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Uptake of ³H-Cortisone in the Rat Incisor a Frozen Tissue Autoradiographic Study

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UPTAKE OF ^{3}H-CORTISONE IN THE RAT INCISOR

A FROZEN TISSUE AUTORADIOGRAPHIC STUDY

by

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DEDICATION

I dedicate this thesis to my wife Aisha,

and my children,

Ibtisam, Ibtihal, Elham and Serage Eddin.

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VITA

Mohammed Mohammed Rahima was born on June 10, 1950, in Garian, a small city in the mountains located a hundred kilometers to the southwest of Tripoli, Libya.

He was graduated from Garian Secondary School, Garian, Libya, in June, 1970. He then began his undergraduate education at Alexandria University School of Dentistry, Alexandria, Egypt. A degree of Doctor of Dental Surgery was awarded him in June, 1975.

In August, 1975, he began working as a dentist at the Institute of National Association Security, Tripoli, Libya. He was head of the INAS Dental Department from March, 1976 until September, 1978. He also worked in his private practice from July 1977 until September, 1978.

In September, 1981, he began a two year graduate program at Loyola University School of Dentistry, Chicago, Illinois, leading to a Master of Science in Oral Biology and a postgraduate Certificate in Pedodontics.

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INTRODUCTION

In spite of a substantial number of investigations, the factors responsible for tooth eruption are still unknown. Theories very much in favor a number of years ago, have been seriously questioned today in light of new experimental research. The continuously erupting incisor of the rat has been the subject of many investigations in this search for the mechanism of tooth eruption. The present study will also utilize the rat incisor in an effort to contribute additional information toward a solution to this problem.

Schour and Rogoff (1936) were first to observe a disturbance in calcification of dentin in the rat incisor following adrenalectomy; however, due to the short survival period, rate of eruption was not measured. Mullinos and Parmer (1942) reported that daily injections of deoxycorticosterone to newborn rats stimulated tooth eruption. Since then, many investigators have also shown an increase in eruption rate of the rat incisor following cortisone administration (Parmer et al. (1951), Domm and Marzano (1954), Goldsmith and Ross (1955), Garren and Greep (1960), Domm and Wellband (1960, 1961), Kiely (1967), and many others).

Sicher (1942), Ulmansky and Shapiro (1963), and Gowgiel (1967) have reported that cell proliferation is at least partly responsible for tooth eruption. Domm and Kiely (1968) observed an increase in number of mitosis in the apical tissues of the rat incisor upon administration of cortisone. They proposed that the positive response of the proliferating tissues to

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the steroid was the cause of the increase in eruption noted by others. Kiely and Domm (1973) used ^{3}H-thymidine to investigate the effect of cortisone on DNA synthesis in the incisor over a period of twenty-four hours. They found that a single injection of cortisone to adrenalectomized rats resulted in a significant increase in the number of the labeled cells in the cervical loop of the maxillary incisor when compared to untreated adrenalectomized animals. These studies indicate that cortisone, or a metabolite thereof, may deposit in the tissues of the cervical loop of the rat incisor.

Although it has yet to be shown that the apical tissues of the rat incisor are specifically receptive to cortisone, much indirect evidence has shown a positive response of this tissue to this steroid. In adrenalectomized and normal rats, the cells of the cervical loop were seen to increase in number after the administration of cortisone. Furthermore, many studies show a direct relationship between rate of eruption of the incisor, and the availability of cortisone.

Nuclear retention of steroid hormone by target tissues appears to be a prominent characteristic which differentiate target and non-target tissues (Jensen et al. 1968). A study by Zigmond and McEwan (1970) indicates that nuclear binding of estradiol in brain and other target tissues reaches a peak at one or two hours and retention remains high even at four hours with the result that the ratio of target to non-target tissues is highest at four hours (longer intervals were not studied).

The application of autoradiography to the detection of diffusible substances, such as steroids, poses particular problems. The threat of

translocation or loss of material is the main stumbling-block in localizing soluble, lightly bound or unbound compounds by autoradiography. In order to obtain a satisfactory autoradiography of labeled diffusible substances, all fluids used in the classical histological techniques must be excluded during the preparation of the autoradiogram in each-and-every instance to the end of photographic exposure. It is necessary to avoid fixation, dehydration, embedding as well as dipping of sections in liquid emulsion or in water. The techniques which were introduced by Stumpf and Roth in 1966 for the study of diffusible substances, namely the dry-mount and the thaw-mount techniques, fulfill these prerequisites. The thaw-mount technique involves sectioning of frozen tissue in a cryostat at very low temperature (-25 to -40°C) and then mounting these frozen sections on dry emulsion coated slides. In recent years the thaw-mount technique has been used more extensively since it is easier to perform than the dry-mount autoradiography. We have, therefore, chose to apply this techniuqe in our present investigation.

The purpose of this study is to examine the tissue of the cervical loop of the rat incisor and surrounding areas for deposition of ^{3}H-cortisone using a frozen section-autoradiographic technique. Through this procedure, we hope to determine the degree of specificity of this steroid to the apical tissues of the rat incisor by observing its loci of deposition and intercellular concentration.

REVIEW OF THE LITERATURE

A. Mechanisms of Tooth Eruption

The theories put forward to explain the mechanism responsible for
axial movement during tooth eruption suggest that the force is generated
by (a) alveolar bone deposition, (b) cellular proliferation (especially
in the basal region of the pulp), (c) root growth, (d) tissue-fluid pres-
sure, (e) tension generated within the periodontal ligament, (f) pressure
from vascular beds, (g) pressure from muscular action on the alveolar
process, (h) growth of dentin and pulpal constriction. The majority of
investigators implicate only one of these systems as being primarily
responsible for eruption - the prime mover.

Until recently, there were two basic school of thought concerning
tooth eruption; one favoring cellular proliferation, while the other
favored the tissue vascularity theory. Most recently, the periodontal
ligament has been implicated as a force in tooth eruption.

Sicher (1942 a, b), on the basis of morphological studies in human
teeth and the rat incisor, thought that tooth eruption was the result of
differential rates of cell proliferation in the apical tissue of the
tooth; primarily, active proliferation of the pulpal cells. A "hammock
ligament" was described by Sicher which separated the periapical tissue
from pulpal tissue. This ligament was seen to traverse the base of the
growing roots and was anchored into both sides of the alveolar bone.
It was postulated that this ligament transmitted the pressure from pulpal

proliferation in a coronal direction causing eruption and at the same time prevented resorption of the apical bone. Realizing that some teeth, such as human canine, travel a distance greater than can be accounted for by the developing root length, Sicher concluded that apposition of fundic (basal) bone causes additional eruption. Therefore, in human teeth, bone growth in addition to pulpal proliferation was considered to provide the force of eruption. Sicher failed to demonstrate a hammock ligament or fundic bone apposition in multirooted teeth. He felt that bone growth at the furcation was responsible for the eruptive force in these teeth.

Taylor and Butcher (1951) interrupted the sympathetic innervation to the rat incisor and ligated major arteries supplying blood to this tooth. From their observation, they concluded that the rate of eruption of the incisor is not sensitive to even fairly noticeable changes in blood flow.

The tissue hydrostatic pressure hypothesis of tooth eruption has been suggested by Bryer (1957). The eruption force is generated in response to tissue hydrostatic pressure in and around the tooth. Three pieces of evidence are now known to support this hypothesis:

1. Human incisor teeth at rest have been shown by Korber (1970) to undergo pulsatile movement consisting of a shift of 0.4 μm from the neutral position toward the labial side, with a very much axial movement, synchronous with the arterial pulse. This illustrates that the hemodynamic system can cause some tooth eruption.

2. The tissue fluid pressure beneath an erupted tooth is greater than the pressure above it. This has been recorded for the erupting,

but still buried, permanent canine of the dog (van Hassel and McMinn, 1972), in which a pressure differential of 15 mm Hg favoring eruption was observed.

3. Once a tooth has erupted into the oral cavity, any tissue fluid pressure above atmospheric pressure in the immediate vicinity of the tooth could contribute to an eruptive force. Evidence suggests that pulp and periodontal tissue fluid pressures are of the order of 25 and 10 mm Hg respectively (van Hassel, 1971 and Lamb and van Hassel, 1972). In most other connective tissue the pressure is 1-3 mm Hg above atmospheric.

Scott (1953), on the basis of gross dissections of fetal, newborn, and young sheep, concluded that active tooth eruption is the result of pulpal proliferation between the calcified part of the tooth and the base of the follicle. He emphasized the role of the dental follicle and gubernacular cord during eruption as a guidance mechanism. Scott states that during active eruption the fibers of the dental follicle organize into the periodontal ligament proper, and into the hammock ligament as described by Sicher. Cahil and Marks (1980) examined the role of the dental follicle of the dog premolar teeth in the eruption process. They found that tooth eruption did not take place in the absence of the dental follicle. Cahil and Marks claimed that the role of the dental follicle may be part of the local control of alveolar bone formation and resorption in tooth eruption. The dental follicle has been shown to function biochemically in collagen breakdown that takes place during the bone remodeling process involved in the eruption of the dog premolar teeth (Woessner and Cahil, 1974).

Ness (1964) in a critical review of the literature on tooth eruption, concluded that the force exerted by blood pressure is primarily responsible for tooth eruption. However, in a later review, Ness (1970) revised his opinion and stated that his favorite hypothesis was now that fibroblasts located in the periodontal tissue exert tension along their length, thus providing the force for movement of the rodent incisor.

Main and Adams (1966) examined the effects of anti-mitotic and hypotensive drugs on the eruption rate of the rat incisor. From their experiments, they concluded that there was no direct relationship between the rate of eruption and blood pressure or cellular proliferation. As an alternative theory, Main and Adams postulated that the mechanism of tooth eruption most likely lies in the periodontal membrane.

Gowgiel (1967) using irradiation to prevent root formation in 10 day old male, albino rats, observed that the crowns continued to erupt. As a result of lack of root formation, he eliminated the periodontal ligament proper and pulpal proliferation from consideration in tooth eruption. He observed changes in cell and fiber size, number, and orientation in the follicular sac which he correlated with eruption. From these observations he concluded that tooth eruption is the result of follicular sac growth.

Kiely (1967) investigated the effect of cortisone on the rate of eruption, DNA synthesis and mitotic activity in the rat incisor. He found an increase in the rate of DNA synthesis of adrenalectomized rats and an increase in the mitotic rate of normal rats in the cells of the cervical loop after injections of cortisone. From these observations,

Kiely concluded that cellular proliferation of these tissues must be considered in any discussion concerning the mechanism of tooth eruption.

Thomas (1964 and 1965) suggested that tractional forces in the periodontal ligament due to molecular cross-linkage and fiber aggregation during collagen maturation must be considered as a force in tooth eruption. He felt that intermolecular and intramolecular cross-linkage produce tension in the oblique fibers of the periodontal ligament thus pulling the tooth coronally.

Beersten et al. (1974) have demonstrated the existence of an ultra-structural system that could account for the active migration of fibroblasts. In these cells, they have observed the existence of microfilaments and microtubules arranged in networks as well as in bundles in the peripheral part of the system. They postualted that these microfilaments and microtubules provide a contractile mechanism whereby the periodontal fibroblasts generate the force of tooth eruption. Thus, it is tempting to suggest that the increased number of microtubules relates to the increased eruption of the unimpeded tooth.

Recent evidence suggests that eruptive mechanism resides within the periodontal ligament (Zajicek (1974), Berkovitz (1975,1976), Beersten (1975) and Pitaru et al. (1976)). Their studies indicate that during the eruptive process, cell movement within the periodontal ligament is accompanied by remodeling and turnover of the periodontal components. More specifically, it has been postulated that the eruptive force is a tensional one, generated by active motility of fibroblasts and transmitted to the tooth by way of collagen (Beersten et al. (1974). The fibroblast,

capable of simultaneously synthesizing and degrading collagen, serve as a cellular representative for this process (Rippen (1976), Sodak (1976) and Ten Cate (1976)). Berkovitz (1981) stated that it is not possible to determine whether the fibroblasts move actively to cause eruption, or the fibroblasts are carried passively with the tooth-related part of the ligament; the eruptive force being generated by another system. A definite proof for either hypothesis is hitherto lacking.

Azuma et al. (1975) implicated intercellular contacts between the cells of the periodontal ligament in the transmission of forces generated by these cells into an eruptive force. They suggested that the increase in simplified desmosomes number and the increased eruption of the unimpeded incisor might be related. However, Shore et al. (1981) observed that the connective tissue adjacent to the enamel of the rat incisor showed a greater number of simplified desmosomes than the periodontal ligament proper. As this connective tissue gains no attachment into the tooth and has never been implicated in generating the eruptive force, they concluded that there may not be a simple relationship between cell contacts in the ligament and eruption.

A fiber distinct from collagen and having some characteristics in common with pre-elastin, the oxytalan fibers, has been described in the periodontal ligament (Fullmer et al. 1974). Using special staining procedures and light microscopy, Beersten et al. (1974) showed that oxytalan fibres with diameters between 0.5 and 2.5 μm are abundant in the periodontal ligament of the rat incisor. They claimed that these fibers may have a possible role in tooth eruption. Shore et al. (1982) showed that

oxytalan fibers, which have a minimum diameter of 0.2 μm and a maximum

diameter of about 1 μm, constitute only of about 0.3 per cent of the

extracellular matrix of the periodontal ligament of the rat incisor.

Shore et al. also found that there was no difference between the oxytalan

fibers in impeded (teeth left in occlusion) and non-impeded rat incisors.

Rippen (1976), Sodak (1976,1977) and many others have shown in their

studies that protein metabolism in the periodontal ligament is high rela-

tive to other body tissues. The collagen in rat molar periodontal tissues

has an unusually high turnover compared to other adult connective tissues

(Sodak, 1977). The reason for the high turnover rate of collagen and

other proteins in the periodontal ligament is unknown. Sodak (1978) sug-

gested that an eruptive mechanism is not responsible for the increased

collagen turnover rate. He observed that the rate of ^{3}H-proline incor-

poration into hydroxyproline of periodontal ligament collagen of fully-

erupted rat molars was three times faster than that for ligament collagen

of the continuously-erupting rat incisor. Limeback et al. (1978) sug-

gested that increased collagen turnover in the periodontal ligament may

be due to mechanical stress resulting from occlusal forces. Plecash and

Bently (1982) in their study concluded that the high rate of collagen

turnover is an intrinsic property of the periodontal ligament tissue and

is uninfluenced by occlusal or eruptive forces.

