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RADIO-AUTOGRAPHIC LOCALIZATION OF Ca^{45} AND P^{32}
IN RAT TEETH

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RADIO-AUTOGRAPHIC LOCALIZATION OF INJECTED CALCIUM^{45} AND PHOSPHORUS^{32} IN GROWING TEETH OF RATS

by

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Thesis

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INTRODUCTION

Introductory remark

Classical investigations conducted on the development and morphology of the rat dentition have clarified the main phases of tooth growth (Addison and Appleton, '15; Andrews, '19; Noyes et al, '38; Schour and Massler, '42; Orban, '44; Maximow and Bloom, '50; Ham, '53). The advent of radio-isotopes provided a new tool, which when used in tracer amounts, is more physiologically compatible to the living body than any of those commonly used in the past. In addition to this advantage, the use of a radio-active element offered increased precision of the experimental results. In calcification studies, the radio-active forms of calcium and phosphorus have been utilized. These two elements, available as Ca^{45} and P^{32} with half lives of 180 days and 14.3 days respectively are β emitting isotopes, Ca^{45} with an energy of 0.25 mev. and P^{32} with 1.69 mev. The properties of these isotopes lend themselves to radio-chemical and also radio-autographic studies.

Purpose of present study

This project was undertaken to investigate the process of the deposition of mineral elements in the teeth of the laboratory rat after administration of tracer doses of either one of these radio-elements. The bulk of the work has been carried out by means of the radio-autographic method of

analysis, which allows fine localizations of the radio-element deposited in the tissues.

Research on the development of the tooth has resorted to histological as well as chemical methods. However, before elaborating on these findings, the gross dentition and an outline of the developmental histology of the teeth of the rat, the animal selected for this study, will be presented.

Gross dentition of the rat

The rat is a monophyodont with a dental formula of I 1/1, M 3/3, that is, it has only one set of teeth consisting of eight in each jaw, two incisors and six molars. A toothless space, the diastema, separates the molars from the incisors.

Incisor

The rat, like other rodents, for instance the mouse, squirrel and beaver, uses its incisors as chisels for cutting hard substances for which purpose these teeth have been especially adapted. The incisors viewed from laterallyare curved; their forms have been analyzed to be separate segments of the same logarithmic spiral and take the form of arcs of spirals of different diameters (Schour, '42; Addison and Appleton, '51). The incisor consists of two parts, a labial or concave surface covered with enamel (usually a yellow-brown colour in the adult), and a lingual or concave surface with cementum. The articulation of the rat jaws allows a forward and backward movement as well as in an upwards and downwards direction due to a modification of the temporo-mandibular joint. This enables the rat to close

its jaw with the lower incisor lingual or labial to its upper jaw. The tip of the incisor tooth is continually lost by attrition, a process of erosion or wearing away by friction. The enamel is less rapidly lost than the dentin and cementum which are not as durable as the enamel and this results in the chisel shaped tip. The length of the tooth is maintained by a continuous growth of its basal end where the odontogenic or tooth forming structures function throughout the life of the animal. The curved form of the incisor is the result of a more rapid cellular activity of the odontogenic base on the labial than on the lingual surface.

Molar

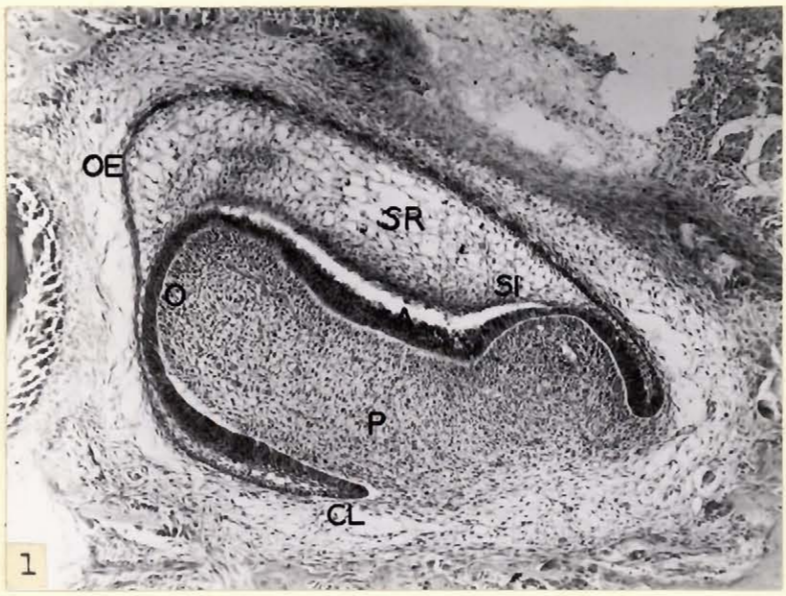
The molars, in contrast to the incisors, are of limited growth and resemble human molars except for the enamel free areas on the tip of the cusps. The first molar is the largest, the third, the smallest and the three develop in numerical order. In the mandible, the limits of the anterior surface of the first and the posterior surface of the third molars lie within the posterior third of the arch of lower incisor. In the maxilla, the anterior limits of the first molar lie posterior to the odontogenic base of the upper incisor.

