated mesenchymal cells with an occasional fibrocyte. The odontoblastic layer was for the most part intact, and there was a lack of cellular degeneration. In some areas many odontoblasts were seen in the dentinal tubules. Much of the pulp tissue even after fourteen days of perfusion appeared almost identical to that seen in immediately extracted teeth. The vascular architecture had largely disappeared, however. The capillary-rich, cell-free zone of Weil, adjacent to the odontoblastic layer, could no longer be clearly recognized. In the areas immediately adjacent to the needles varying degrees of regenerating and degenerating activity could be seen. In some locations fibroblasts were in abundance; in other areas tissue histiocytes were present in great numbers. In some regions, particularly in the root portion of the pulp, atrophy, probably a result of pressure, was evident.

In non-perfused and unsuccessfully perfused teeth the histologic findings were similar. There was a general loss in cellular and collagen integrity. There was a noticeable absence of nuclei, and the few which

were present appeared pycnotic. The odontoblastic layer was virtually gone with only a few ghost cells remaining. Tissue histiocytes and fibroblasts were rare except in the apical tissue of the perfused teeth. Much of the pulp exhibited frank necrosis, and in a number of the teeth the entire pulp was necrotic making histologic sectioning impossible.

2. Specific

The following photomicrographs were from two contralateral teeth from the same patient. Tooth A was considered to be successfully perfused. Tooth B was considered to be unsuccessfully perfused. The length of perfusion was fourteen days. (All cells referred to by letter are located directly above the letter).

Figure 3.

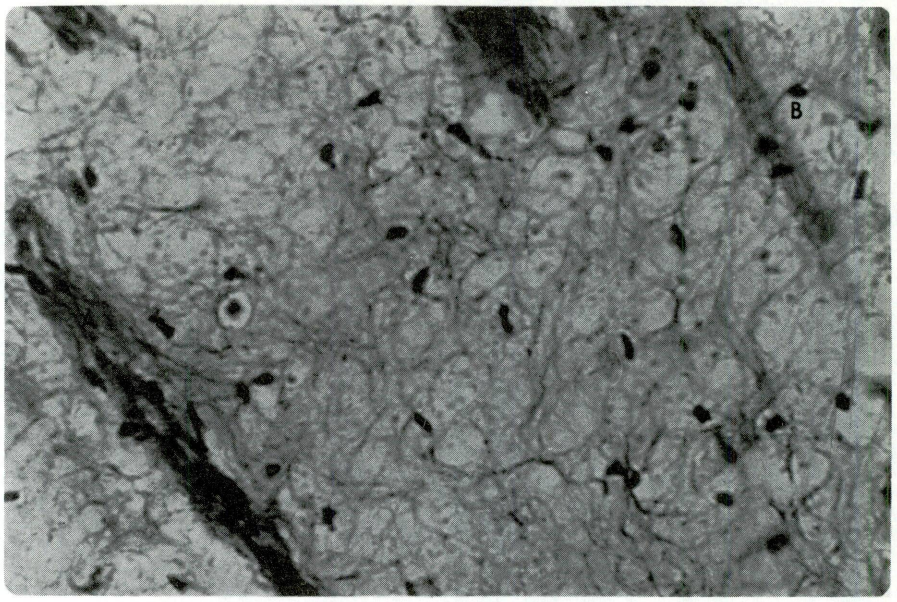
a. Typical mesenchymal pulp tissue from the successful perfusion (450X).

The cellular detail was good and the collagen fibers appeared intact, discrete and abundant.

A) Collagen fiber
B) Undifferentiated mesenchymal cell

b. Typical mesenchymal pulp tissue from the unsuccessful perfusion (450X).

Frank necrosis was present everywhere. There were few nuclei present and cellular integrity was completely gone.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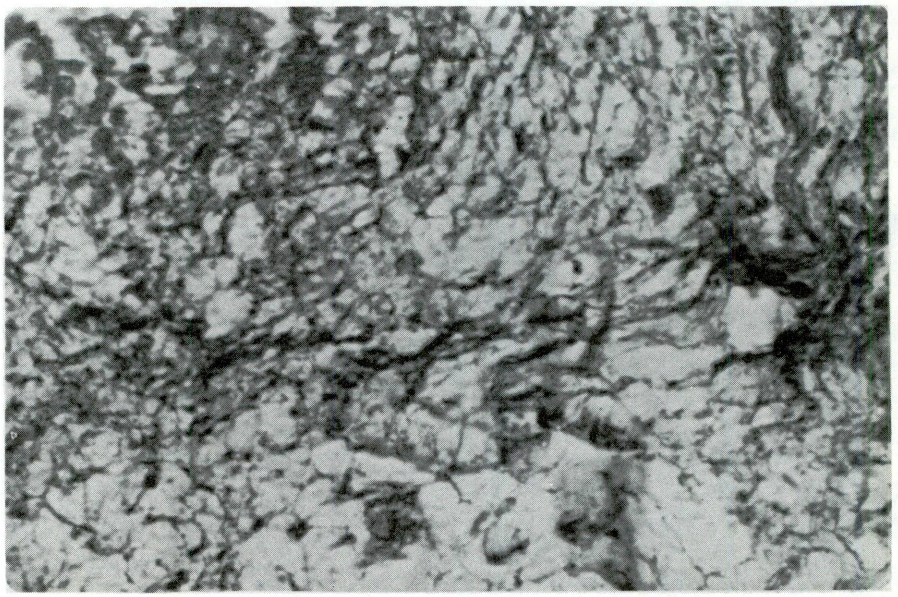


Figure 4.

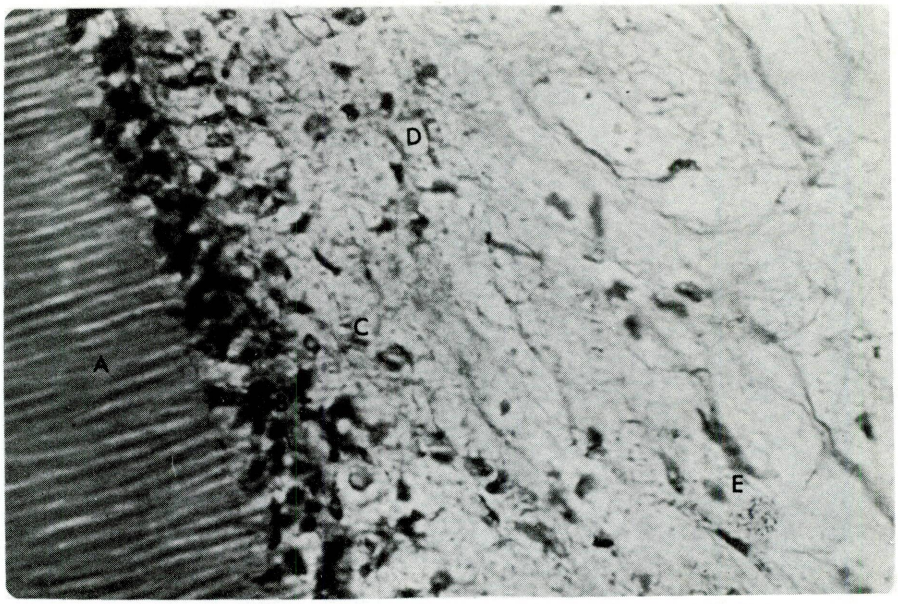
a. A typical area of the pulp in the odontoblast region of the successfully perfused tooth (450X).