To determine whether remodeling of collagen occurs predominantly

in a specific area of the periodontal ligament or this process extends

uniformly across the entire width of the ligament, several investigators

have suggested that the most active site of collagen metabolism in the

rodent incisor is in the intermediate plexus, whereas in molar teeth, collagen turnover appeared to be evenly distributed throughout the width of the periodontal ligament (Garant (1976), Ten Cate et al. (1976), Rippen (1976, 1978) and Beertsen et al. (1978)). All these studies were based upon autoradiography or electron microscopy. Conflicting results were also reported regarding the amount and site of greatest apparent remodeling in the periodontium (Beertsen and Everts, 1977 and Diaz, 1978).

In summary, no one theory of eruption is yet supported by sufficient experimental evidence for it to be identified as the prime mover. The overall process of eruption probably required the integrated activity of all the biological systems within the periodontium.

B. Role of the Basal Tissue and the Dental Pulp in Rat Incisor Eruption

The role of the basal tissue and the dental pulp in eruption of the rat incisor has been studied by many investigators. Herzberg and Schour (1941) either removed the pulp of the rat incisor or cut away the odontogenic base but found no alteration in eruption. Massler and Schour (1941) after transecting the rat incisor observed a "normal" eruption rate of the coronal half while the base half did not erupt. Bryer (1957) in repeating these three experiments, reported that the rate of eruption was initially much decreased but a subsequent recovery to the normal rate was observed. In other experiments Lefkowitz et al. (1947) removed a large portion of the enamel organ from the lower incisors of young dogs and later observed a retarded rate of eruption. Following removal of the odontogenic epithelium in the lower incisors of rats, Mohammed and Mardfin

(1962) observed a degradation of pulp cells, a reduction in vascularity and rate of eruption reduced to one-half of normal. Atrophy of the ameloblasts was noted four weeks after the operation. Reith and Cotty (1962) found the rate of eruption to be significantly reduced in rats in which the lower incisors were filled with zinc oxide and eugenol at either the basal end or in the center. Similarly, Eccles (1965) removed the pulp of the lower incisors in rats and then filled the cavity with a mixture of zinc oxide and eugenol and finally inserted a gutta percha point into the cavity. In the animals in which the pulp canal was filled to within 1-2 mm of the basal end, eruption ceased and did not resume. When the filling was more than 4 mm from the basal end, eruption continued, slowly at first but finally returned to normal. Upon histological examination the periodontal membrane of the unerupted teeth was found to be uninjured, thus supporting the concept that this structure is not involved in eruption but that the pulp is. Ulmansky and Shapiro (1963) noted an increase in the eruption rate of the amputated right incisor of rats. However, when the pulp in these teeth was damaged by the insertion of a metal reamer into the pulp cavity, the eruption rate was delayed, slowed, or completely stopped. Many pathological changes were observed in these incisors such as regression of the ameloblasts and papillary layer, edema and hemorrhage in the basal area, atrophy and calcification of the pulp, disorganization of odontoblasts and congestion in the periodontal membrane.

Berkovitz and Thomas (1969) removed the proliferative odontogenic base of the rat incisor. They observed the eruption rate of these

root-resected teeth to obtain control levels in 4-7 days after the opera-
tion and remain at this level for approximately 12 days after which time
exfoliation occurred or eruption ceased. From this experiment they con-
cluded that the following mechanisms do not appear to be essential to
eruption: (1) root elongation; (2) pulpal proliferation and dentin forma-
tion; (3) fundic bone deposition; (4) tissue fluid pressure. Berkovitz
and Thomas, however, suggested that the force of eruption is located in
the periodontal ligament.

Berkovitz (1972) studied the effects of anti-mitotic drugs upon the
eruption rates of normal and root-resected lower rat incisors. A similar
fall in eruption rates was observed in both the normal and the root-resec-
ted incisors of treated rats. From these experiments, Berkovitz concluded
that the eruptive mechanism following root resection was "physiological"
and that growth of the periodontal ligament is most likely the important
component in the eruptive process.

C. Cortisone and the Incisor Eruption

Numerous studies have shown cortisone to stimulate the rate of
eruption in the rat incisor. Following the administration of cortisone
to pregnant rats, Goldsmith and Ross (1956) observed precocious develop-
ment and eruption of the lower incisor in the fetal rats. They also re-
ported that the incisors of cortisone-treated neo-natal rats erupted
earlier than controls. Parmer, Katonah, and Angrist (1951) noted that
cortisone stimulated incisor eruption in newborn rats while inhibiting
body growth. In a study on adult male and female rats, Domm and Marzano

(1954) reported that administration of cortisone accelerated the eruption rate of both maxillary and mandibular incisors. In a group of hypophysectomized rats, they noted a profound decrease in eruption. However, an increase in rate was observed when these animals were administered cortisone. Domm and Leroy (1955) and Leroy and Domm (1955) observed premature eruption of the incisor in the newborn rat when cortisone was given to the pregnant mother, to newborn rats or to the fetus in utero. Garren and Greep (1960) also noted a reduced incisor eruption rate in hypophysectomized rats. Like the results of Domm and Marzano, cortisone was also found to restore the eruption rate to normal. Garren and Greep (1960) made the observation that cortisone administered to normal adult male rats resulted in an acceleration of the eruption rate of the incisor.

Following adrenalectomy, Domm and Wellband (1961) observed a decrease in the rate of eruption of the rat incisor. However, with the administration of cortisone the rate was increased above normal. After thyroidectomy and thyroadrenalectomy a reduction in the rate of eruption was also noted. Again, cortisone therapy increased the eruption rate of these animals. Kiely (1967) found that the eruption rate of the rat incisor increased with cortisone administration and so did the rate of mitosis in the normal incisor (Domm and Kiely, 1968) and DNA synthesis in the incisor of the adrenalectomized rats treated with cortisone (Kiely and Domm, 1973). Kiely and Domm (1977) found that incisors of Mg-deficient rats showed a marked inhibition of eruption and a decrease in mitosis of apical tissue. Cortisone administration resulted in some increase in eruption rate and cell division. Kiely and Domm (1977) suggested that

the effect of cortisone on incisor eruption is mediated through a stimu-
lation of cell division. Daily injections of 1.25 mg/100g body weight of
hydrocortisone also has been found to increase the eruption rates of the
mandibular unimpeded root-resected incisors of the rat (Berkovitz, 1971)
and of normal mandibular unimpeded incisors (Sobkowski and Smithgall,
1960; and Moxham and Berkovitz, 1980).

It is an accepted fact that incisor reduced out of occlusion erupt
at an accelerated rate (Hoffman (1939), Taylor and Butcher (1951), Ness
(1956), Bryer (1957), Ulmansky and Shapiro (1963), and Wellband and Domm
(1964). With the administration of cortisone, Wellband and Domm (1964)
observed a further increase in the eruption rate of the amputated inci-
sors. From the preceding review, it is apparent that no disagreement
exists regarding the observation that cortisone does indeed enhance the
eruption of the rat incisor. The reasons for this effect, however, are
still to be discovered.

D. Effects of Cortisone

1. General Tissues Effects

The effects of cortisone on a variety of tissues have been reported.
Parmer, Katonah and Angrist (1951) found body growth to be inhibited in
rats treated with cortisone. Fleming (1953) noted a decrease in the body
growth of cortisone treated guinea pigs. Glickman, Stone and Chawla
(1953) found that 4 week old white mice treated with cortisone were less
active, smaller and have more hair loss than controls. They found that
cortisone administration resulted in retardation in vertical growth of

long bones, a narrowing of the epiphyseal plates, cessation of cartilage proliferation, and osteoclastic bone trabecule resorption in the metaphyses. D'Arcy and Howard (1961) injected cortisone acetate to albino rats for six weeks and found that cortisone caused a retardation in body growth. Adrenal and pituitary weights were less than controls without consideration for loss of total body weight. Nakamoto and Wilson (1968) found that cortisone (40mg/kg) administered to young rats resulted in retardation of weight gain, skin damage, hair follicle defects and increased mortality. The erythrocyte count remained normal but the lymphocyte count dropped markedly. Atrophy of the adrenal cortex and the lymphoid tissue of the thymus was apparent. After eight days, there was reduced hematopoietic tissue in the spleen, liver, and femur. It was concluded that immunosuppressive doses of cortisone could not safely be used in the very young animal.

Layton (1951) showed that high doses of cortisone inhibited the synthesis of chondroitin sulphate. This effect may be secondary to the alteration in protein metabolism indicated by a large negative sulfur balance. He claimed that a failure to form hexosamine could be partly responsible for the disappearance of chondroitin sulphate from the tissue; a simultaneous decrease in all mucopolysaccharides would then be expected. Cortisone inhibits both hyaluronidase and streptococci (Birk, 1953), increases the amount of alkali soluble and neutral salt soluble collagen in the chicken (Kivrikko, 1963), delays the development of granulation tissue (Murota et al. 1975), inhibits the synthesis of DNA of the mouse liver (Amaral and Werthamer, 1970), and the ability to alter collagen metabolism

has been attributed to cortisone (Russel et al., 1978). Cortisone has also been found to inhibit the uptake of oxygen by rat liver mitochondria, to reduce the activity of the enzyme cytochrome oxidase, and to uncouple the process of oxidative phosphorylation which occurs in mitochondria (Karppala and Pitkanen, 1960). DeVenuto et al. (1968), by the use of cell fractionation and steroids which were labelled with radioactive tritium and carbon-14, showed that corticosterone and cortisone exhibit a definite interaction with the nuclear and mitochondrial fraction of rat liver cells.

Davidson (1965) found that cortisone induces the synthesis of a number of new proteins in the liver cells of adrenalectomized rats. Among these proteins are enzymes required for the synthesis of glucose (but not the breakdown of glucose) and enzymes involved in the metabolism of amino acids. Moreover, cortisone increases the total synthesis of protein by the liver cells. The effect of cortisone on the synthesis of messenger RNA is apparent as soon as five minutes after the hormone has been administered; within 30 minutes the amount of RNA produced has increased two to three fold and probably includes not only messenger RNA but also ribosomal RNA. These events are followed by an increase in enzyme activity. In this study, the effect of cortisone on DNA synthesis was not examined.

Roberts (1969) and Kenner (1970) found that the effect of hydrocortisone on bone is dose dependent. Low doses of cortisol (1-5 mg/kg/day) stimulated bone resorption and high doses of cortisol (20-75 mg/kg/day) inhibited bone resorption. The low doses of cortisol reduced the bone volume by increasing the number of osteoclasts and decreasing the number of osteoblasts. Over a long period, dosages over 2.0mg/kg/day

inhibited bone resorption (Roberts, 1969). Roberts also noted at low doses the corticosteroid action is characterized by increased progenitor cell proliferation, increased number of osteoclasts and increased resorption of alveolar bone.

Henderson et al. (1971) found that the administration of cortisone to rapidly growing rats results in a prompt and virtually complete inhibition of liver DNA synthesis. There is a concomittent stimulation of liver RNA, protein and glycogen synthesis. Inhibition of DNA synthesis is sustained for as long as the hormone is administered and rapidly returns to normal upon cessation of hormone treatment. The inhibition was observed in animals receiving as little as 0.3mg/100g/day of cortisone, and the doses required for inhibition of DNA synthesis and for suppression of somatic growth are similar.

2. Oral Tissue Effects

The dental literature contains a number of reports in which the effects of cortisone on oral and dental tissues have been studied. Schour and Rogoff (1963) were the first who reported that adrenalectomy disturbs the calcification process in the developing incisor of the rat. Fleming (1953) studied the effect of daily injections of cortisone acetate (2.5mg) on tooth germs of 20 day old guinea pig embryos transplanted into the anterior eye chamber of guinea pig adults. The results were as follows:

1. Transplants vascularized in 24 hours as compared to four days in the controls.

2. Growth of the tooth germs was initially more rapid than in controls.

3. This growth was surpassed by controls after vascularization occurred.

4. Many transplants resorbed upon discontinuation of cortisone injections. This was due to lymphocytic phagocytosis.

5. Control transplants were observed to make attachment to the iris of the host, while this process did not occur with cortisone treatment.

Glickman, Stone and Chawla (1953) observed the effect of cortisone on the periodontium of white mice. One half of the litter mates were injected initially at 4 weeks of age, with 0.5mg of cortisone daily for 14 days. Grossly, the oral tissues appeared similar in the control and experimental groups. Histologically, the height of interdental alveolar bone was reduced with cortisone treatment, however, when the photographs were examined no differences were apparent. The cortisone group showed minimal osteoblastic or osteoclastic activity, enlarged osteocytes, an edematous periodontal ligament, fibroblasts that were reduced in number and size, and a reduction in the number of collagen fibers. These fibers had a wavy appearance and were loosely arranged due to intercellular edema.

Applebaum and Seelig (1955) studied histologic changes in the jaws and teeth of young rats treated for one or two months with cortisone. These animals showed alveolar bone loss and pulpal blood vessel dilatation in the molars.

Goldsmith and Ross (1956) studied the histological and histochemical effects of cortisone on the lower incisor of fetal and postnatal rats. They found an increase in the alkaline phosphatase and ribonucleic acid content of ameloblasts, as well as increased glycogen content in the stratum intermedium of the fetal rats. In the cortisone treated postnatal rats they observed premature differentiation of the incisor. In the older postnatal rats an overgrowth of alveolar bone, degradation of ameloblasts and disorganization of the periodontal ligament was observed.

Blackey and Johannessen (1964) found that the jaws of immature albino rats were reduced in size after receiving 0.2mg cortisone/gm of food for 20 days followed by a normal diet for 20 days. In another experiment, (Johannessen 1964), weanling rats received 0.2 mg cortisone/gm of food for 20 days. One group received a total of 20gm of food, a second group, 40gm and a third control group received food ad libitum. Cortisone was observed to inhibit apposition of dentin, especially on the occlusal surface of the pulp chamber. An inhibition of body growth and food intake was seen in the cortisone treated rats.