Developmental histology of the teeth

The sequence of events involved in odontogenesis are initiation, proliferation, histodifferentiation, morphodifferentiation of the dental lamina, apposition of enamel

and dentin, and cementum matrices, calcification and tooth eruption. Of these stages, the first, initiation, is the shortest (Noyes et al, '38; Orban, '44; Schour, '52). The ectodermal portion of the tooth bud is formed by a rapid proliferation and invagination of the ectoderm of the oral cavity into the dental arch. An accumulation of mesenchymal cells under the ectodermal cells forms the primordium of the papilla. The cap stage follows. The cap is formed by a shallow invagination on the deep surface of the tooth bud. The phase of histodifferentiation follows during which the enamel organ begins to develop. The convex surface of the cap consists of a single layer of cuboidal cells--the outer enamel epithelium--while the concave surface is formed by a layer of columnar cells--the inner enamel epithelium. The group of cells between these two epithelia is the stellate reticulum or enamel pulp, formed of cells of a branched reticular type. The cells of the inner enamel epithelium soon change their polarity and develop into ameloblasts or ganoblasts, the enamel forming cells. The stratum intermedium, consisting of several layers of flat cells then appears between the stellate reticulum and the inner enamel epithelium in the amelogenic regions. These changes are followed by a differentiation, under the influence of the ameloblasts, of the peripheral cells of the mesenchyme forming the papilla, which become the odontoblasts, the dentin forming cells. Differentiation into odontoblasts has been claimed to occur only under the influence of

PLATE 1



Molar tooth germ. EO: outer enamel epithelium; SR: stellate reticulum; SI: stratum intermedium; A: ameloblasts; P: pulp; CL: cervical loop. The dental sac surrounds the tooth germ. Magnification × 102

pre-ameloblasts, while differentiation into ameloblasts occurs only after the deposition of dentin (Schour, '52). Concomitant with the development of the enamel organ, the cells surrounding the future tooth form the dental sac which later develops into periodontal fibers. These changes alter the shape of the enamel organ to that of a bell. Morphodifferentiation at the bell stage defines the outline of the future dentino-enamel and dentino-cemental junctions (Fig. 1). The junction of the inner and outer enamel epithelia at the basal margin of the enamel organ, in the region of the future cemento-enamel junction is called the cervical loop which proliferates and gives rise to the epithelial root sheath of Hertwig. This epithelial sheath plays a role in shaping the root but does not begin to exert its influence until the enamel matrix has been completely formed and the site of the cemento-enamel junction established (Diamond and Applebaum, '42). The odontoblasts differentiate from the dental papilla in the region of the future root under the influence of the inner layer of Hertwig's epithelial root sheath, which disappears after the root is formed. The persisting remnants of the latter are known as the epithelial rests. Each of the changes which have been described occurs gradually and there is an overlapping of the different phases.

The apposition phase in which the dentin, enamel and cementum matrices are formed then follows. The formation of dentin commences with thickening of the membrane (membrana

preformativa) between the ameloblasts and the odontoblasts. Precollagenous fibrils known as Korff's fibers originating from and continuous with those in the pulp, merge with the fibers of the membrane. These fibrils change to the collagenous type and become embedded in the interfibrillar cementing substance forming the organic dentin matrix. The uncalcified form of this matrix is known as predentin. The odontoblasts play a role in the formation of this matrix. Calcification follows matrix apposition and by the time a succeeding layer of predentin is formed, the layer preceding it is already calcified. As more matrix is deposited, the odontoblasts recede from the dentin. A fine process of these cells, however, remains in the dentin, forming the dentinal fibers which are present in the dentinal tubules. These processes are found to run throughout the whole thickness of the dentin to the dentino-enamel junction.