The odontoblast layer was of normal appearance with good cellular integrity.
The capillary-rich subodontoblastic region (cell-free zone of Weil) was conspicuously void of capillaries.

A) Dentin
B) Odontoblast layer
C) Cell-free zone of Weil
D) Possible capillary
E) Fibroblast

b. An area of the pulp in the odontoblast region of the unsuccessfully perfused tooth (450X).

The odontoblast layer was completely gone and cellular integrity was absent.
No collagen fibers were evident.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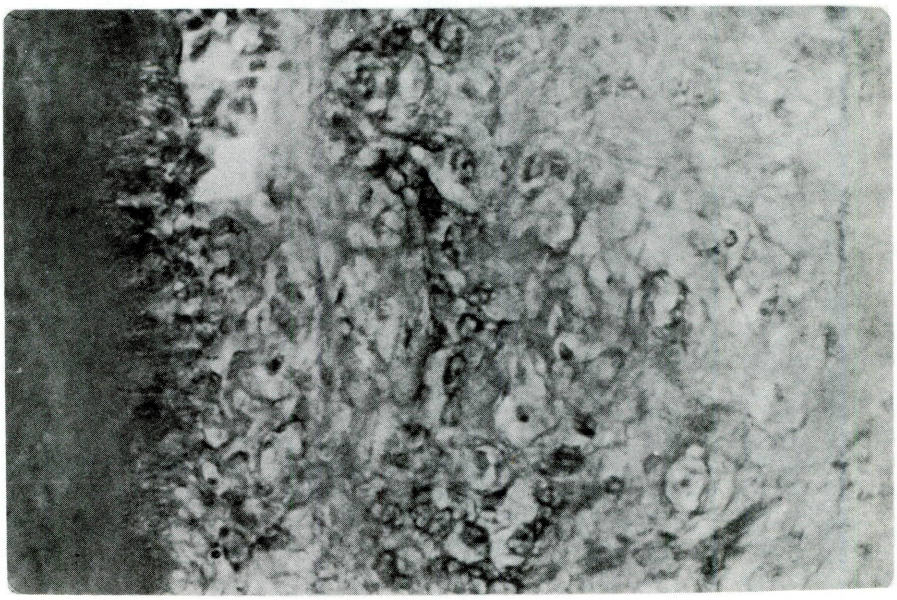


Figure 5.

a. Low power (150X) of the area where the needle was placed.

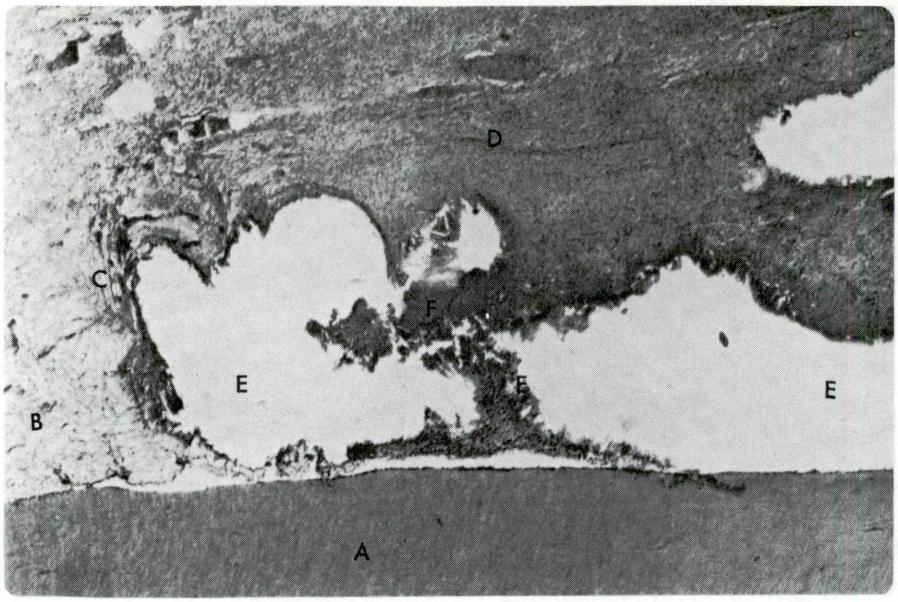
This was the most traumatic looking area for needle placement of all the successfully perfused teeth. Normal tissue was in the coronal region.

A) Dentin
B) Normal tissue
C) Area of collagen deposition
D) Area of inflammation
E) Area occupied by needle
F) Necrotic tissue

b. High power (450X) of the inflammed area of I.

There was marked tissue histiocyte infiltration.

G) Necrotic tissue
H) Area of inflammation
I) Tissue histiocytes
J) Fibrocyte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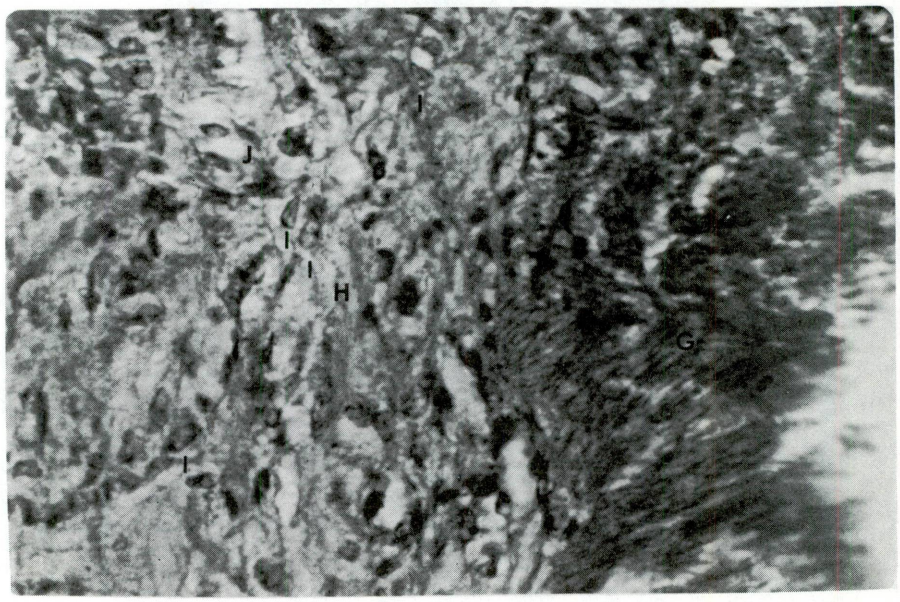
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Figure 6.