Anneroth and Bloom (1966) observed that cortisone treatment (50mg/day) resulted in a disorganization, increase in cellularity and inhibited differentiation of rat incisor odontoblasts. The pulp showed an increase in cellularity. Pulp stones, with cellular and vascular inclusions were also found. The authors felt that these changes were similar to those observed in vitamin A and C deficiency.

Nakamoto and Wilson (1968) administered cortisone (40mg/kg) to young rats and noted that tooth germ growth was retarded, the ameloblast

and odontoblast layers were thinner, although the dentin was of normal
thickness.

Davidovitch (1971) observed the mandibular condyles of cortisone
treated (0.2 mg/gm of food or 1.9 mg/day) young rats to be sclerotic due
to continuous matrix formation and lack of resorption. The cartilage
cells were reduced in size and number in the proliferating and hyper-
trophic zones. The osteoblasts were also smaller and fewer in number.
Cessation of cortisone therapy resulted in normal condyles.

3. Effect on the Mitotic Activity

The effect of cortisone on cell division in various tissues and
organs has been the subject of a number of investigations. Dustin (1963),
after comprehensively reviewing the literature, reported that cortisone
has been shown to inhibit cell division in some tissues; whereas in
others, it has either had no effect or even acted in a stimulatory capa-
city. He also mentioned that this hormone has been demonstrated to be
antagonistic to certain spindle poisons.

Bullough (1952) suggested that cortisone has a general antimitotic
action. He stated that disturbances in carbohydrate metabolism may be a
factor in depressing cell division. Roberts et al. (1952) found that
the effect of cortisone on mitosis varys from one type of tissue to
another and that it does not seem likely that one of the fundamental,
universal actions of cortisone is to inhibit mitosis. Roberts et al.
(1952), after injecting young white mice with 2.5mg of cortisone over a
two day period, found a decrease in the mitotic activity of regenerating
liver. However, no difference was noted in the rate of cell division

of the crypt's of Lieberkuhn and the lymph follicle of the large and small intestine between treated and control animals. Fat and glycogen deposition was seen to increase, DNA synthesis to decrease and RNA content to remain the same in the regenerating liver of the cortisone treated animals. Watson and Morimoto (1961) also reported a significant decrease in the mitotic activity of the liver cells of cortisone treated chicks on the 10th, 11th, and 13th day of incubation.

In contrast to the above observations, Babick and Gatz (1952) reported an increase in mitotic activity in the epidermis of cortisone treated rats when compared with controls. Sigelman et al. (1954) also found that rats given daily injections of cortisone over a period of five days, showed a significant increase in the mitotic activity of the corneal epithelium.

Kiely (1967) investigated the effect of cortisone on the rate of eruption, DNA synthesis and mitotic activity in the rat incisor. He found an increase in the rate of DNA synthesis of adrenalectomized rats and an increase in the mitotic rate of normal rats in the cells of the cervical loop after injection of cortisone. Kiely (1967) noted that the dosage, frequency, and duration of administration are important factors in the response of tissues to cortisone. Domm and Kiely (1968) observed that injections of cortisone (0.5 and 1mg) twice daily, over a two-day period, resulted in an increase in the number of mitosis of the stratum intermedium, the preameloblasts, preodontoblasts and adjacent pulp in the maxillary incisor of the rat at 7 A.M., 1 P.M. and 1 A.M. but not at 7 P.M. Kiely and Domm (1973) also showed an increase in the number of

^{3}H-thymidine labeled cells in the cervical loop tissue of adrenalectom-

ized rats following the administration of cortisone over a 24 hour period.

E. Cortisone Synthesis, Structure and Metabolism

Cortisone is principally synthesized in minute quantities by the

mammalian adrenal cortex. Hydrocortisone is the main secretion from the

adrenal cortex in man, while corticosterone is the chief glucocorticoid

found in rodents. Cortisone, as any other steroid, is characterized by

the twenty-one carbon pregnan nucleus, i.e., cyclopentanoperhydrophen-

anthrene nucleus. This common central structure consists of four inter-

connected rings of carbon atoms; three of the rings have six carbon atoms

and the other ring has five atoms. Cortisone has a ketone at C3, C11,

and C20, a C4 C5 double bond, and a hydroxyl group at C17. The only dif-

ference between cortisone and cortisol is that cortisol has a hydroxyl

group at C11 instead of ketone.

In general, glucocorticoids typically have a molecular weight of

about 300 (peptide hormones have a large molecules with a mulecular weight

of about 10,000). All of them are synthesized from the same precursor,

cholesterol. The difference between these steroids are determined by the

pattern of chemical bonds with the rings and by the nature and orienta-

tion of the side groups attached to the rings. Because of their small

molecular structure, glucocorticoids can easily diffuse into cells of

many kinds, and they just as easily diffuse back out into the blood stream.

They are effective even in very small quantities. Their concentration in

blood reaches only about 10^{-9} mole per liter (O'Mally and Shrader, 1976).

Cortisone metabolism occurs mainly in the liver (Goodman and Gillman,

1970).

Cortisone or (compound E), cortisol or (compound F), as well as other corticosteroids produce a number of metabolic effects. They are involved in carbohydrate, protein, fat and purine metabolism; water and electrolyte balance, and the functional capacities of the kidney, skeletal muscle, cardiovascular system and the nervous system. The corticosteroids also provide for cells to have the ability to resist many forms of noxious stimuli and environmental changes. Corticoids may induce enzymes, may alter RNA synthesis or their action may effect DNA transcription into messenger RNA. Studies on culture cells showed that some enzymes have been produced by hydrocortisone stimulation (Kinny et al. 1965). Feigelson et al. (1962) have shown that cortisone acetate causes a net increase in the number of tryptophan pyrrolase enzyme molecules in rat liver and that increased synthesis reaches a maximum four hours after a single dose of 0.5 mg/100g body weight of the hormone, coincident with the maximal increase in RNA synthesis (Feigelson et al. 1962). Actinomycin inhibits the cortisone-induced synthesis of tryptophan pyrrolase (Weber et al. 1963). These investigations have led to the inference that the induction of enzyme synthesis by cortisone is regulated at the level of DNA transcription into messenger-RNA (Kidson and Kirby, 1964).

It is well known that cortisone and hydrocortisone are regulated by a series of feedback mechanisms. They have in common the interaction with cytoplasmic receptors of their tissue target cells (Burns, 1974). It has been shown by Burton et al. (1968) that the enzyme 11-beta hydroxydehydrogenase is the primary catalyst for the biotransformation of

cortisol to cortisone in the liver of U.B.C. Swiss Mice. This is a reversible reaction in most mammals. This transformation can occur in the liver and in lymphocytes. Although this conversion produces a compound (cortisone) which from the medical point of view has less anti-inflammatory effect, the potential for reversal remains. Cortisone may have non-specific effects that are unrelated to glucocorticoid activity. These non-specific effects are produced through mechanisms, and probably binding sites, that differ from those responsible for specific glucocorticoid effects (Munck, 1965).

Hydrocortisone acts directly on thymus cells and it produces a block at the level of glucose transport or phosphorylation, or both (Munck, 1965). This action rapidly leads to decreased rates of glucose uptake. This mechanism clearly precedes effects on protein and nucleic acid metabolism. Rat thymus cells contain binding sites, about 5000 per cell, specific for hydrocortisone and related glucocorticoids (Wira and Munck, 1970). These authors also found that the specific binding sites for cortisol dissociate slowly (time constant of 3 minutes at 37°C) while the specific binding sites of cortisone dissociate rapidly (time constant of less than 3 seconds).

F. Postulated Mechanisms of Action of Cortisone

According to Burns (1974), O'Mally and Schrader (1975), Goodman and Gilman (1975), Katzenellenbogen (1980), and McMurray (1982), all of the steroid hormones exert their metabolic effects, more or less, through the cytoplasmic receptor-nuclear acceptor system. It is hypothesized

that steroids are capable of penetrating the cell membrane and combining with a highly specified protein receptor in the cytoplasm. This conjugated steroid-receptor complex, in turn, is translocated to the nucleus where it combines with a specific acceptor located in chromatin. This steroid-receptor acceptor complex now causes nuclear genetic material to produce messenger ribose nucleic acid (m-RNA), which then signals and directs the fabrication of some highly specific enzymes or other proteins whose action is directly responsible for producing the physiologic expression of the hormone's activity (Kidson and Kirby, 1964; O'Mally and Shrader, 1976). Specificity of this system resides in the conformational structure of the receptor protein to the steroid hormone and this complex condensing to the nuclear chromatin acceptor (McMurray, 1982). This system may be visualized in Figure 1 (from Burns, 1974).

There is considerable evidence to show that hormone retention by the cell is a primary step in the tissue hormone interaction since in its absence, the effects attributable to the presence of the hormone are not observed (Stumpf, 1968 and Lisk, 1971). The interaction of steroid hormones with their receptors is generally specific at physiological levels of the natural hormone (Stumpf and Sar, 1976a). This interaction is mediated by receptor molecules found only in cells that respond to the hormone (target cells). In non-target cells the hormone diffuses freely into the cell and out of it, and the concentration remains quite low (O'Mally and Shrader, 1976). Hormone retention by various target tissues has been demonstrated for a number of steroids. Retention of the steroid by the target tissue appears to be specific and saturation occurs at

Figure I. Receptor-Acceptor System
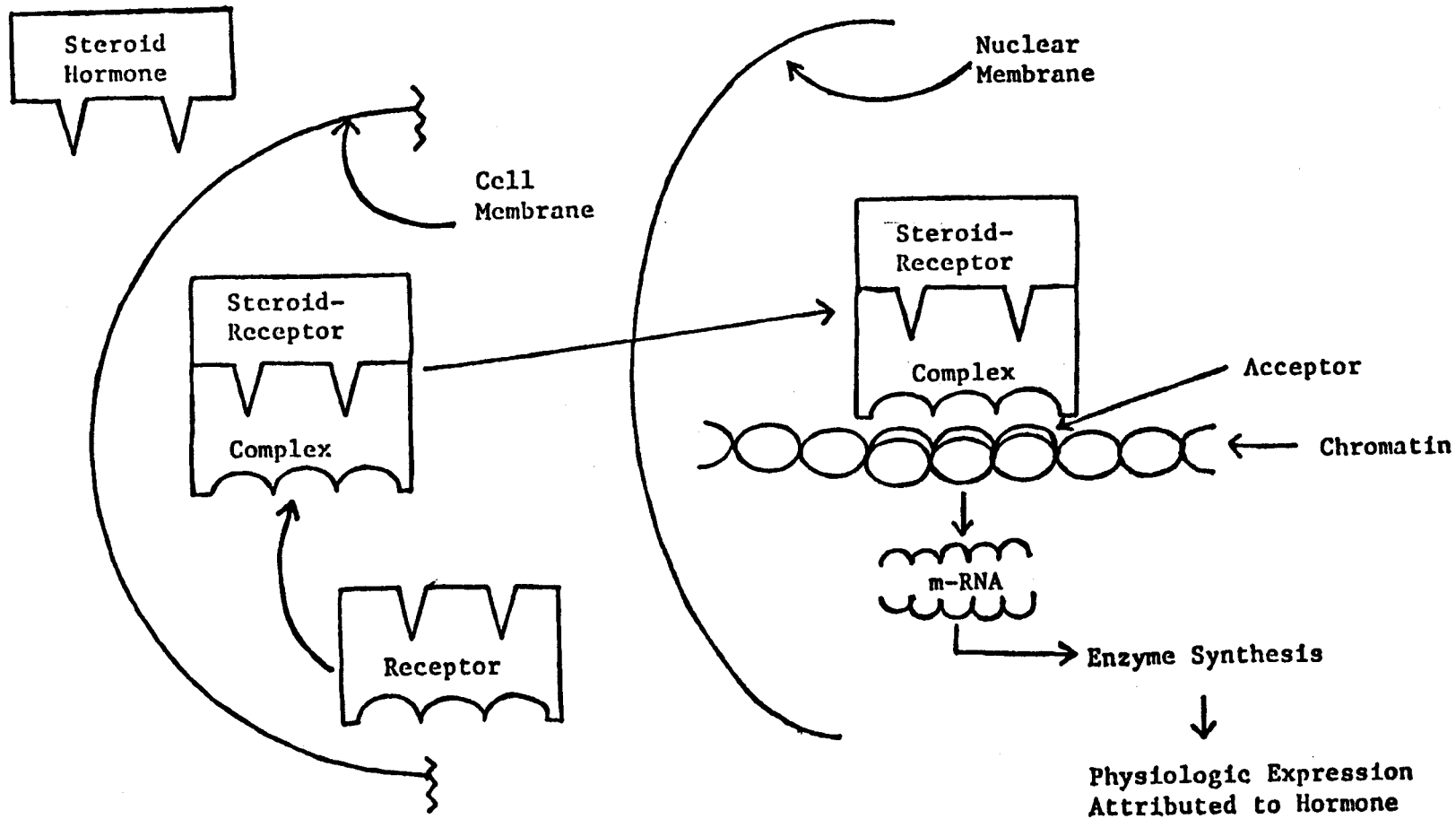
physiological levels of hormone (Zigmond and McEwan, 1970). Thus, target tissues are said to possess specific receptors for the hormone. Only the estrogens have been extensively studied, and both a cytoplasmic and nuclear receptor have been described for various estrogen target tissues (uterus, pituitary, hypothalamus, and vagina). Progesterone retention has also been demonstrated for some target tissues.

It is evident that only certain types of interactions are possible between free steroids and other molecules. Except for the phenolic OH group in the estrogen, there is no possibility for true ionic bonds. All of the steroids have at least one oxygen function which usually takes the form of a primary or secondary alcohol or a ketone (aldosterone is exceptional in having an aldol group). All these groups are capable of forming hydrogen bonds with suitably placed reactant groups.