The ameloblasts which form enamel matrix enter their formative stage only when the first layer of dentin has already been formed. The dentin is necessary to induce the beginning of enamel matrix formation just as it was necessary for ameloblasts to come into close contact with the connective tissue of the pulp to induce differentiation of the odontoblasts and the beginning of dentin formation. The first result of the activity of the ameloblasts is the formation of a thin membrane, the dentino-enamel membrane, on the enamel side of the membrana preformativa. In later stages of enamel development, the dentino-enamel membrane is continuous with the

interrod substance; its function is probably that of cementing together the enamel and dentin. This new membrane calcifies after its formation. The ameloblasts then produce short processes known as Tomes' processes which are demarcated from the cell body by terminal bars at the basal end, that is, the end nearest the dentin. These processes may be considered as the primordia of the enamel rods. Tomes' processes which are hexagonal in shape, are granular at first, but later become homogenized and become basophilic in reaction. The homogenized Tomes' processes is then transformed into pre-enamel. The latter begins to calcify, accompanied by a change in which it becomes acidophilic and may henceforth be referred to as young enamel matrix. All of the changes described proceed from the dentinal end. The end of the matrix formation phase is indicated by a gradual reversal of the young enamel matrix into a slightly basophilic state. As the enamel matrix is deposited the ameloblasts, like the odontoblasts, recede from the enamel. The maturation phase then follows, which commences after the final thickness of the enamel matrix has been reached. This phase is characterized by a gradual influx of almost three quarters of the ultimate content of its mineral salts and by a simultaneous loss of water from the matrix. When the enamel has completely developed and matured, the ameloblasts become cuboidal and can no longer be differentiated from other cells of the enamel organ. They then form a stratified epithelial covering over the enamel, the so called

reduced enamel epithelium which is protective in function.

The development of cementum, cementogenesis, occurs over the dentin formed under the influence of Hertwig's epithelial sheath. The covering of the latter over the newly formed dentin is soon disrupted by an invasion of connective tissue surrounding the developing root. When the cells of connective tissue, the fibrolasts, establishes contact with the root surface, cementum is formed in the following manner. Precollagenous or argyrophil fibers which later changes to collagenous fibers appear at right angles to the root surface and attach to the surface of the dentin. Cementoblasts, which form from fibroblasts are presumed to secrete an organic cementing substance. The collagenous fibers are bound by this product to form the cementum matrix. Such fibers are known as Sharpey's fibers. Calcium salts are then deposited in this matrix. Cementum which is thus formed is acellular; however, cells, cementocytes, may come to lie in the cementum, thus resulting in the cellular type. The function of the cementum is the attachment of the tooth to the surrounding tissues.

The area of mesenchyme enclosed by the odontoblasts becomes the pulp. The original cells retain their star-shaped appearance even in the adult. In addition to these cells, the pulp contains precollagenous fibers, blood vessels, phagocytic cells and myelinated nerve fibers.

Eruption of the tooth commences at the time of root formation and continues throughout the life of the tooth. The

term was at one time applied to the appearance of the tooth in the oral cavity but it is now understood to apply to the movement of the tooth which occurs even after its appearance in the mouth. The process of tooth eruption involves mechanisms which are too complex to be elaborated here; root elongation, growth of the alveolar bone and dental sac are some of the factors responsible for tooth eruption.

History

The study of dental structures has proceeded in many phases of development, growth, and function under various conditions, not excluding some of clinical significance. It is impossible to give here a complete summary of investigations in these fields with fair credit to all the investigators. Therefore, only those of special interest to a study of development and calcification of teeth will be noted.

The development and growth of a tooth, as in other organs, requires the balance of intricate mechanisms, synchronized and occurring in a harmonious sequence. Experimental efforts to demonstrate the intrinsic growth potential of rabbit and rat molars were carried out in vitro in hanging drop preparations (Glasstone, '38; '52). Molar teeth germs were found to possess ability to form cusps when they were extracted from embryos prior to cusp formation. This capacity was lost when odontoblasts and dentin appeared in the tooth. A similar study in vivo (Fleming, '52), involving transplants of whole

or parts of tooth germs to the anterior chamber of the eye of guinea pig, rabbit and mouse revealed that these tissues survived when the transplants were from man to lower animals and between lower animals, except for homotransplants of dental anlagen of 14 or 15 day mice embryos. Morphology of the tooth was lost when ameloblasts failed to survive while odontoblasts separated from ameloblasts were capable of forming dentin. The odontogenic potency was lost with time, and osteoid tissue resembling bone were found to replace the transplants.