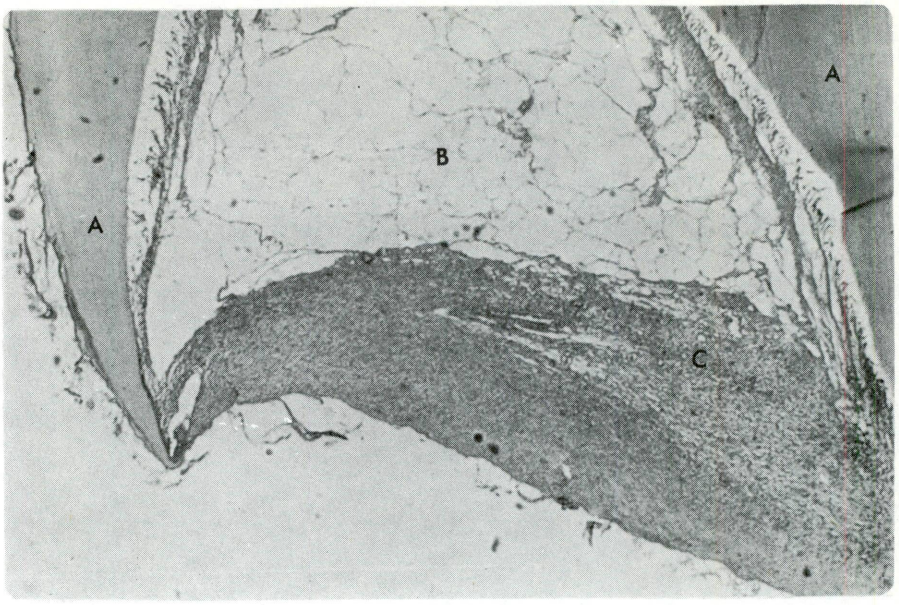
a. Low power (150X) of the apical area of one of the roots in the unsuccessfully perfused tooth.

Apart from a small band of partially viable tissue at the apex all the tissue appeared necrotic.

A) Dentin
B) Necrotic tissue
C) Area of higher magnification in II below

b. High power (450X) of the area C. in the unsuccessfully perfused tooth.

D) Fibroblast
E) Tissue histiocyte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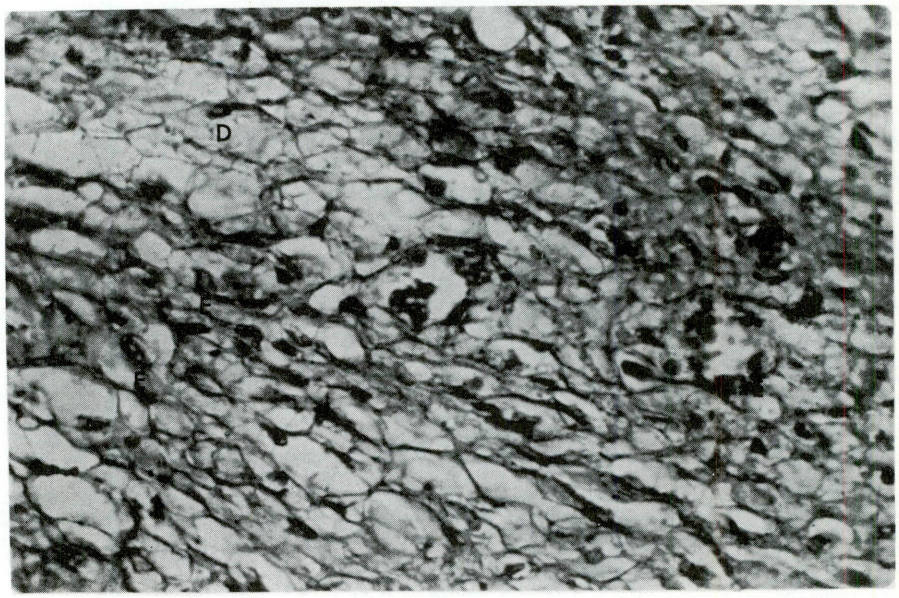
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C. The Monolayer Outgrowth Technique

After 48 hours of incubation in the Rose multipurpose chamber, numerous fibroblast-like cells were seen to have grown out readily from explants obtained from the 72 hour perfused teeth. In the 72 hour non-perfused teeth, explant outgrowth was limited to a few fibroblast-like cells which were seen occasionally. Outgrowth from the pulp tissue of freshly extracted teeth always occurred and was of the magnitude of the perfused teeth. After 7 days incubation explants from the perfused teeth had developed extensive monolayer outgrowth, while the non-perfused controls exhibited little increase in cell number.

Figure 7.

a. Phase contrast photomicrograph of outgrowth from previously perfused pulp tissue (400X).

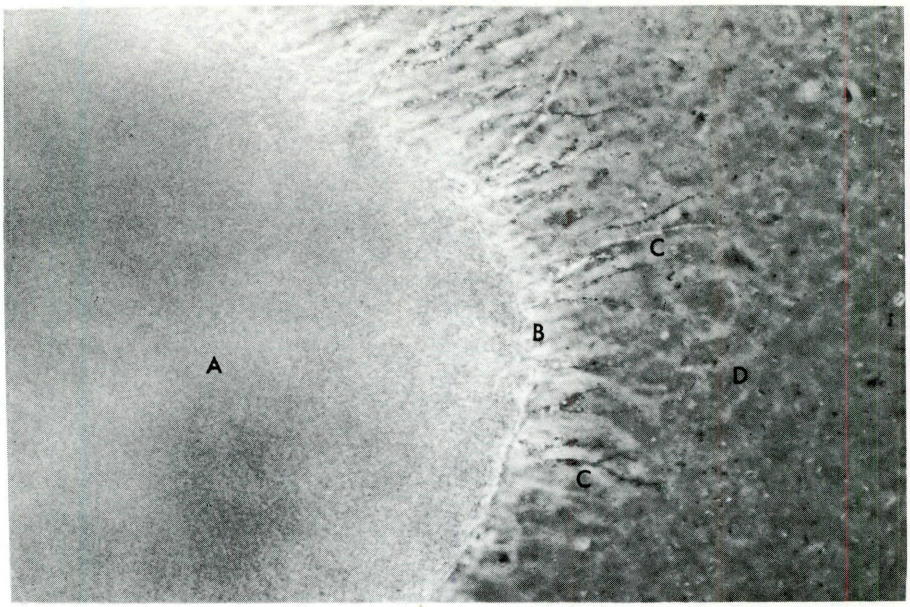
Fibrous (odontoblastic?) processes could be seen radiating from the entire outer circumference of the explant just peripheral to the monolayer outgrowth of fibroblast-like cells.

A) Explant
B) Area of monolayer fibroblast-like cells
C) Fibrous process
D) Plasma clot

b. Phase contrast photomicrograph of outgrowth from a non-perfused control pulp tissue (400X).

A few isolated cells and or processes could be seen.

E) Fibroblast or its process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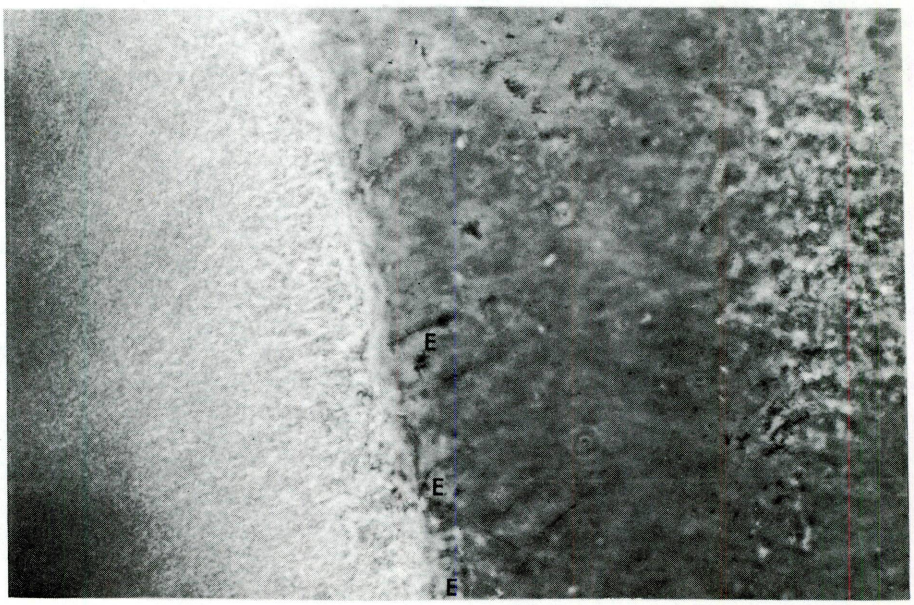
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DISCUSSION