For many years a receptor-steroid complex of some sort has been proposed to explain specificity of steroids for certain tissues. Jensen et al. (1968) reported specific binding sites in the nucleus of rat uterus for estradiol. This nuclear estradiol receptor complex seemed to be preceded by formation of a cytoplasmic complex by which the hormone was transferred to the nucleus. The binding was found to be very specific, but easily dissociated through histological processing. This was theorized to be due to the weak binding capabilities of the steroid. Similar results were reported by Wira and Munck (1970) with regards to cortisol binding receptors in rat thymus cells.

G. Frozen-Tissue Autoradiography and Localization of Steroids

The exact mechanisms by which steroids activate the gene is not known (Davidson, 1965). Initially it was demonstrated that the steroid hormones have a particular affinity for their target cells (Stumpf and Sar, 1976b). However, since the hormone is present in such low concentration in the cell, the methods of conventional analytical chemistry are inadequate for the study of hormone physiology.

Highly radioactive steroids were first prepared in the early 1960's by E.V. Jensen of the University of Chicago. By injecting female rats with a radioactive preparation of estrogen, Jensen et al. (1968) showed that the hormone is retained longer in the uterus than it is in other tissues such as muscle and blood. The uterus is considered a "target" organ of estrogen, whereas muscle and blood are not. Edelman et al. (1963) using autoradiography, localized ^{3}H-aldosterone in the nuclear and perinuclear areas of the epithelial cells of the toad bladder. The hormone appeared in the target tissue within minutes after it was administered to the animal. Moreover, it was retained long after all radioactive molecules left the non-target cells.

In 1968 W.E. Stumpf proceeded one step further and showed that the hormone tends to accumulate in the nucleus of target cells. This observation provided additional evidence that the mechanism of steroid action is a genetic one. Using autoradiography, Stumpf (1968) also found the movement of the hormone into the nucleus to be extremely rapid, and the concentration in the target cells to increase with time. Numerous autoradiographic studies have been undertaken since then to determine the target tissues for various hormones.

In autoradiography, the radiation effect from an isotope in the tissue is stored as latent image in a photographic emulsion during exposure. It is, therefore, possible to amplify tracer related information; i.e., small physiological quantities of exogenously administered radioactive agents can be followed in the body and associated with individual cells and subcellular particles. This technique is relatively straight forward, if the tracer molecule is chemically bound to tissue constituents and therefore, not readily subject to translocation during the necessary manipulations of the tissues for microscopic examination. However, it has been shown by Williams (1950) and Holt and Warren (1951), that when paraffin tissue sections containing non-covalently bound isotope-labelled compounds, such as steroids, are floated on a photographic emulsion, some of the activity is lost. Leblond (1953) and many others proposed that translocation of a radioactive label is unavoidable with any soluble or slightly bound isotope during normal liquid fixation, solvent dehydration, embedding, or overlying of wet emulsion prior to development of the photographic emulsion.

Stumpf and Roth (1965, 1966, 1969) have shown that steroid hormone protein complexes can not be demonstrated by normal autoradiographic techniques; but only by autoradiographic techniques designed for freely soluble compounds. Stumpf and Roth (1966, 1969) developed a freeze-drying technique that has been demonstrated to be highly successful for use with soluble compounds. Appleton (1968, 1972) has also developed a slightly different freezing technique that has also proven quite successful. Through the use of these techniques there have been many

steroid hormone target tissue investigations. Attramadal (1969) studied localization of ^{3}H-estradiol in the uterus, vagina, and the anterior pituitary glands using the freeze-drying technique. Waremburg (1974b), Rhees, Grosser, and Stevens (1974), and others have observed corticosteroid deposition in the rat brain using the thaw-mount technique. Waremburg (1974a) reported localization of ^{3}H-progesterone in the uterus of the guinea pig. O'Mally, Taft, and Sherman (1971) reported progesterone binding in the chick oviduct. Sar and Stumpf (1973, 1977, 1979, 1981), Keefer et al. (1976), Sheridan and Herbert (1979), and others were able to demonstrate nuclear receptor sites for various steroid hormones in the brain, pituitary, and other target tissues. Gasc, Sar, and Stumpf (1980) observed estrogen and androgen binding in target cells in the anterior pituitary of the chick embryo. Hernandez, Wenk, and Vittek (1982) demonstrated nuclear localization of ^{3}H-dexamethasone in the secretory duct cells of the rabbit submindibular gland. Kim et al. (1983) demonstrated target cells for ^{3}H-1,25(oH)$_2$ Vitamin D$_3$ in developing rat incisor teeth. To our knowledge no one has ever reported localization of ^{3}H-cortisone or any other steroid hormone in the periapical tissues of the rat incisor.

MATERIALS AND METHODS

Twenty-eight female rats (Sprague-Dawly strain)[1] were utilized in this investigation. The rats were housed, one per cage, and were fed a normal pellet diet of Purina Lab Chow. During the first week, all animals received tap water ad libitum.

At 35 days of age, twenty animals were bilaterally adrenalectomized and maintained for one week on a 1.0% sodium chloride solution. The remaining eight animals served as controls and were given tap water.

At periods of 30 minutes, 1 hour, 2 hours, and 4 hours before sacrifice, four adrenalectomized animals each period were injected subcutaneously with 1.0 microcurie/gm body weight of $(1,2-^3H)$ cortisone[2] (specific activity, 29Ci/mmol) in a 25 - 75% ethanol-isotonic saline solution. The original solution of toluene: ethanol- (9: 1v/v) had been evaporated using a slow vacuum technique. One adrenalectomized rat was also sacrificed at 6 hours following injection. Eight animals served as controls and were injected (two rats for each sacrifice period) with 1.0 microcurie/gm body weight of $(1, 2-^3H)$ cortisone and immediately followed with 4 mg/kg body weight of non-labeled cortisone acetate.[3] In addition, three

1. Locke-Erikson, Chicago, Illinois
2. Amersham, Arlington Heights, Illinois, 60005
3. The Upjohn Company, Kalamazoo, Michigan, 49001

adrenalectomized animals were injected subcutaneously with 1.0 microcurie/ gm body weight of $(1,2-^3H)$ cortisol[4] (specific activity, 40Ci/mmol, prepared in a 25 - 75% ethanol-isotonic solution) and sacrificed at 1 hour, 2 hours, and 4 hours. All rats were sacrificed after receiving an overdose of Nembutal.

Upon sacrifice, the animals were quickly decapitated and both the upper and lower jaws were removed. The lower jaws were placed in a 10% formalin solution buffered with calcium carbonate and utilized later for liquid scintillation counting. The upper jaws were immediately bisected longtudinally between the incisors. Excess tissue was then removed from the upper incisors. This was accomplished by visualizing the root of the incisor within the bone and then cutting much of the excess soft tissue and bone posterior to the root tip. The remaining bone around the tooth was then fractured by exerting pressure along the line of the border of the root. A thin plate of bone was allowed to remain in contact with the root. The root tips (2-3 mm) were then slightly embedded in minced liver on a tissue mount and immersed immediately in liquid propane (-180°C) for several seconds. The freeze mounted specimen was then transferred rapidly to a 50 ml wide-mouth plastic bottle filled with liquid nitrogen. The bottle was then placed in liquid nitrogen storage until cryostat sectioning.

Prior to the above procedure, the tissue mounts had been numbered

4. Amersham, Arlington Heights, Illinois, 60005

and placed in a dry petri dish on ice. Also liver from a non-experimental animal had been minced with a razor blade within a petri dish and kept in an ice tray. This finely minced liver, served to adhere the root tips to the mount.

Liver specimen were taken from four animals, selected randomly from the ^{3}H-cortisone treated adrenalectomized animals, representing the 30 minutes, 1 hour, 2 hour, and 4 hour groups. These liver specimens were mounted on tissue mounts, frozen in liquid propane, and then stored in liquid nitrogen until sectioning.

Previous to cryostat sectioning, slide preparation was performed. Utilizing a darkroom, glass slides were dipped in Kodak NTB2 liquid photographic emulsion.[5] A Thomas duplex super sage light with a sodium vapor lamp and x-ray film filters, was used in the darkroom. The slides were air-dried on the darkroom table and placed into light-proof boxes containing small bags (made from tissue paper and scotch tape) of 15-25 gm of Drierite. The boxes were wrapped securely with black tape. These boxes were stored in the refrigerator (4°C) until use.

The specimens in liquid nitrogen were transferred to the cryostat (-25 to -35°C). Using a precooled forceps, a tissue mount was removed from the plastic bottle within the cryostat and inserted into the tissue holder of the microtome and kept for one to two hours, under conditions of low humidity, before sectioning. The tissue was always sectioned

5. Eastman Kodak, Rochester, NY

within four hours of placement into the cryostat. Tissue sections of 3
to 4 microns were made with a well sharpened knife under safe light
conditions. At this point, the thaw-mount technique introduced by Stumpf
(1975) was followed. Under the safe light, the sections were directly
mounted onto an emulsion coated slide. The slides were then numbered
accordingly, placed into black light-tight boxes containing Drierite bags
and wrapped with black tape.

Exposure was accomplished at low temperature (-10°C) in the dry,
light-tight slide boxes. After one month of exposure, some test slides
representing the different groups, were tested for the presence of silver
grains. This procedure was repeated every month for seven months.

The light-proof dessicator box was removed from the freezer and
allowed to warm to room temperature before opening, since precipitation
of moisture could damage the sections or cause artifacts. Under a safe
light, the slides were removed from the boxes and breathed on briefly
thereby securing the contact between sections and the photographic emul-
sion. The slides were then developed in Kodak D19 developer[6] for one
minute at 20°C, briefly dipped in running tap water, fixed in 24% sodium
thiosulfate solution for about five minutes, and subsequently stained
with hematoxylin eosin.

In the second part of this investigation, the right mandibular
incisor was analyzed, utilizing the method of liquid scintillation

6. Eastman Kodak, Rochester, NY

counting. This procedure was carried out in order to measure the amount of radioactivity in the root tips of these incisors. Following fixation in 10% formalin buffered with calcium carbonate, the incisor was decalcified in a formic acid-sodium citrate solution, trimmed of excess tissue, washed in tap water and then put in 75% ethyl alcohol. Under a binocular dissecting microscope (10X), a 2 mm length of the cervical or root end of each incisor was carefully dissected free. Special care was taken to keep the size of the tissue as constant as possible. The root tips were then taken from 75% ethyl alcohol, air dried for several hours and then placed in a dessicator at room temperature for a period of ten days after which they were individually weighed, folded in Whatman #1 filter paper, and then crushed under light pressure. Both sample preparation and sample counting were kindly carried out by Dr. Walter E. Kisieleski at the Argonne National Laboratory, Division of Biological and Medical Research, Argonne, Illinois. By utilizing a Packard Tricarb sample oxidizer (Model 305), the samples are automatically combusted. The tritrated water is then dispensed into a counting vial to which is added a measured amount of scintillator. Sample counting was then carried out using a Packard Tri-carb fully automatic liquid scintillation spectrometer (Model 3375). The counts were recorded as counts/minute/sample which was then converted to disintegrations/minute/sample using an efficacy factor of 30.0%. Disintegrations/minute/sample is then calculated as disintegrations/minute/ mg of sample weight.

RESULTS

A. Autoradiographic Observations

Thirty minutes following injection of ^{3}H-cortisone, silver grains were randomly scattered over the periapical tissues of the rat incisor. The label was observed to be quite heavy over the tissue without any definite localization. It appears that the hormone was obviously present in the tissues of the incisor. Silver grains were scattered over the proliferating cells of the cervical loop, over the pulp cells, and also over the preodontoblasts, odontoblasts, preameloblasts, ameloblasts, stratum intermedium, stellate reticulum, and fibroblasts as well as the intercellular spaces (Figure 4 and 6). The minor salivary glands were also heavily labeled with radioactivity with no clear localization. Silver grains were especailly concentrated over predentin, dentin, and over the bone surrounding the periapex of the tooth. Radioactivity was also observed over the endothelial cells and lumen of the blood vessels. Similar observations were noticed for the 30 minute control group (Figure 5). Liver tissue representing the 30 minute time interval, also showed a very dense label with no apparent localization.

At the 1 hour after ^{3}H-cortisone administration, the periapical tissues of the incisor of the adrenalectomized rat continued to show a heavy distribution pattern of radioactivity without clear localization to any type of cells (Figure 7). Distribution of silver grains over these tissues was almost identical with that seen for the 30 minute group. The

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periapical tissues of both the experimental and the control groups at this time interval showed the same silver grain distribution pattern (Figure 7 and 8). Liver tissue also showed no evidence of silver grain localization. Heavy distribution of silver grains were observed over the minor salivary glands of the palate without definite localization. These same observations were noted for the ^{3}H-cortisol treated animal.

At 2 hours, relatively less silver grains were observed over the tissues of the incisor than at 30 minutes and 1 hour. Also the control slides showed much less silver grains than the experimental group. The proliferating cells of the cervical loop showed relatively less concentration of silver grains than did the pulp tissue. Localization of silver grains over the nuclei of any type of cells of the periapical tissue was lacking (Figure 9, 10, and 11). Minor salivary glands of the palate as well as liver cells also showed no definite intracellular localization of silver grains. The ^{3}H-cortisol treated animal sacrificed at 2 hours also revealed the same pattern of radioactivity over the periapical tissue cells as previously described. A very heavy condensation of silver grains was still noticed at this time interval over predentin, dentin, and over the bone surrounding the periapex of the rat maxillary incisor.

At 4 hours, the periapical tissues of the rat maxillary incisor showed an almost identical distribution and concentration of silver grains for both the experimental and the control groups. The cells of the cervical loop demonstrated less radioactivity than any other tissue (Figures 12 and 13). Dense clustering of silver grains over predentin, dentin, and over the bone surrounding the root apex was still observed at this

time interval (Figure 15). Localization of silver grains was not apparent over any cells in the tissue under study. The ^{3}H-cortisol treated animal sacrificed at 4 hours revealed a somewhat heavier distribution of silver grains over the periapical tissue than the 4 hour ^{3}H-cortisone treated animals, especially those of the pulp, but there was still no apparent localization (Figure 14). Minor salivary glands of the palate as well as the liver tissue showed a lesser amount of silver grains than in the previous time intervals. No silver grain localization was noticed over the acini of the minor salivary glands of the palate or over the cells of the liver. Similar observations were recorded for the ^{3}H-cortisone treated animal sacrificed at 6 hours.