A. The Perfusion Procedure

1. The media

For many years after the classic paper of Fell and Robinson (1929) all organotypic culturing was done on solid media such as the plasma clot. Recently however, fluid media have become more popular due largely to the work of Trowell (1959). NCTC 109 (McQuilkin, Evans, and Earle, 1957) is the most complex synthetic medium which is commercially available (Microbiological Associates). In our work it was supplemented with horse serum and embryo extract and used as the first perfusing medium. However, the cost of NCTC 109 was prohibitive and so CMRL-1066 (Parker, Castor, and McCullock, 1957), also obtained from a commercial source (Microbiological Associates), was substituted. The additions of embryo extract and horse serum were discontinued because of the time involved in their preparation since it was found that CMRL-1066 alone could maintain pulp ATP levels in perfused teeth.

Later, tissue culture medium MB 752/1 (Waymouth, 1959) obtained from the same commercial source was substituted for CMRL-1066 because of the suggestion of Pomerat (1962) that, in general, tissues concerned with mineralization do better in a medium richer in glucose. Medium MB 752/1 contains five times as much sugar as CMRL-1066. However, since MB 752/1 was a simpler medium, and since perfusion periods of up to two weeks were to be attempted, it was felt that an additional supplement should be included in the perfusing medium.

Serum is necessary for the growth of many cell types, but it may be toxic and attack the cells in a number of ways (Terasaki and Chamberlain, 1962; Saksela, Saxen and Penttinen, 1960). Paul (1960) states that there appears to be no advantage in using autologous or homologous serum except in a small number of adult tissues, and,as a rule of thumb, serum is either toxic or non-toxic, irrespective of its origin. Despite this apparent danger, organ culture techniques which employ serum still appear to be favored by most investigators. In an effort to partially overcome the disadvantage of the use of regular serum and yet retain its unknown beneficial aspects, fetal bovine serum was used. It is known to contain lower amounts of fat and gamma globulin and higher amounts of feteum (a growth promoting substance for certain cell types) and was found by Puck, et al (1958) to be quite satisfactory for fibroblast-like cells.

Ito, et al (1963) found when cultivating chick embryo femora by the roller-tube method without plasma, that a serum supplement resulted in maximum values for dry weight, hydroxyproline and phosphorus levels. It was impossible to obtain their results without the use of the serum. The results of these studies indicated that the use of fetal bovine serum as a supplement to the MB 752/1 would promise better perfusion results in our work.

The histologic examination of the perfused pulp tissue revealed a possible chronic type inflammatory process occurring. This response was particularly noticeable in the area immediately adjacent to the place-

ment of the needles. This inflammatory response might be lessened by incorporating some anti-inflammatory steroid into the perfusing medium. These steroids have been shown to be capable of suppressing and modifying fibroblasts in culture (Kline, et al, 1957) and of increasing the success of skin organ cultures later autographed (Gillette, Findley and Conway, 1961). Gillette, Goulian and Conway (1965) actually observed better cellular integrity and tissue architecture in cultures after several weeks when cortisone acetate was added to the medium in the organotypic culturing of skin. A similar observation was made by Weissman and Fell (1962).

In the last few years workers in the field of organ culture have begun to add various hormones, in addition to those mentioned above, to the culturing medium. The organ response, generally based on histologic observation, has been encouraging. For example, Rivera (1964) observed marked changes in mammary gland cultures when a variety of hormones including aldosterone, progesterone, and growth hormone were included in the medium. It has been found that insulin is essential for maximal survival of breast tissue of mice and rats in organ culture (Elias, 1961; Rivera and Bern, 1961). In addition reports have appeared indicating that insulin affected growth in a variety of explanted tissues (Chen, 1954a; Franks, 1961).

It is possible that the addition of certain hormones to the perfusing medium likewise might produce a more "normal" tissue.

2. The delivery of the medium

Thirty gauge needles were used in the course of this work primarily because smaller size needles have less predictable flow rates. Small air bubbles can exert, by surface tension phenomena, great resistance to flow in small diameter tubes (DeVincenzo and Jeffries, 1965). However, if a peristaltic pump were used to deliver the medium to the pulp horns, much smaller size needles could be used and the amount of trauma caused to the pulp by the needle insertion would be considerably reduced.

A completely different approach to the delivery of the medium, although not applicable to organ culture in general, might be a negative pressure suction of medium from the apices to the pulp horns. This could perhaps be accomplished in a manner similar to that used by Kramer (1951) to study the vascular architecture of the pulp. He drilled a small hole at the coronal end of the pulp chamber, and connected this hole to a suction pump. The apices of the extracted teeth were placed in an India ink solution, and when the suction was applied all blood vessels of the pulp were filled with the solution. This procedure would have the advantage over the needle insertion approach of being less traumatic to the pulp tissue. However, Kramer (1960) stated that the technique does not work well if the apices are large, and this is quite frequently the case in young adult teeth. A more serious disadvantage of this technique would be the creation of a negative pressure in an area which normally has a high positive pressure (Brown and Yankowitz, 1964).

The validity of results of physiological studies on the pulp tissue or odontoblasts using a perfusion procedure with negative pressure could be seriously questioned.

3. The rate of perfusion

Fell (1963) stated that there is an optimal relationship between the size of the explant in organ culture and the volume of the medium. Too much medium in relation to explant size might allow necessary metabolic products to diffuse away from the tissue. If this is correct, it would appear that the perfusion of teeth in vitro should be conducted with as small a volume flow as would allow for adequate oxygenation. The importance of flow rate in influencing pulp tissue ATP levels has not been investigated. Mechanical limitations to slow flow rates in terms of small air bubbles-surface tension interactions make this, technically speaking, a difficult task (DeVincenzo and Jeffries, 1965) which, however, could be overcome by substituting a peristaltic pump for the present gravity flow delivery system.

Meyer, Weiner and Grim (1964) using isotope fractionation with K^{42} reported that the average blood flow of 26 canine teeth was 0.98 ml/min-gm. If the assumption is made that the average weight of the pulp in a young human tooth is 12 mg and that the flow rate is the same in human and dog teeth, then the normal flow volume would be 0.65 ml per hour per tooth. However, Meyer and Tschetter (1966) using microspheres in place of isotope fractionation reported an average blood flow

of approximately 0.55 ml/min-gm. in canine teeth. Meyer (1966) attributed this lower blood flow to more accurate technique with the use of microspheres and stated that the higher value using K^{42} was an expression of potassium exchange with the mineralized portion of the tooth. Using the same assumptions as previously the normal volume flow would be about 0.36 ml per hour per tooth.

Thus perfusion rates of 4 ml per hour per tooth would be about 10 times as fast as normal blood flow. It is possible that a slower perfusion rate would be more acceptable, but the amount of oxygen delivered to the pulp tissue via the medium with a slower perfusion rate would have to be considered.

4. The oxygen content of the medium

Parker (1936) was the first investigator to draw attention to the importance of oxygen in the culture of adult tissues, although the advantage of culturing in oxygen was discovered by Loeb in 1897 (Loeb and Fleisher, 1919). In the standard watch-glass method of Fell and Robinson (1929) and in all the subsequent modifications of that technique attempts were made to replace the interrupted blood supply by placing the tissue as close as possible to the atmosphere-medium interface thus allowing for a maximum supply of oxygen. The perfusion procedure does not make allowances for this gas-medium interface except at the level of oxygen dissolved in the perfusing medium.

The solubility of oxygen in biological media is very low and this

results in no small problem in organ culture. Trowell (1959) states that with his organ culture technique twenty organ culture explants consume in one hour all the oxygen dissolved in 2.5 ml of medium. If it is assumed that each of his explants is a sphere of maximum size i.e., 2 mm diameter, then an average tooth pulp (12 mg) might approximate 4 such spheres. If the further assumption is made that the pulp has a Q O_2 similar to that of Trowell's explants, it becomes apparent that pulp tissue would be capable of consuming, at most, all the dissolved oxygen in 0.7 ml of medium per hour. (It is interesting to note that our perfusion rate was about 4 ml per hour which implies that the medium was capable of delivering adequate oxygen to the tissue.)

The diffusion of oxygen from the gas phase is a critical factor in the survival of adult organ cultures. When Parker (1936) made the observation that organ culture explants maintained with 80% O_2 resulted in far better cell preservation than when 21% O_2 was used, he concluded that fluid cultures containing large amounts of organized tissue have very high oxygen requirements. However, his results could be interpreted to mean that he was dealing with the problem of diffusion of O_2 into the center of the explant.

As oxygen diffuses through an explant some of it is utilized by the cells and the least amount of diffused oxygen will be present at the center of the explant. Gerard (1931) in an interesting report discussed the diffusion of oxygen into single cells. His work might be applicable to explants in

organ culture. He concluded that oxygen consumption is quite likely dependent on oxygen concentration up to considerable values. Trowell (1959), making the assumption that the diffusion of oxygen into the cell nucleus model of Gerard (1931) is the same as the diffusion of oxygen into the interior of an explant, concluded that, theoretically, internal necrosis should occur if the diameter of the explant exceeded 1.4 mm. Actually the limiting size is closer to 2 mm. Obviously the limiting size of an explant depends on the normal oxygen consumption of the organ. If air was used instead of O_2 in calculating the maximum thickness of an explant before internal necrosis occurs, a value of 0.64 mm was obtained.

Theoretically, it should be possible to markedly increase the oxygen content of the medium employed. In fact, it is somewhat surprising that with all the concern and effort to develop organ culture procedures which allow for maximal diffusion of oxygen into the tissues, no one has suggested or attempted to increase the O_2 content of the medium.

In the perfusion procedure the O_2 content of the medium could be increased in the following ways: 1) The solubility of O_2 decreases with increased temperature. For example, at 25°C Henry's Law Constant for O_2 in water is 3.30×10^7 which is nearly twice that at 0°C (1.91×10^7). If the medium in the flask, large tubing, and manifold could be kept at or near 0°C, oxygen release would occur in the pulp tissue. 2) Dalton showed that the solubility of the individual gases in a mixture of gases is directly

proportional to their partial pressures, the solubility of each gas being nearly independent of the presence of the other. Thus if the medium were deposited in the perfusion reservoir, the reservoir were evacuated under vacuum, and nearly pure O_2 (with enough CO_2 to maintain the bicarbonate buffer) were redelivered as the gas phase, the partial pressure of the O_2 would more than quadruple (at the expense of N_2). 3) The partial pressure of O_2 in the medium delivered to the pulp horns could be further increased by locating the Erlenmeyer reservoir below the perfused teeth and using the pressure of O_2 in the flasks to determine the flow rate. 4) The solubility of gases in water is usually decreased by the addition of other solutes. The extent of this "salting out" varies considerably with different salts. Perhaps the substitution of one salt for another in the composition of the medium might increase the solubility of O_2 in the perfusing medium.

5. Intrapulpal pressure

Intrapulpal pressure would be expected to be an important factor in successful perfusion. It can be regulated by altering the pressure head of the medium reservoir by varying the height. What is the most desirable intrapulpal pressure? Brown and Yankowitz (1964) drilled a small hole through the enamel and dentin to the dentino-pulpal junction in canines and third incisors of anesthetized dogs. A threaded cannula filled with mammalian Ringer's solution was screwed into the hole and connected to a microsyringe and a pressure transducer of low compliance.

They found that equilibrium pressure was fairly constant for a given tooth, with an average of 58 mm Hg (range 38-78 mm Hg) in the ten tested teeth. The authors concluded that this magnitude of pressure implied a high capillary pressure and thus a relatively high resistance in the venous side of the pulp circulation.

In the perfusion procedure the medium reservoir was located a distance above the teeth, sufficient to give a pressure equivalent to 20 mm Hg. The net intrapulpal pressure should approximate 17 mm Hg. This is considerably lower than the value found in dogs' teeth by Brown and Yankowitz, but is more in the range reported by Beveridge (1965) on human teeth. Beveridge noted blood pressure variations of from 42 mm Hg. to 11 mm Hg. with an average of about 30 mm Hg. It would be interesting to investigate the possible role of pressure in the perfusion procedure.

6. Atmosphere of the Incubator

Into the incubator was continuously delivered a gas mixture of humidified CO_2 and O_2 as described in the section on methods and materials. The CO_2 gas served two functions. First, it was used to control the pH of the carbonate-bicarbonate buffering system used in the medium. Second, it satisfied the requirement of living cells for CO_2, postulated in the early 1950's (Werkman, 1951) and later demonstrated by several investigators. (Harris, 1954; Swim and Parker, 1958). There now exist elaborate methods for CO_2 delivery (e.g., Carpenter and Prater, 1964).

However, because of a lack of this type of equipment and since the dental pulp was not directly exposed to the gaseous environment of the incubator, it was felt that a much simpler delivery system would be adequate. Should it become essential to have a more elaborate method of CO_2 delivery such as that of Carpenter and Prater, or should it become essential to monitor CO_2 levels during perfusion (Searcy, Siddings and Bordon, 1964), methods and materials are available to do so.

7. Temperature of the Incubator

The temperature of the incubator was maintained at 36°C for all the perfusions. However, there have been reports which indicate that, in some cases at least, tissues survived best at a temperature below 36-37°C. Sidman (1956) observed that adipose tissue in vitro was almost always necrotic in 48 hours at 37°C. However, at 33°C the tissue survived much better for up to 15 days. He found that, for a given medium, survival was prolonged with younger donors and lower incubation temperatures. His conclusions were based on a histologic evaluation.

Sarkany, Grice and Caron (1965) reported that growth was optimal at 31°C for their organ culture method of skin and that no growth occurred at 40°C. Gerstner and Butcher (1958) in culturing tooth germs of fetal rats found at temperatures of 34° - 35°C that the germs reached "the most advanced development stages seen in these experiments." However, a temperature of 30° - 34°C was less desirable. It is interesting to note that they found markedly different tissue responses depending on

the temperature used in the range of 20°C to 37.5°C. For example, intense mitotic activity occurred if the explants were first incubated at 20° - 25°C.