B. Liquid Scintillation Counting Analysis

The amount of radioactivity in the root tips of the right mandibular incisor was the highest at 30 minutes following injection of ^{3}H-cortisone. The average (202.67 ± 37.5) disintergrations per minute per milligram (DPM/mg) of the adrenalectomized rats was less than that of the control group (222.67 ± 79.2). Statistically there was no significant difference between the two groups. The values for the experimental and control groups measured as DPM/sample are almost identical. See Tables 1 and 2 and Figures 2 and 3.

At 1 hour, the amount of radioactivity is relatively less than that of the 0.5 hour group. The mean values of the 1 hour experimental group (275.75 ± 103.9 DPM/sample and 178.69 ± 45.3 DPM/mg) were greater than those of the control group (148.5 ± 171.8 DPM/sample and 135.0 ± 156

DPM/mg). Statistically, there was no significant difference between the experimental and the control groups. The ^{3}H-cortisol treated animal sacrificed at 1 hour showed higher values (709 DPM/sample and 283.6 DPM/mg) than the average values of ^{3}H-cortisone treated adrenalectomized animals.

The rats sacrificed 2 hours after injection showed the mean number of DPM/sample and of DPM/mg to be less than the values of the previous groups. The mean values of the 2 hour experimental group (80.0 ± 35.2 DPM/sample and 54.17 ± 23.4 DPM/mg) were greater than those of the control group (29.0 ± 10.0 DPM/sample and 13.08 ± 3.6 DPM/mg). A significant difference was found between the values of the experimental and the control groups measured in DPM/mg (P = 0.05) but not between those measured in DPM/sample. The ^{3}H-cortisol animal sacrificed at 2 hours showed a higher value (62.0 DPM/mg) in comparison to the ^{3}H-cortisone treated animals. See Tables 1 and 2 and Figures 2 and 3.

At 4 hours, the mean values of the ^{3}H-cortisone treated adrenalectomized rats (26.18 ± 26.1 DPM/sample and 31.25 ± 41.0 DPM/mg) are still higher than are the control averages (9.65 ± 5.3 DPM/sample and 7.18 ± 0.17 DPM/mg). However, the level of activity is less than the previous time intervals. The mean values of the 4 hour control group were even less than the background radioactivity which was calculated to be 15.7 DPM/mg. The DPM/mg of the ^{3}H-cortisol animal at 4 hours showed a value of 14.17; almost background level. At 6 hours, the single adrenalectomized rat treated with ^{3}H-cortisone showed a DPM/mg value of 9.09 which again was below the level of background radioactivity (Table 1).

DISCUSSION

This investigation was carried out in an attempt to determine the site of action of cortisone on the proliferating tissues of the rat incisor. It was postulated that cortisone may directly effect the undifferentiated cells of the cervical loop and adjacent cells in the apical region of the rat incisor. Localization of this steroid at the cellular level might then account for the observed increase in DNA synthesis and mitosis in the cells of the cervical loop of rats injected with cortisone (Kiely and Domm, 1973).

Based on the above postulations, it was felt that by employing a frozen tissue thaw-mount autoradiographic technique, any localization of ^{3}H-cortisone in the periapical tissue of the maxillary rat incisor would be apparent. Analysis of the tissue utilizing liquid scintillation counting was also undertaken.

The thaw-mount autoradiograms representing the 30 minute post-injection time interval showed a generalized distribution of silver grains over the cervical loop and pulp tissue cells without any significant intracellular localization. From Tables 1 and 2, and Figures 2 and 3, it can be noted that the amount of radioactivity as measured by liquid scintillation is higher at 30 minutes than at any other post-injection time interval. This strongly indicates that ^{3}H-cortisone, or its metabolite thereof, has reached the tissues of the root apex in an appreciable quantity. Therefore, the cells of the root apex would presumably be able

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to retain much of the labeled hormone if they are, in fact, target cells.

At 1 hour, the scintillation counting analysis showed relatively high levels of radioactivity and the periapical tissues still revealed a large number of silver grains without any obvious intracellular localization. Both the experimental and the control groups showed a similar silver grain pattern of distribution over the entire tissue. These observations indicate that a significant amount of hormone is still available to the tissues at one hour (See Figures 2 and 3).

At 2 hours, a significant difference was found in DPM/mg between the experimental and the control groups (P = 0.05). The mean value for the experimental groups was 54.17 DPM/mg and that of the control group 13.02 DPM/mg. This value for the controls was equivalent to background levels and would indicate a saturation by the non-labeled cortisone of presumed receptors in the cells of the apical tissue. The significantly higher level of radioactivity in the tissues of the adrenalectomized rats indicate a possible intracellular concentration of the labeled steroid by 2 hours after injection.

Autoradiograms representing the 2 hour sacrifice time show a similar contrast between the control and experimental rats after 7½ months of exposure time. By careful observation of these autoradiograms, the tissues of experimental animals definitely showed more silver grains, especially over the pulp cells, than the control autoradiograms. These tissues should have demonstrated localization if they possess target receptors for cortisone, however, little specificity of label was noted. Longer exposure time may verify if these tissues are target or not. Other

investigators have shown localization of ^{3}H-corticosterone (Waremburg, 1974) and ^{3}H-testesterone (Sar and Stumpf, 1973) in the rat brain tissue at 1 and 2 hours after injection.

The least number of radioactivity disintegrations was noticed for the 4 hour post-injection time interval. Nevertheless, the mean value of the experimental group was found to be higher than that of the control group, though not statistically so. This may indicate that the cells of the periapical tissues retain some radioactivity. Longer exposure times for the autoradiograms would be necessary to determine whether this postulation is true, since intracellular localization was again not evident after exposure time of 7½ months.

The minimal number of silver grains observed over tissue taken at 2 and 4 hours after injection may be related to the low specific activity of the ^{3}H-cortisone (29 Ci/mmol). Many more silver grains were observed over the periapical tissue of the ^{3}H-cortisol treated animal representing the 4 hour interval with the same amount of exposure time. The obvious difference between the two hormones would be the higher specific activity of ^{3}H-cortisol used (40 Ci/mmol). Also the cells of the root apex may have a higher affinity for cortisol than cortisone. Metabolism of the excess hormone by the liver (Goodman and Gilman, 1970) would also be expected with passage of time after injection. As a result, decrease of radioactivity in the rat incisor periapical tissue with the passage of time would be expected. Most of the radioactivity remaining would be that retained by the intracellular compartment of the presumed target cells of the root apex.

An adequate explanation for the heavy labeling of silver grains over calcified tissues such as dentin, and bone surrounding the root apex of the rat incisor is lacking. Since this labeling was found to be most dense at 30 minutes and somewhat lesser at 4 hours, just as was the observation for the soft tissues, it seems unlikely that this localization is an artifact.

Cortisone is known to stimulate bone resorption (Glickman et al., 1953; Applebaum and Seeling, 1955; Roberts, 1969; and Keener, 1970). In contrast, Goldsmith and Ross (1956) described an overgrowth of bone formation in cortisone treated post-natal rats. Davidovitch (1971) also observed the mandibular condyle of cortisone treated young rats to be sclerotic due to continuous matrix formation and lack of resorption. Therefore, the heavy labeling of silver grains over the bone surrounding the tooth periapex may indicate that cortisone, or its metabolite, has a direct influence on bone. Whether this is a stimulatory or an inhibitory effect, remains to be told.

Controversial observations were reported concerning the effects of cortisone on dentin of the rat incisor. Goldsmith and Ross (1956) studied the histological and histochemical effects of cortisone on the lower incisor of fetal and post-natal rats. They found an increase in the alkaline phosphatase and ribonucleic acid of ameloblasts and odontoblasts of the fetal rats. In contrast Johannessen (1964) found that cortisone administration inhibited apposition of dentin especially on the occlusal surface of the pulp chamber of the rat incisor. Nakamoto and Wilson (1968) observed normal dentin thickness of cortisone-treated young

rats, whereas ameloblast and odontoblast layers were thinner. Therefore, the heavy labeling of silver grains over dentin may indicate that cortisone, or its metabolite, has a direct effect on dentin calcification. The question needs to be answered, however, how does cortisone enter the dentin? The most plausible answer seems to be that cortisone was taken up by the odontoblast where it was transferred to secretory vesicles and deposited into its matrix (containing collagen embedded in a ground substance rich in glycosaminoglycans), then reached the mineralizing front through the odontoblastic process.

Cho and Garant (1981) studied the localization of ^{3}H-colchicine in the oral tissue of mice. They unexpectedly noted a heavy labeling of ^{3}H-colchicine at the mineralization front of bone and dentin, and less binding over osteoblasts, odontoblasts and the fibroblasts of the periodontal ligament. They found ^{3}H-colchicine to be present at the mineralization front, 10 minutes after injection. In our investigation, the heaviest concentration of silver grains over dentin was at 30 minutes after injection, however, dense labeling was still observed even at 6 hours after ^{3}H-cortisone administration. Cho and Garant (1981) also showed the number of silver grains to be the greatest just proximal to the predentin-dentin junction. The same observation can be seen in Figure 14 for the ^{3}H-cortisone. At the electron microscopic level, Cho and Garant observed ^{3}H-colchicine to be localized in the extracellular space and not over the odontoblastic process. As a result, they suggested that colchicine is rapidly bound by some component of the bone and dentin matrix and that its binding occurs extracellularly and not by

incorporation into the osteoblast and odontoblast as part of the synthesis and/or secretory process. Therefore, a similar electron microscopic study using ^{3}H-cortisone would be important. A study of this nature should verify whether ^{3}H-cortisone finds its way to the mineralization front through the odontoblasts, after incorporation into a matrix component during its synthesis, or instead, directly through extracellular routes.

The results of this study are not in agreement with the theory which postulates that cortisone increases the rate of eruption through a direct stimulation of cells in the periapices undergoing mitosis. In other words, based on the results of this investigation, the proliferating tissues of the rat incisor do not appear to be targets for this steroid. Cortisone, therefore, may affect the eruption rate of the rat incisor through other more indirect mechanisms such as its action on dentin formation or growth of the surrounding bone.

However, the possibility remains that certain aspects of the procedure may account for the reported observations. The thaw-mount technique used in this study may be inadequate to properly visualize the localization of cortisone at the cellular level. A thorough search of the literature, failed to reveal any articles concerning localization of cortisone by means of frozen tissue autoradiographic techniques. Numerous articles can be found dealing with the localization of other steroids in various tissues with the use of this technique (Sar and Stumpf, 1977, 1979, and 1981; and Hernandez, et al., 1982). The thaw-mount technique has proven to be as accurate as the dry-mount technique, and has, therefore, become the procedure of choice (Stumpf, 1975 & 1976; and Sar and

Stumpf, 1981). It may be that the cortisone-receptor complex is less stable than that of other steroids and is, therefore, more susceptible to dissociation.

It is also possible that the apical tissues of the rat incisor may, in fact, possess receptors for cortisone and, that a longer exposure time will be necessary in order to observe intracellular localization. This possibility becomes more important when one considers the low specific activity of ^{3}H-cortisone used in this investigation. Some investigators have shown localization of other steroids in different tissues, using the thaw-mount technique with exposure times ranging from 11 to 28 months (Sar and Stumpf, 1981; Hernandez et al., 1982). Also since the specimens used for scintillation counting were kept in 10% formalin solution for an extended period of time before dehydration, an undetermined loss of radio-activity from the tissue due to dissociation of the steroid-receptor com-plex may have taken place; thus resulting in reduced disintegrations per minute.

SUMMARY AND CONCLUSIONS

Twenty-eight female rats were utilized in this investigation. At 35 days old, twenty animals were adrenalectomized and maintained for one week on 1.0% sodium chloride solution. The remaining eight animals served as controls.

At periods of 30 minutes, 1 hour, 2 hours, 4 hours, and 6 hours before sacrifice, four animals each period (one at 6 hours) were injected subcutaneously with 1 μc/gm body weight of $(1,2-^3H)$ cortisone. The controls were injected (two rats each sacrifice period except 6 hours) with 1 μc/gm body weight of $(1,2-^3H)$ cortisone followed immediately with 4 mg/kg body weight of non-labeled cortisone. In addition, three adrenalectomized animals were injected subcutaneously with 1 μc/gm body weight of $(1,2-^3H)$ cortisol at 1, 2, and 4 hours before sacrifice. All rats were sacrificed after receiving an overdose of Nembutal.

Upon sacrifice, the animals were quickly decapitated and both the upper and lower jaws were removed. The lower jaw was placed in a 10% formalin solution buffered with calcium carbonate and utilized later for liquid scintillation counting. The root tips of both upper incisors were removed from the surrounding tissue, slightly embedded in minced liver on a tissue mount and immersed immediately in liquid propane (-180°C) for 5 seconds. The freeze-mounted specimen was then transferred rapidly to plastic bottle filled with liquid nitrogen. The bottle was then placed in liquid nitrogen storage until cryostat sectioning. Liver specimens

were also taken from four adrenalectomized animals representing 30 minute, 1 hour, 2 hour and 4 hour sacrifice times.

Cryostat sectioning was performed in a darkroom under a safe light. Tissue sections of 3 to 4 microns were made at -25 to -35°C. The thaw-mount technique introduced by Stumpf (1975) was followed. Sections were directly mounted on emulsion coated slides. The emulsion used was NTB2. The slides were put into light tight boxes and stored in a freezer. Exposure was accomplished at low temperature (-10°C). Each month, some test slides were developed. After 7½ months, the final group of slides were developed.

For liquid scintillation counting, the right mandibular incisor was utilized. Under a binocular dissecting microscope (10X) a 2 mm length of the root end of each incisor was carefully dissected free. After thorough drying, the root tips were individually weighed, placed in filter paper, and crushed under light pressure. By utilizing a Packard Tricarb Sample Oxidizer (Model 305), the samples were automatically combusted into a counting vial to which was added a measured amount of scintillator. Counts were recorded as disintegrations/minute/sample and disintegrations/minute/mg of sample weight.