Probably the lower the temperature of the organ culture technique, the lower would be the oxygen requirements for the tissue. Perhaps a lower incubation temperature for the perfusion procedure might prove to be desirable, particularly if the oxygen supply to the pulp tissue were adequate.

8. Microbial Contamination

For perfusion periods longer than 3 days microbial contamination often becomes a serious problem. It is most likely that the contamination is present at the time the tooth is removed from the patient. Bacitracin, effective against many oral microorganisms, might be a valuable adjunct to the antibiotic solution. Reaven and Cox (1965), in the culture of human skin, soak the skin in a double strength antibiotic solution of penicillin-streptomycin for one hour before actually preparing it for organ culture. This could be done with teeth.

B. The Adenosine Triphosphate Values

1. The Determination of ATP

The basic procedure used in the ATP determination was that outlined by Strehler and Totter (1954). They suggested that for many tissues a simple boiling water procedure was an acceptable method for the extraction of acid-soluble phosphorus compounds.

The pulp tissue of the tooth, being encased in a calcified shell, is not immediately accessible upon removal of the tooth from its alveolus. A series of cuts with a pair of wire cutters was the most rapid method found for liberating the pulp tissue, but even this required one to two minutes. At times small pieces of tooth structure could be found in the liberated pulp tissue as a result of the procedure.

Once the pulp tissue was freed from the tooth, two possibilities existed in regard to a wet weight determination. The pulp could be weighed and then placed in the boiling water bath, or it could be placed immediately in the boiling water bath and weighed at a later, more convenient time. Since a number of the teeth were collected at private offices, the transportation of an analytical balance would be difficult. In addition, it was found that any chunks of calcified material that happened to stick to the removed pulp tissue would become separated from the pulp during the boiling water procedure. Thus, the likelihood of calcified tissue giving spurious weight recordings would be greatly reduced by weighing after boiling. It was for these two reasons, convenience and less calcified tissue contamination, that the boiling and then weighing procedure was adopted.

However, as can be seen in Table 2, the placement of the tissue in boiling water with subsequent freezing drastically reduces the "wet weight." But, fortunately, the amount of reduction is fairly constant, so that the true wet weight can be estimated with a fair degree of accuracy.

The data of Table 1 clearly demonstrate the importance of plunging the tissue into 100°C. water in the test tube as stated by Strehler and Totter (1954).

It is possible that during the period the ATP samples are stored at -15°C. non-enzymatic hydrolysis could reduce the ATP content of the boiled extract. In a very interesting report Tetas and Lowenstein (1963) showed that the pH of an ATP solution markedly affects the rate of ATP hydrolysis. They found that the rate of ATP hydrolysis increases with decrease in pH and that bivalent metal ions accelerate the rate of hydrolysis of ATP in the acid pH range rather than in the alkaline range. For example, if a solution containing 20 millimoles ATP plus buffer was heated to 80°C. for 5 minutes in a solution containing Cu^{++} at pH 5.5, 156 micromoles of AMP plus ADP were formed. However, in a solution of the same acidity with Ca^{++}, two hours' heating at 80°C. released 5.2 micromoles of AMP plus ADP. The pH of the distilled water used in our extraction procedure was also approximately 5.5, and, since the presence of calcium ions from the pulp tissue would be expected, it is possible that a loss of ATP could occur from hydrolysis at -15°C. in several days.

There is no ready explanation for the large standard deviations which are a part of the results in all the ATP determinations of tooth pulp. That this large standard deviation is an expression of a poor ATP extraction procedure is not supported by the finding reported in Table 3. For example, the mean and standard deviation for the 8

femoral nerve determinations performed on dog B is 0.22 ± 0.02, and the per cent standard deviation is 7.3%; but using the same dog B for pulp tissue ATP determinations (Table 4), a mean and standard deviation for the coronal pulp is 0.54 ± 0.25, and the per cent standard deviation is 46.8%. Likewise, the apical pulp tissue (1.21 ± 0.09) gives a per cent standard deviation of 42.3%. If one considers each tooth a whole organ and realizes that the magnitude of trauma and function in the teeth varies greatly within the same mouth, a larger standard deviation would be expected.

The 30, 60 and 120 second readings of phosphorescense on the fluorometer were made to indicate the approximate relative amounts of ATP and ADP present. The 30 second reading was indicative of the amount of ATP initially present, while the 60 and 120 second readings could indicate a relative difference in the ATP-ADP ratio. Myokinase present in the firefly lantern extract catalyzes the conversion of ADP to ATP, but this source of ATP would not produce noticeable amounts of phosphorescense until after 60 and 120 seconds. It was found that the ratio of ATP to ADP was constant for all samples. Thus, 60 and 120 second readings were not used in any calculations.

The boiling water procedure as used in this study does not appear to yield the same amount of ATP as other standard methods. If the mean ATP level in the femoral nerve of dog B (Table 3) is considered representative, studies by other investigators indicate considerably higher

ATP content for nerve tissue. Gerard and Tupikova (1939), with trichloroacetic acid extraction, found 0.33 micrograms ATP/mg wet weight. Greengard, Brink and Colowick, using hot Tris as extractant, found approximately 0.77 micrograms ATP/mg wet weight. Goldman found the amount of ATP to be 0.65 microgram ATP/mg when perchloric acid extraction was used. Greengard and Straub (1959) found 1.01 micrograms ATP/mg. Cheng (1961) using perchloric acid extraction and luciferin-luciferase system reported 0.48 micrograms ATP/mg wet weight. The levels of ATP obtained from the boiling water procedure appear even smaller if "wet weight" as measured is actually half of the true wet weight.

The boiling water procedure utilizes at least two steps in which the tissue ATP levels might be reduced. First, no homogenization of the tissue could result in entirely equal exposure of all areas of the tissue to the boiling water; the more poorly exposed portions might not yield as much ATP. Second, if the heat denaturation of the ATP-destroying enzymes by the boiling water was not instantaneous, ATP would continue to be hydrolyzed until such time as enzymatic inactivation had occurred. It might take several seconds for the denaturation to occur.

Despite the severe inadequacies of the boiling water procedure, the results obtained in this investigation using ATP tissue levels are valid because the results are comparative in nature.

2. Pulp tissue levels of ATP

There is considerable variation in the concentration of ATP in the pulp tissue of freshly extracted teeth. In an early report (DeVincenzo and Steinman, 1963) it was shown that these variations could not be correlated with age, sex or caries activity. These variations might be a function of the length of time required to remove the teeth from their respective alveoli. The rocking of the tooth in the alveolus produces a volley of impulses in the nerves of the pulp tissue. The resynthesis of acetylcholine after a nerve impulse requires ATP. Perhaps repeated rocking or other forms of surgical trauma to the tooth could deplete the ATP supply of the tissue by an increase in nerve impulses which are an expression of this trauma. However, the ATP level could not be correlated with the length of time required to extract the tooth (DeVincenzo and Steinman, 1963).

The variation in pulp tissue ATP concentration may be caused in part by the length of time required to prepare the pulp tissue for extraction in the boiling water bath. These time variations were not recorded for individual teeth, but were of the order of 30-120 seconds. However, Osawa, Allfrey and Mirsky (1956) and Stecker (1961) have reported that for cell nuclei of thymus tissue the ATP concentration in vitro is reduced 50% in 5 minutes at 30°C. Assuming a similar rapidity of loss of ATP from pulp tissue, it is not likely that a 10 fold difference could be attributed to the 30-120 second time interval required to prepare pulp for

boiling water extraction. In fact, recent work (DeVincenzo and Parker, 1966) using an entirely different ATP extraction procedure indicates that there is little change in the pulp tissue ATP level from 2 to 8 minutes after extraction.