Scintillation counting analysis showed that:

1. The greatest amount of radioactivity for the ^{3}H-cortisone treated animals was at the 30 minute time for both the experimental and control groups, and at 1 hour time interval for the ^{3}H-cortisol animal.

2. Average disintegrations for both the experimental and control groups dropped sharply thereafter and reached the minimum at the 4 hour period.

3. However, there was a statistical significant difference at 2 hours between experimental and control groups.

Similar results were observed autoradiographically.

1. The 30 minute thaw-mount autoradiograms were heavily labeled with silver grains with no definite localization to the cells of the apical tissue of the incisor.

2. The 4 hour sections showed the least number of silver grains over the tissue with still no intracellular localization apparent.

3. Liver sections at 4 hours also demonstrated minimal silver grains distribution without any apparent localization.

4. A similar pattern of silver grain distribution was demonstrated for the minor salivary glands of the palate.

5. The thaw-mount autoradiograms of the 6 hour animal also showed minimal silver grains distribution.

6. Heavy condensation of silver grains were observed over dentin and the bone surrounding the root apex of the rat maxillary incisor at 30 minutes after injection. This condensation was also demonstrated to a somewhat lesser extent at the 4 and 6 hours sacrifice time.

Based on the results of this investigation, it is concluded that the periapical tissues of the albino rat incisor may not possess receptors

for cortisone. Therefore, cortisone may not directly affect the proliferating cells at the basal region of this tooth through the usual mechanism of action of glucocorticoid hormones. In other words, the stimulatory effect of cortisone on incisor eruption may not be the result of a direct influence on the cell cycle events of the periapical tissues. Cortisone, therefore, may affect the eruption rate of the rat incisor through other more indirect mechanisms such as its action on dentin formation or growth of the surrounding bone. However, other considerations in this investigation are worth mentioning:

1. The thaw-mount technique used may not be suitable for localization of cortisone in the incisor. Despite the processing of tissue at low temperature, dissociation of the steroid from the target cells receptors may have occurred. To our knowledge, no one has reported localization of cortisone in any tissue by using any frozen tissue autoradiographic technique.

2. The low specific activity of the ^{3}H-cortisone (29 Ci/mmol) used may require further exposure time for the tissue sections in order to reveal any presumed localization within the cells of the periapices.

3. Specimens used for the liquid scintillation counting analysis were preserved in 10% formalin solution for some time before further processing thereby possibly causing some removal of radioactivity from the tissue.

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Table I. Weight and Disintegrations per Minute of Individual

Root Tips of the Right Mandibular Incisor of

^{3}H-Cortisone Treated and Control[1] Rats

Rat #	Post-Injection Time Interval	Sample Weight in grams	DPM/Sample[2]	DPM/mg of Tissue Sample
1 X	30 Minutes	.0017	350	205.22
2 X	"	.0020	455	227.50
3 X	"	.0022	503	228.64
4 X	"	.0022	327	148.64
5 C	"	.0015	418	278.67
6 C	"	.0024	400	166.67
7 X	1 hour	.0013	227	170.00
8 X	"	.0026	379	145.77
9 X	"	.0014	343	245.00
10 X	"	.0010	154	154.00
11 C	"	.0011	27	24.55
12 C	"	.0011	270	245.45
13 X	2 hours	.0015	101	67.33
14 X	"	.0012	89	74.17
15 X	"	.0013	28	21.54
16 X	"	.0019	102	53.68
17 C	"	.0025	39	15.60
18 C	"	.0018	19	10.56
19 X	4 hours	.0007	65	92.86
20 X	"	.0007	8.7	12.43
21 X	"	.0013	14	10.77
22 X	"	.0019	17	8.95
23 C	"	.0017	12	7.06
24 C	"	.0010	7.3	7.30
25 X	6 hours	.0033	30	9.09
27 Cortisol	1 hour	.0025	709	283.60
29 "	2 hours	.0010	62	62
31 "	4 hours	.0024	34	14.17

1. Controls were injected with ^{3}H-cortisone together with non-labeled cortisone
2. Disintegrations per minute
X. Experimental
C. Control

Table II. Mean Number of Radioactive Disintegrations in

the Root Tip of the Right Mandibular Incisor

of ^{3}H-Cortisone Treated and Control[1] Rats.

Group	Sacrifice Time Following Injection	Number of Root Tips	DPM/Sample[2]		DPM/mg	
			Mean	SD[3]	Mean	SD
1. Experimental	30 Minutes	4	408.8	±84.0	202.67	±37.5
Control		2	409.0	±12.7	222.67	±79.2
II. Experimental	1 hour	4	275.75	±103.9	178.69	±45.3
Control		2	148.5	±171.8	135.0	±156.2
III. Experimental	2 hours	4	80.0	±35.2	54.17	±23.4
Control		2	29.0	±10.0	13.08	±3.6
IV. Experimental	4 hours	4	26.18	±26.1	31.25	±41.0
Control		2	9.65	±5.3	7.18	±0.17

1. Controls were injected with ^{3}H-cortisone together with non-labeled cortisone

2. Disintegrations per minute

3. Standard deviation

FIGURE 2. Graph showing the mean number of disintegrations/
minute/mg in the root tips of experimental and
control rats sacrificed at various intervals following
injection.
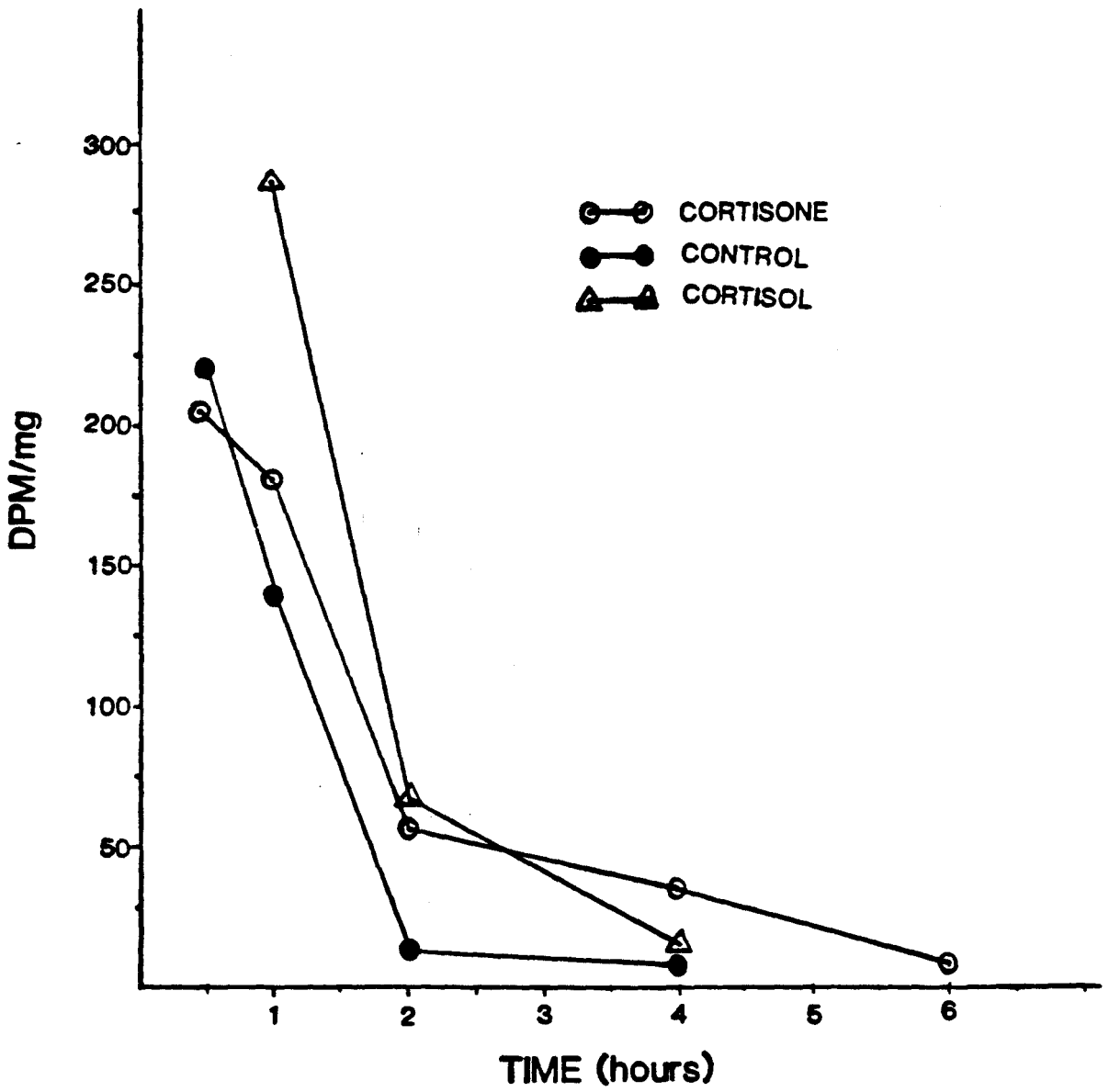
FIGURE 2

FIGURE 3. Graph showing the mean number of disintegrations/
minute/sample in the root tips of experimental and
control rats sacrificed at various intervals after
injection.
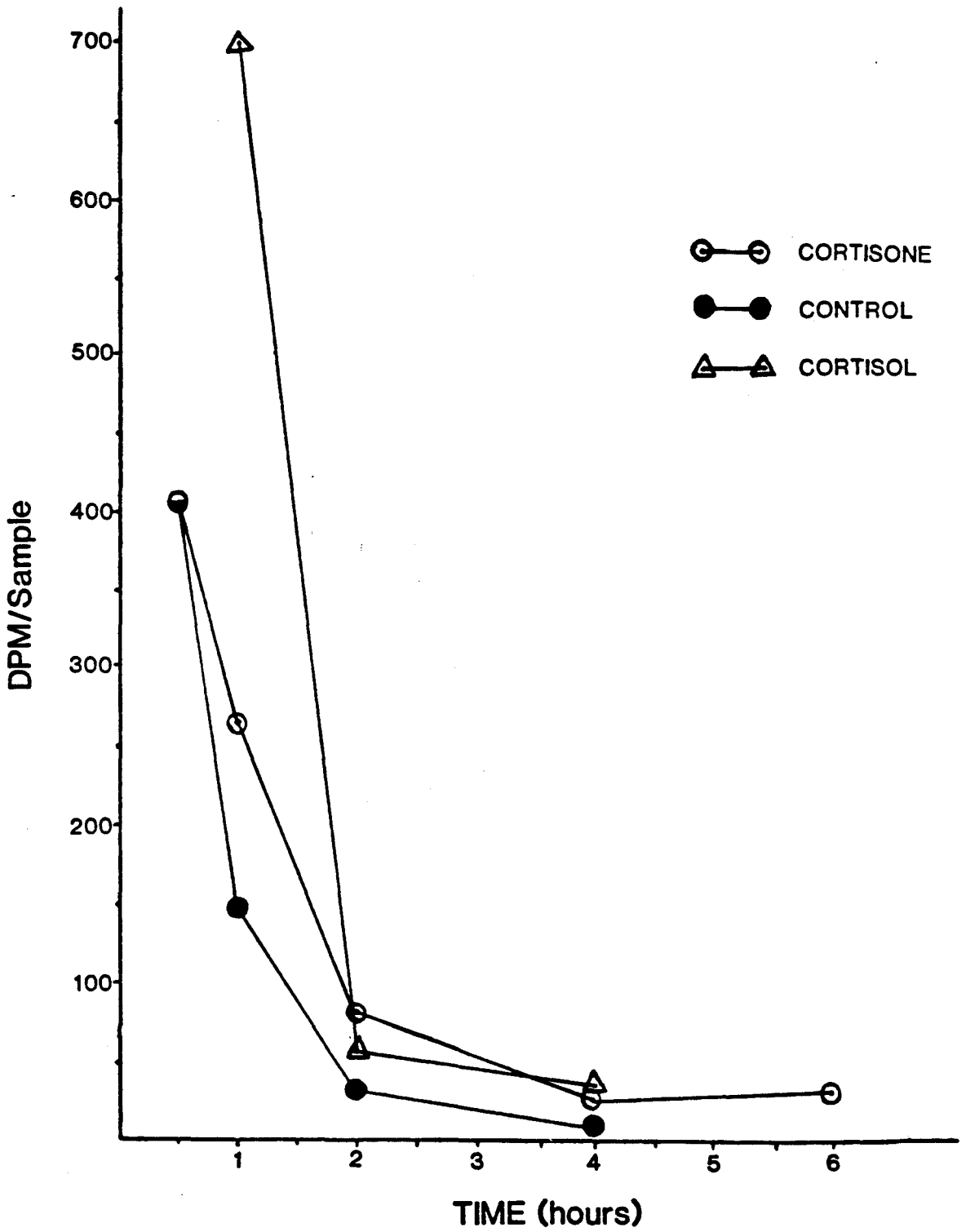
FIGURE 3

FIGURE 4. Thaw-mount autoradiogram showing random distribution of
silver grains over the cervical loop and the pulp cells
of the rat maxillary incisor of a ^{3}H-cortisone treated
adrenalectomized animal 30 minutes following injection.
Seven and one-half months of exposure time. (X45)