The large variation in pulp tissue ATP might be in part an expression of the function of this tissue as an entire organ. Perhaps certain areas of the pulp or certain cells in this tissue maintain higher ATP levels than the adjacent intrapulpal tissue. Provenza (1958) using routine histologic techniques found that "the density of the capillary bed is greatest toward the periphery of the pulp, where the cell population is also greatest." The following year Cheng and Provenza (1959) reported that in the coronal region of the pulp the arteries were of a larger diameter than the veins, and they suggested that this might indicate an area of greater metabolic activity. Kramer (1960), using an ingenious staining technique employing negative pressure, was struck with the marked vascularity of the dental pulp. He also observed that the capillary plexus tended to be present on only one aspect of the root canal in molar teeth, usually in conjunction with the odontoblasts. Avery and Rapp (1959), utilizing a histochemical technique, noted a relatively high concentration of acetylcholinesterase in that portion of the pulp tissue opposite the enamel. Steinman (1962), also using histochemical techniques, reported greater metabolic activity in the pulp tissue in the same area. Fisher (1965) arbitrarily divided bovine

tooth pulps into coronal, body, and apical areas, as done in this work, and noted a progressively increasing QO_2 from the apical to the coronal tissue. It is not surprising, then, that marked differences in ATP concentrations were found in various areas of the pulp (Tables 4 and 5). This variation in ATP with location indicates the importance of taking all the pulp from a given tooth for the analysis. If only a portion were taken one might not know whether it was from a relatively high or low ATP area.

The rate of loss of ATP from pulp tissue of unperfused extracted teeth is seen in Table 7. The number of teeth represented is small. However, to this number might be added the saline and gauze controls of Table 8. The data of Table 7 indicate that little or no pulp tissue ATP remains after 30 minutes. The saline controls of Table 8 demonstrate that nearly all the ATP is utilized within 1 hour. However, chloride ions are known to be an important inhibitor of the luciferin-luciferase reaction, and it is possible that their presence in the physiologic saline used for the saline controls could prevent phosphorescence. This would lead one to the erroneous conclusion that little ATP was present. However, ATP standards, using crystalline ATP were made up with physiologic saline and no light inhibition was observed.

The data of Table 8 show the marked difference in the ATP levels of perfused and control pulp tissue. This difference in ATP concentration could come from three sources, namely, the perfusing

medium, microbial contamination, or pulp tissue metabolism. The medium employed contained no ATP or other high energy phosphate compound that could be utilized by the luciferin-luciferase system. In addition, samples of medium before and after perfusion were tested for ATP with negative results. Microbial contamination could occur at the time the sterile 30 gauge needles are passed from the apices to the pulp horns. The tooth was placed in medium containing a high concentration of antibiotics as it was carried from donor to apparatus (approximately 30 seconds duration). Although the tooth apices were probably not sterile at the time the needle was threaded into position, the amount of microbial contamination was kept to a minimum. The perfusing medium contained enough antibiotics to inhibit microbial multiplication for the first 24 hours. The phenol red indicator did not change color during the first 24 hour period of perfusion, suggesting that there was no gross contamination. Bacteria could have been introduced, but the environment was not conducive to their multiplication. It is evident from Table 8 that a relatively high level of ATP was recorded after 1 1/2 hours of perfusion and it is doubtful that microbial contamination could account for such a level of ATP in this length of time.

When long periods of perfusion were attempted, microbial contamination, as determined by the phenol indicator and turbidity of the solution surrounding the tooth, was frequently present. However, very

often the pulp tissue from these teeth contained no ATP. This finding would not be expected if microbial contamination were accounting for the tissue ATP levels.

The data of Table 8 indicate that, on an average, the longer the period of perfusion, the lower the pulp tissue ATP level. The data also demonstrate a marked difference in the ATP level of the saline and gauze control teeth as compared to the perfused teeth. If it is assumed that a tissue with 0.03 micrograms ATP per milligram or less is metabolically inactive, an assumption which is supported by the saline and gauze ATP levels, then the perfused teeth contained on the average 17 times the amount of ATP in the saline controls and 8 times the amount of the gauze controls.

A comparison of the results in Table 6 with those in Table 8 shows that immediately extracted human teeth contain 0.15 micrograms ATP/mg as compared to 0.14 micrograms ATP/mg for perfused teeth.

A comparison of the results in Table 7 with those in Table 8 indicate that for teeth not perfused but immediately immersed in medium, the pulp tissue ATP levels are negligible after 30-60 minutes while the perfusion procedure can maintain levels of ATP for long periods.

There are two hypotheses which could account for the ATP levels of perfused teeth. First, the teeth are functioning in the same manner

as they did in the host. That is, there is a delicate balance between ATP-synthesizing and ATP-destroying reactions with a net expression in some type of dynamic ATP pool. Or second, the teeth do not function in the same metabolic manner, resulting in a net decrease in the ATP-destroying reactions, which would also be expressed as a high tissue level in the perfused teeth.

Direct evidence as to which of these hypotheses is applicable to the perfusion procedure is lacking. However, the tissue ATP levels in the saline control teeth of Table 8 indicate that ATP-destroying reactions predominate over ATP-synthesizing reactions. This same conclusion is derived when the data of Table 8 are analyzed. Despite the myriad ATP-ases and pyrophosphatases present in tissues that should break down ATP at a rapid rate at 36°C., high ATP levels are found. Hence, it is concluded that the perfusion procedure is capable of maintaining pulp tissue levels of ATP within the range of those of freshly extracted teeth for periods up to 11 days. The ATP levels in perfused teeth for up to 3 days have been reported (DeVincenzo, 1966). The levels of ATP indicate that the pulp tissue maintains a high degree of energy metabolism during the period of perfusion.

C. The Histology

The results of the histologic study show the marked differences which exist between successfully perfused teeth and others. In non-perfused or unsuccessfully perfused teeth frank necrosis is the rule.

In the successfully perfused teeth a variety of tissue responses are evident, and much of the tissue is of a normal histologic appearance.

The needles definitely cause trauma, and it is in the area adjacent to the needles that the tissue histiocytes are most abundant. In normal tissues few histiocytes are present. Their appearance is one of the classical signs of a chronic inflammatory process. Because histiocytes are seldom seen in normal pulp tissue their presence in the perfused pulp is of interest. They must have originated from a cell present in the pulp when the perfusion began. The most likely precursor is the undifferentiated mesenchymal cell which is so prevalent in normal pulp connective tissue. The presence of tissue histiocytes strongly suggests that the tissue was viable, for these cells had to differentiate and then migrate to the area of inflammation.

In normal pulp few fibroblasts are seen, but in the perfused pulp some areas contained large numbers of this cell type. These fibroblasts, too, are considered to have come from undifferentiated mesenchymal cells. Their presence and also possible collagen deposition are also indicative of viability.

D. The Monolayer Outgrowth

The immediate and extensive outgrowth of pulp tissue explants which had first been perfused as compared to the paucity of outgrowth in non-perfused explants indicated that perfused pulp tissue responds in a manner similar to the pulp tissue from immediately extracted teeth.