FIGURE 5. Thaw-mount autoradiogram from a control animal
sacrificed at 30 minutes after ^{3}H-cortisone
injection comparable to Figure 4. (X45)
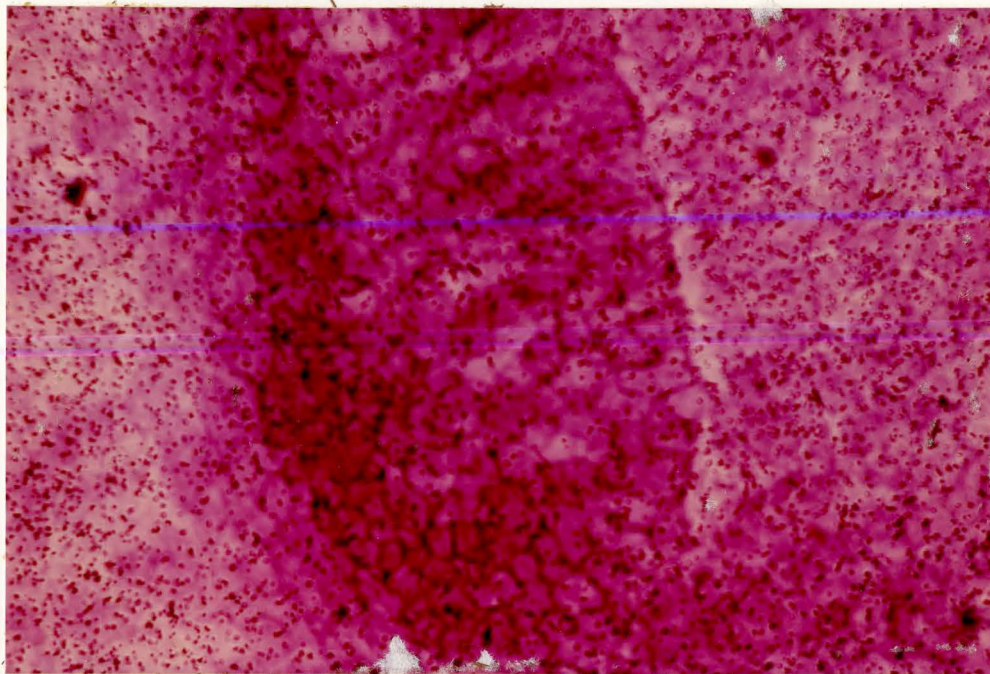
FIGURE 4
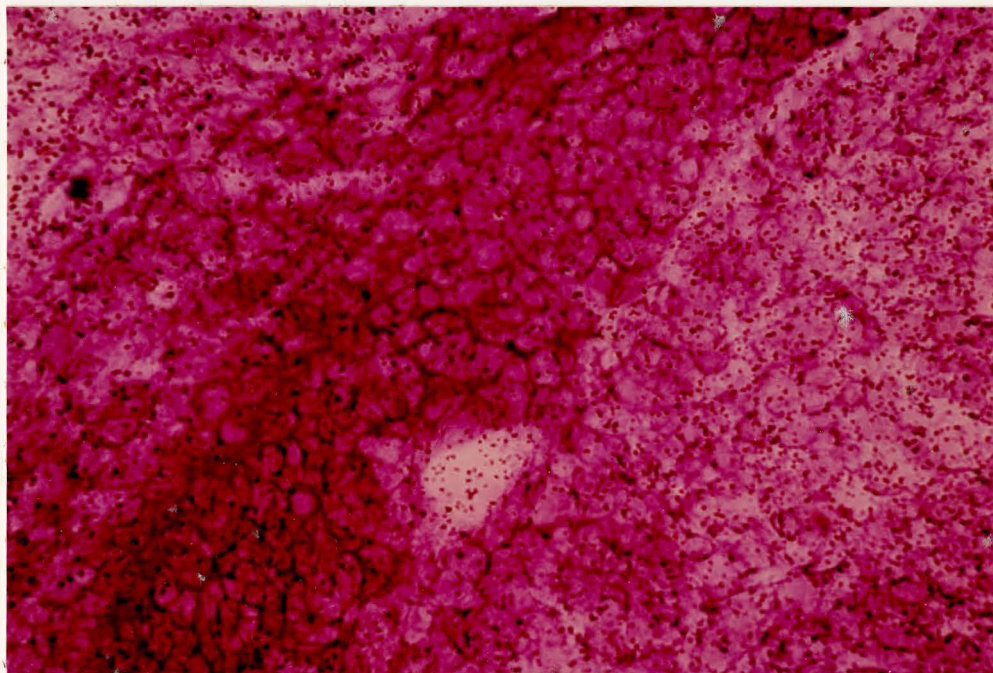
FIGURE 5

FIGURE 6. Frozen tissue autoradiogram showing silver grain

distribution over the pulp cells of the rat

maxillary incisor from an adrenalectomized rat

sacrificed 30 minutes after ^{3}H-cortisone

injection. (X45)
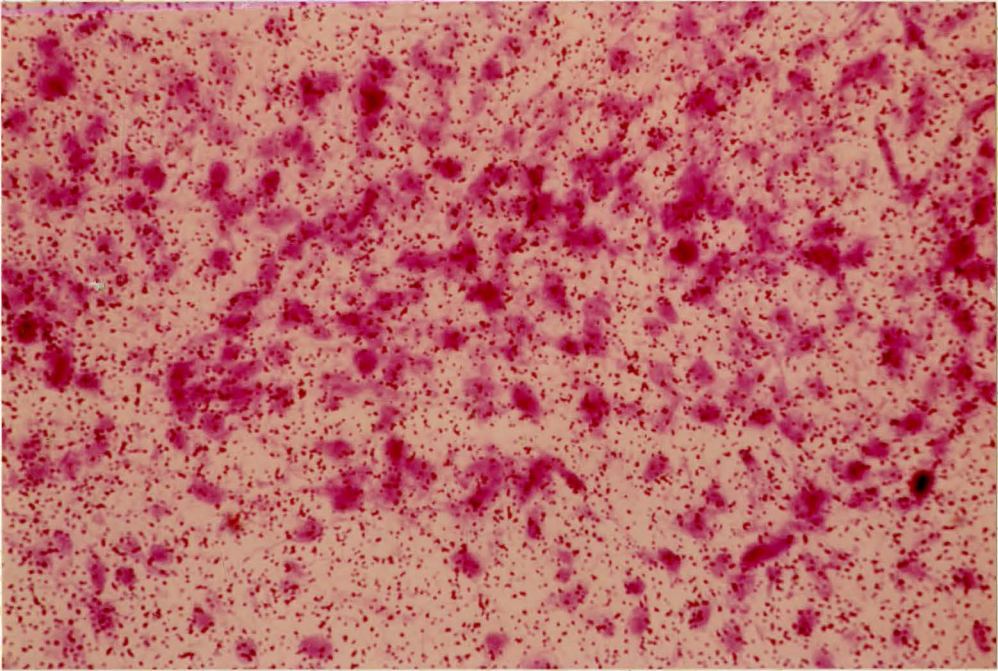
FIGURE 6

FIGURE 7. Thaw-mount autoradiogram of an 1 hour ^{3}H-cortisone

treated adrenalectomized rat showing silver grain

distribution over the cervical loop of the rat

maxillary incisor. No definite localization can

be seen over these cells after 7½ months of exposure

time. (X45)

FIGURE 8. Comparable section to Figure 7 representing a

control rat sacrificed at 1 hour. (X45)
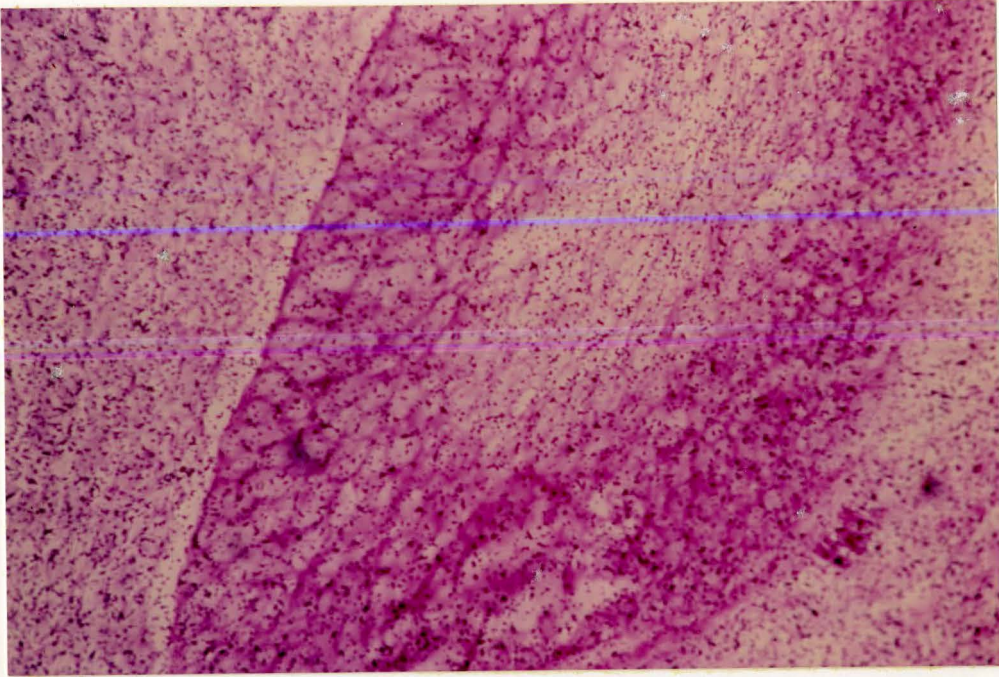
FIGURE 7
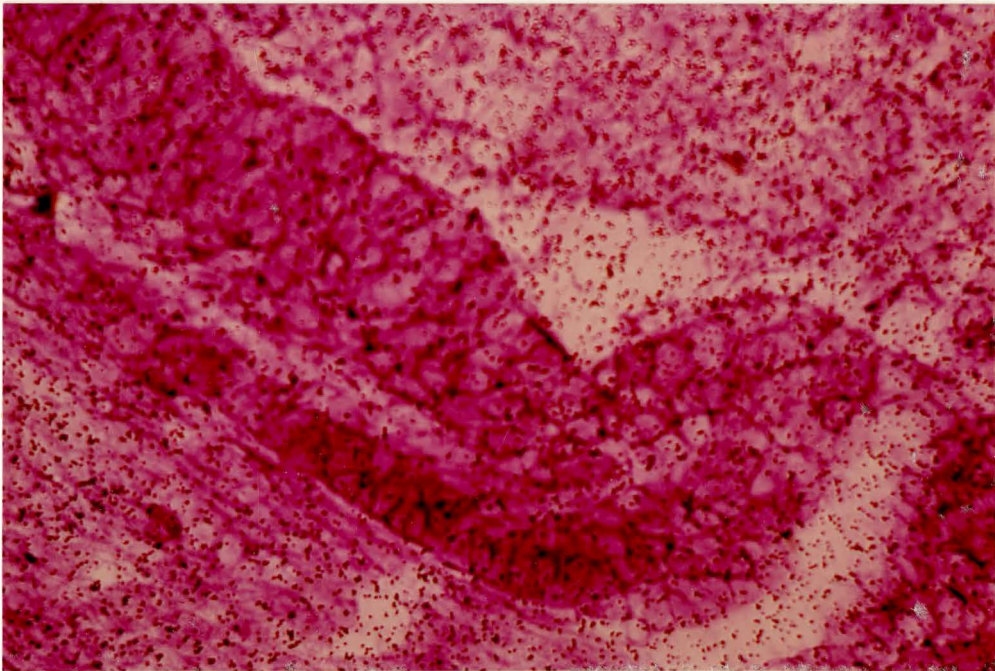
FIGURE 8

FIGURE 9. High power section showing silver grain distribution over the cervical loop of the rat maxillary incisor from a ^{3}H-cortisone treated adrenalectomized rat at 2 hours post-injection. No localization of silver grains are obvious. (X45) Exposure time: 7½ months.

FIGURE 10. Section of cervical loop from control rat comparable to Figure 9. (X45)
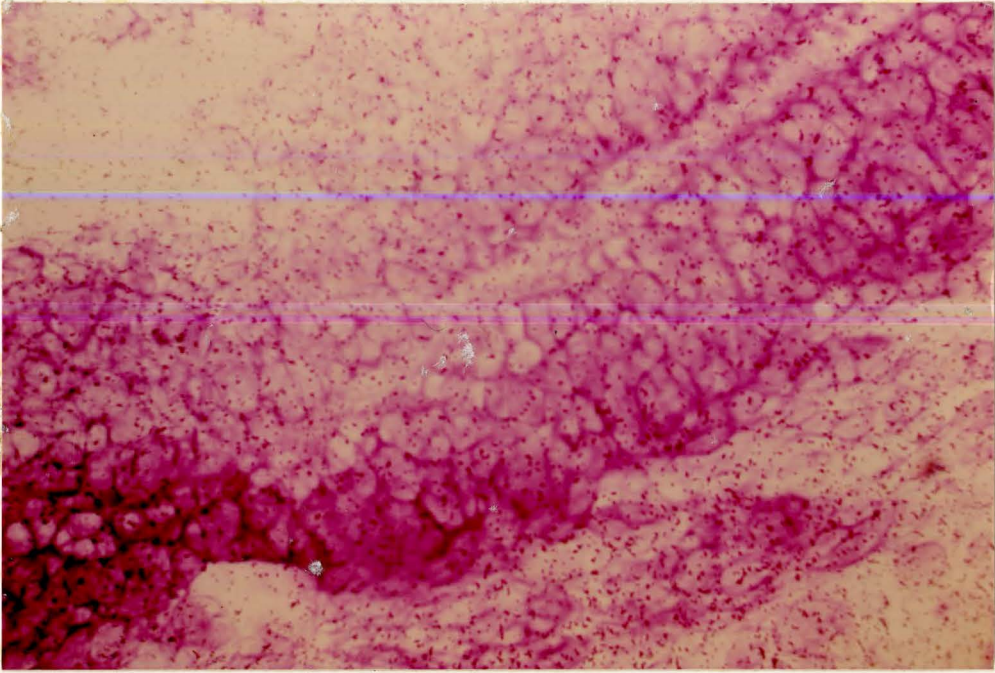
FIGURE 9
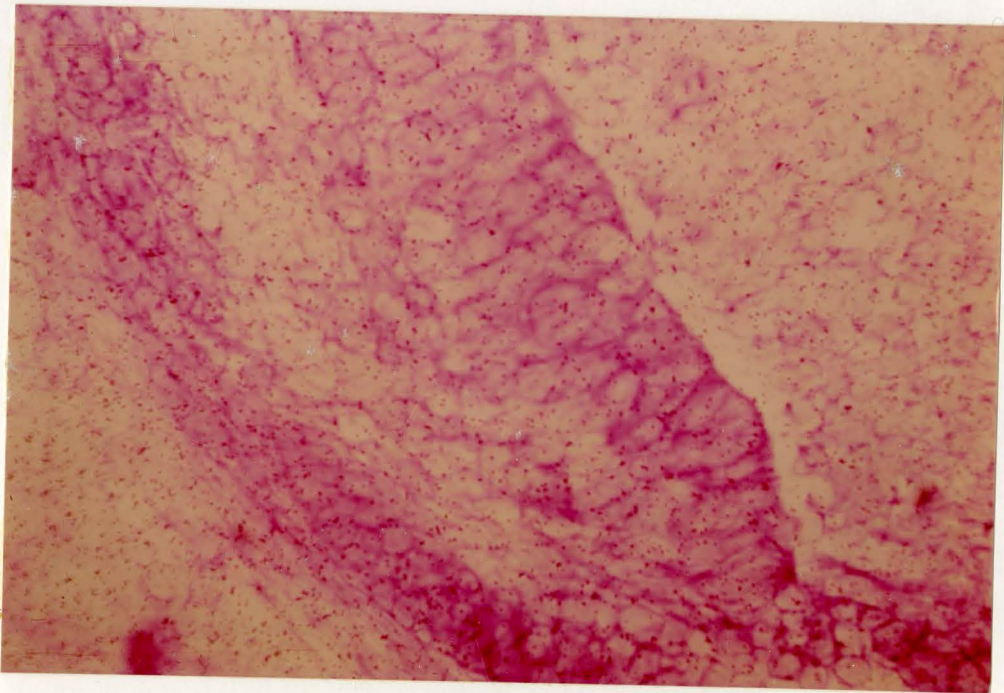
FIGURE 10

FIGURE 11. Oil immersion thaw-mount autoradiogram from an
adrenalectomized rat given ^{3}H-cortisone 2 hours
prior to sacrifice showing cells of the cervical
loop. No definite localization of silver grains
over these cells can be seen. (X100)
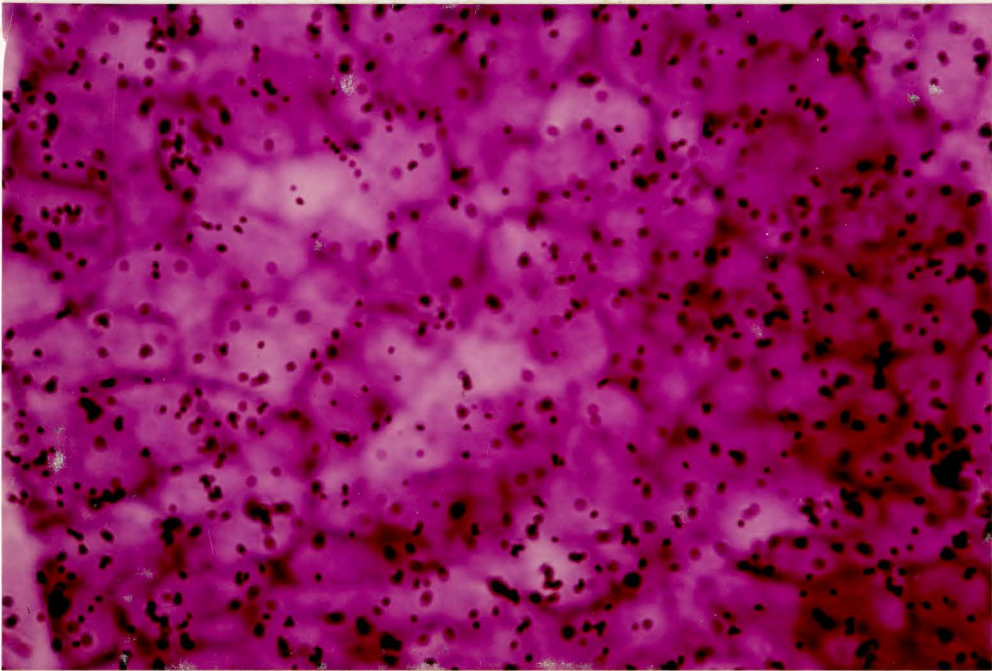
FIGURE 11

FIGURE 12. Thaw-mount autoradiogram of a ^{3}H-cortisone experimental
animals at 4 hours showing the cervical loop of the
maxillary incisor. No obvious intracellular localiza-
tion of silver grains can be seen after 7½ months of
exposure. (X100)

FIGURE 13. Frozen tissue autoradiogram of a control rat sacrificed
at 4 hours after injection showing the cervical loop
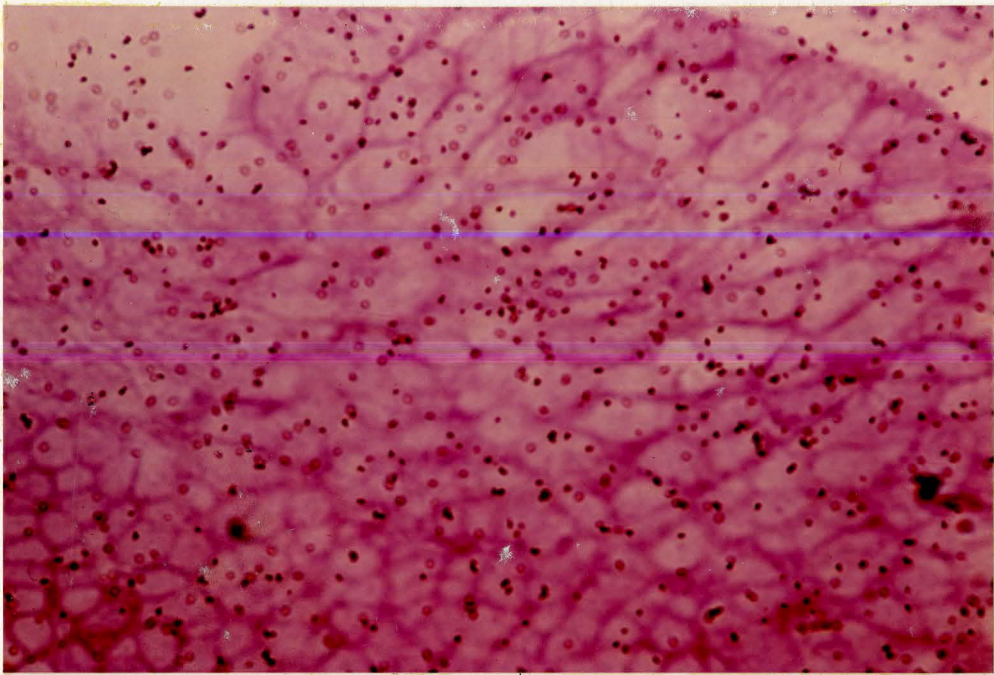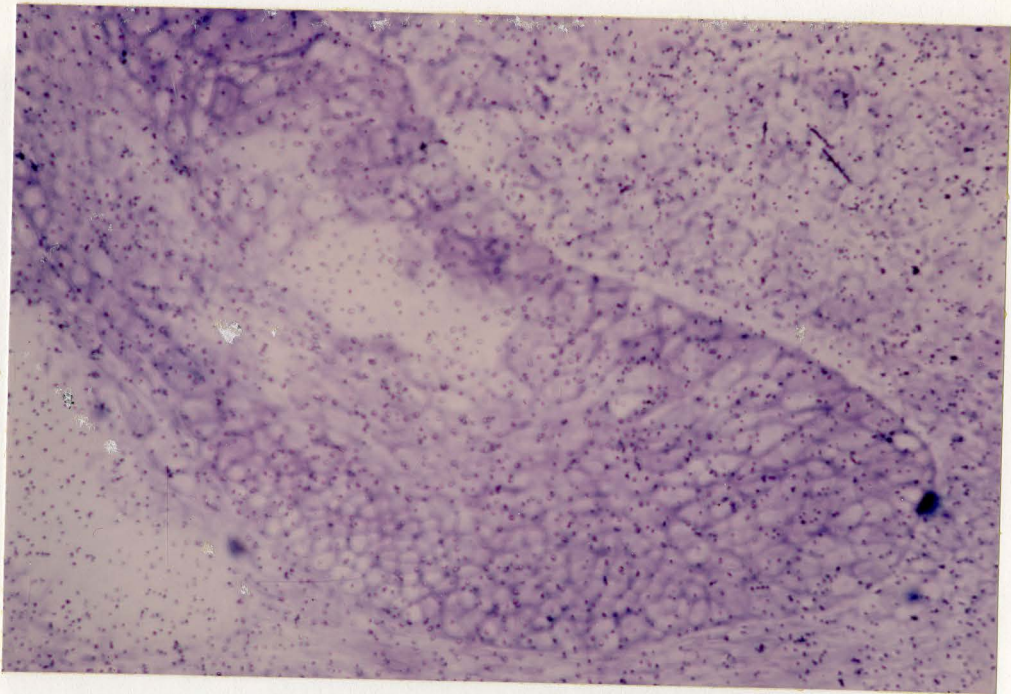of the rat maxillary incisor. (X45)

FIGURE 12

FIGURE 13

FIGURE 14. Thaw-mount autoradiogram of the ³H-cortisol treated
animal sacrificed at 4 hours showing a high magnifica-
.tion of the pulp cells of the rat maxillary incisor.
Localization of silver grains over the nuclei of some
of these cells can be seen (arrows). (X100)
Exposure time: 7½ months.

FIGURE 15. Thaw-mount autoradiogram of the labial aspect of the
rat maxillary incisor showing heavy concentration of
silver grains over dentin. (X45) The animal was
sacrificed 4 hours after ³H-cortisone injection.
Abbreviations: O - Odontoblast
P - Predentin
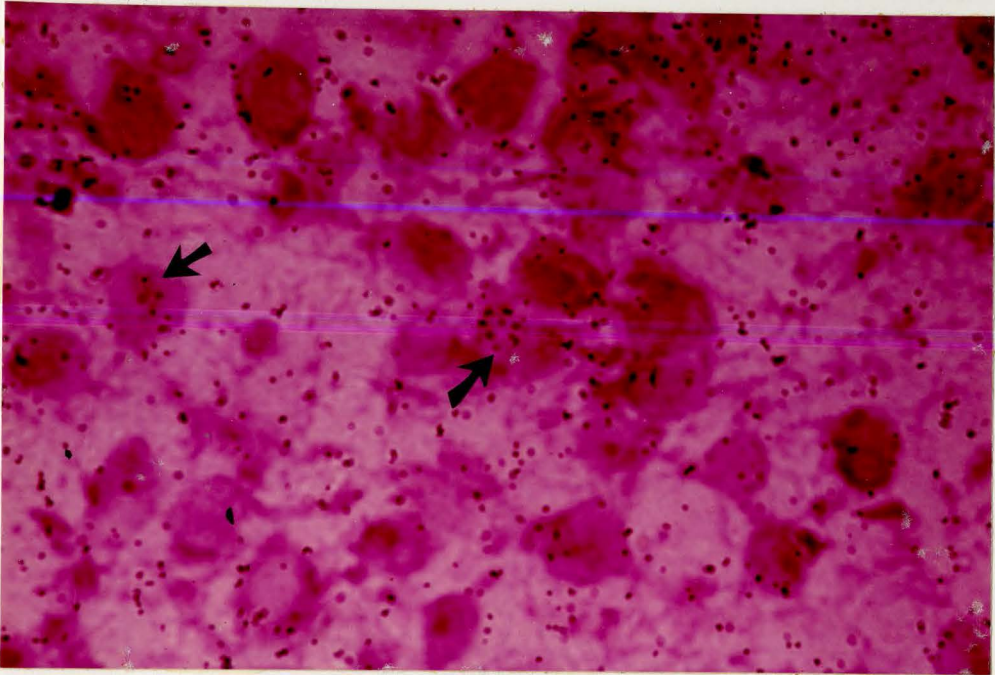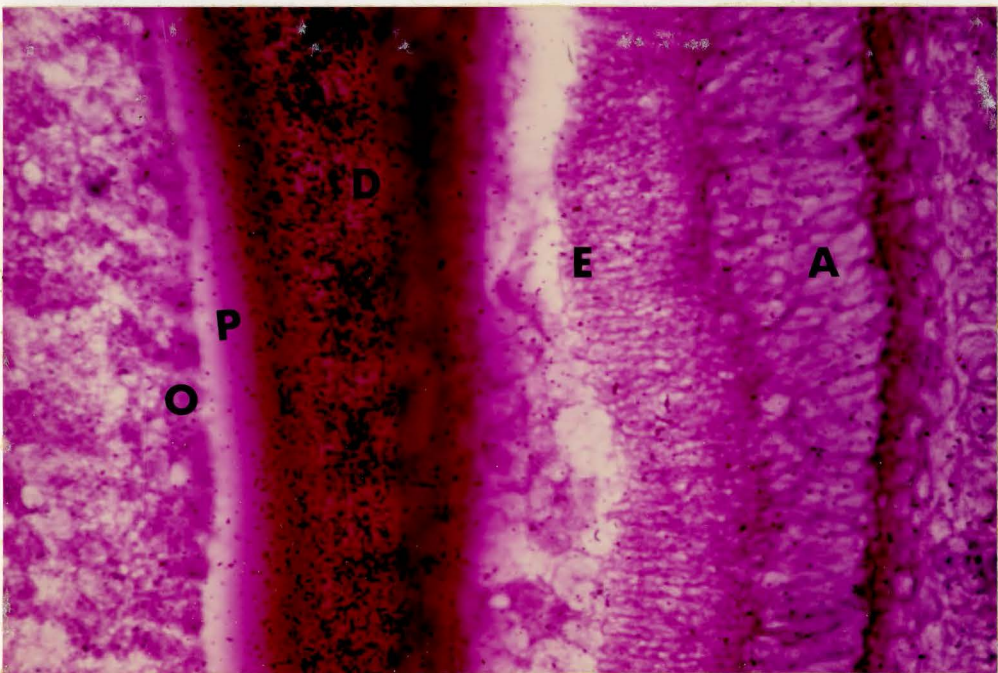D - Dentin
E - Enamel
A - Ameloblasts

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FIGURE 14

FIGURE 15

APPROVAL SHEET

The thesis submitted by Mohammed Mohammed Rahima, has been read and approved by the following committee:

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

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

12-7-83
Date

Director's Signature

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