When no additional outgrowth occurred in explants from non-perfused pulp tissue after 7 days incubation, it can be assumed that the lack of outgrowth after 48 hours incubation was not the result of slow adaptation of tissue to the perfusing medium.

E. Possible Uses of the Perfusion Procedure

When the limitations of the perfusion procedure and the requirements for maximal success of perfusion become more fully known this procedure could be valuable in a wide range of research investigations. For a procedure not limiting the size of explants could open for study the areas of culture of adult tissues and entire organs, whereas to date organ culture has been limited primarily to embryonic tissues because of their smaller size and lower oxygen requirement.

The author once attempted to perfuse whole hemi-sections of new born rat mandibles using the principles developed for the perfusion procedure. Although there appeared to be continued eruption of the incisor teeth during the 7 days of perfusion, growth was not quantitatively measured. Although an ATP analysis of a perfused mandible after one week was negative, this could have been the result of calcium inhibition of phosphorescence or calcium induced, non-enzymatic hydrolysis of the ATP (Tetas and Lowenstein, 1963).

The perfusion procedure could be of value in a number of dentally related research areas. The influence on the tooth organ of a specific constituent or a variety of constituents placed in the perfusion medium

could be studied. Fell and Mellanby (1952) used this approach in their important study of hypervitaminosis A and bone maturation. A variety of studies on the permeability of enamel and dentin and the factors influencing this permeability could be conducted. An adaptation of the perfusion procedure to tooth permeability studies and preliminary results using Na^{22} and I^{131} were reported by Smith and DeVincenzo (1964). A number of the principles involved in the perfusion procedure were adapted into a self-contained apparatus which could be worn by young rats (DeVincenzo and Jeffries, 1965). A variety of "artificial mouths" e.g., Pigman and Newbrun (1962), have been used for the study of *in vitro* caries none of which are as correct physiologically or biochemically as the perfusion procedure here described. Other attempts at providing a simulated "normal" environment for the study of teeth *in vitro* have not closely simulated the *in vivo* situation.

F. Concluding Remarks

The level of ATP in perfused teeth is in the same range as that for immediately extracted teeth. This probably indicates that the perfused pulp tissue is metabolizing at a rate comparable to that of the normal pulp. (ATP levels also indicate pulp metabolic rates to be of the same order of magnitude as those of nerve and liver). The histologic evidence indicates that successfully perfused teeth give much the same histologic picture as normal teeth. In some areas repair and inflammatory processes, both indicative of viability, are

evident. The monolayer outgrowth technique demonstrated that the outgrowth from perfused pulp readily occurs but that this outgrowth fails to develop in non-perfused teeth. But these combined data for ATP, histology, and monolayer outgrowth do not necessarily indicate that the tissue is being maintained near its normal state.

A serious and frequent error of tissue culture workers is the failure to realize the tremendous adaptability which cells possess. For example, it is frequently assumed that since the histologic picture appears normal, the tissue culture must be a success. Gillette (1963) has clearly shown this is not the case, and yet he falls into error with a similar notion that if his explant can grow when placed back into the host it has "functional viability." This might only indicate that it can grow in the host as well as in tissue culture; it does not imply that all functions are normal. Tissue culture workers often are elated to detect mitotic activity in cultures, and they err in assuming that this proves the particular explant is functioning normally. Perhaps the observation of Gerstner and Butcher (1958) that mitotic activity increases if the explants are first incubated at 20-25°C. might imply that increased mitotic activity may not be an expression of normality.

To answer the question as to what constitutes a normal tissue response under in vitro conditions ultimately brings one to the question as to what constitutes normality in vivo--normality histologically, biochemically, and physiologically--which is in itself an overwhelming question.

SUMMARY AND CONCLUSIONS

The classic organ culture method of Strangeways and Fell with all its subsequent modifications, which in fact comprise all existing organ culture techniques, strictly limits the size of explants which can be grown. In the perfusion procedure here described the limitation in explant size has been overcome by perfusing the tissue with nutrient through small needles placed within the tissue mass. The test organ used in this study was the human tooth. Perfusion periods were up to 14 days. Three criteria were used to evaluate the success of the perfusion procedure. First, comparison of pulp tissue ATP levels in perfused, non-perfused, and immediately extracted teeth, showed immediately extracted pulp to contain 0.15 micrograms ATP, non-perfused saline or gauze control pulp to contain virtually no ATP and pulps of all successfully perfused teeth to contain 0.14 micrograms ATP/mg tissue. Second, histologic observations on the pulps of perfused, non-perfused and immediately fixed teeth revealed marked differences between the perfused and non-perfused teeth. In the perfused teeth, the connective tissue and odontoblast cells appeared intact, and collagen fibers were both plentiful and clearly marked. In some areas numerous tissue histiocytes could be seen, indicating a chronic inflammatory response. In non-perfused teeth frank necrosis, with little evidence of any cellular or collagen integrity except at the apices, was seen. Third, monolayer outgrowth from pulp tissue explants of

perfused teeth showed rapid and prolific growth, while explants from non-perfused teeth displayed no such growth.

The results of the three types of studies indicated that significant biological differences existed between perfused and non-perfused teeth.

It is concluded that the perfusion procedure maintains viability of the pulp tissue of intact teeth for extended periods of time.

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LOMA LINDA UNIVERSITY

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AN ORGAN CULTURE TECHNIQUE FOR MAINTAINING THE PULP TISSUE OF INTACT HUMAN TEETH

by

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ABSTRACT

The size of explants in organ culture is limited by their ability to feed, respire, and excrete by diffusion. The perfusion procedure described in this report is not limited to a particular explant size. The test tissue was the adult human tooth.

In order to maintain the pulp tissue of the intact human tooth in organ culture, special precautions were taken to prevent internal necrosis and to allow diffusion of nutrients and waste products. Using aseptic technique, a standard tissue culture medium plus appropriate antibiotics were delivered to a glass manifold housed in an incubator with an atmosphere at 100% humidity containing about 5% CO_2. Tubing from the manifold led to small needles which were threaded from the apex of each tooth to the pulp horns. The teeth covered with a layer of saturated gauze rested in modified petri dishes.

Adenosine triphosphate concentrations in perfused, non-perfused and immediately extracted teeth were compared to determine the success of the procedure. The mean ATP level in 25 immediately extracted teeth was 0.15 micrograms ATP/mg wet weight. The mean ATP level in 24 teeth successfully perfused for up to 11 days was 0.14 micrograms ATP/mg wet weight, while the ATP level in all non-perfused teeth was 0.01 micrograms ATP/mg.

Eighty-six percent of all teeth were considered successfully perfused.

A histologic study on perfused, non-perfused, and immediately fixed teeth revealed good cellular detail, collagen fiber integrity, and normal appearing odontoblasts in many areas of the pulp in teeth perfused for periods up to 14 days. The non-perfused counterparts demonstrated little cellular detail, no odontoblastic layer, and frank necrosis in many areas. In some areas of the pulp of perfused teeth numerous tissue histiocytes could be seen.

Using standard tissue culture techniques, explants of pulp perfused for 72 hours showed extensive monolayer outgrowth of fibroblast-like cells. Non-perfused pulp tissue after 72 hours in saline showed no such outgrowth.

It is concluded that the perfusion procedure maintained viability of the pulp tissue of intact teeth for extended periods of time.