The intrinsic forces in the developing tooth may be affected by alteration in metabolism, which cause disturbances reflected in the tooth. Thus, experiments involving deprivation of minerals, vitamins, and hormones have been conducted and reported as producing marked changes in the teeth (Leicester, '49). In some cases, replacement therapy was applied and reparative changes noted.

Diets lacking or consisting of unfavourable ratios of mineral elements, most notably calcium and phosphorus, reflect the disturbances in retarded rate of growth and in altered composition of the teeth. It is generally accepted that growing teeth are favoured at the expense of bones under such conditions but they are not totally uninfluenced; the mechanism of calcification is hindered while the processes of matrix formation remain unaffected. Deprivation of magnesium, or administration of strontium, fluorine and manganese have been

tried. All of these showed effects on the teeth either in the matrix or calcification process or both. The fact that the teeth readily reflect these changes has been used in studies on the rate of apposition and calcification of dentin and enamel.

Of the vitamins, A, C and D are known to affect the teeth during the course of their growth. The fundamental function of vitamin A is to control the development of epithelial tissues. Thus the ameloblasts, which are of ectodermal origin, do not develop properly in vitamin A deficiency. This in turn produces secondary effects in the dentin, resulting from the fact that odontoblasts depend on the proper function of ameloblasts in order to produce dentin matrix. Vitamin C is intimately connected with mesenchymal tissue development from which the odontoblasts are derived. During prolonged vitamin C deficiency, these cells degenerate, accompanied by other symptoms of scurvy and in turn produce drastic changes in the dentin. Secondary changes follow wherever enamel was in the process of being formed since enamel formation is dependent on the presence of dentin. Vitamin D deficiency produces symptoms which are not distinct from those associated with altered calcium and phosphorus ratios or deficiencies of these mineral elements, while toxic symptoms caused by excessive doses are accompanied by a rise and then a fall of blood calcium. Both types of disturbances are recorded in the teeth, especially in the dentin.

Hormones have been found to possess little direct effect on growing tooth except for the parathyroid hormone. Deficiency of the latter causes disturbances similar to calcium deficiency or vitamin D deficiency in low calcium diet, while overdoses of the hormone produces hypo- followed by hypercalcification of the developing dentin. The enamel is also observed to undergo hypoplasia.

Research on the chemical and physical properties of the teeth have contributed much to the understanding of the behaviour of the teeth, both during and after growth (Orban '44; Leicester, '49).

The chemical composition of the dental structures has been investigated by direct chemical analysis of calcium and phosphorus and other constituents. Elements analyzed to be present in small quantities in the teeth include sodium, potassium, lead, iron, copper, uranium, chlorine, fluorine, lithium, and strontium. Much of the work has been conducted on human teeth; average figures are 36% calcium, 17% phosphorus, 0.4% magnesium and 2.5% carbon dioxide while for the dentin the corresponding figures are 27% calcium, 13% phosphorus, 0.8% magnesium and 3% carbon dioxide. These values were obtained from dried, unashed samples. Ashing raises the values for dentin to nearly those for enamel, that is, 35% calcium, 17% phosphorus, 1.2% magnesium and 4% carbon diosice. Ash values of cementum are 35.5%, 17%, 0.9% and 4.4% respectively for the same components. It has been found that human teeth appears to be more completely calcified than

those of other animals. The mineral contents of rat incisors were examined in detail (Matsuda, '27). There was a steady increase in the inorganic residue with increasing age.

Studies into the physical properties of the tooth have yielded data which have contributed to elucidating the form of the crystal composing the mineral salt of the teeth. It is now generally agreed that the crystal structure of bone and teeth most resemble the apatite form of minerals (Sobel et al, '49; McConnell, '52a; '52b; Trautz et al, '53).

In recent years particularly, the presence of various substances in the organic moiety of the oral structures has been revealed, namely alkaline phosphatase (Bevelander and Johnson, '45; '46; '49; Greep et al, '48; Harris, '50; Belanger, '51), cholesterol (Leopold et al, '51), chondroitin sulfuric acid (Hess and Lee, '52) and sulphur (Verne et al, '52), polysaccharides (Engel, '48; Wislocki et al, '48; Wislocki and Sognnaes, '50; Verne et al, '52), ground substance (Engel, '51), proteins and amino acids (Carter et al, '51; Hutton and Nuckolls, '51; Stack, '51; Hess et al, '52; Stack and Williams, '52; Verne and Weill, '53). The interrelationships of these substances and the significance of the role which they play in the development, growth and maintenance of the dental structures are not completely understood at present.

Radio-chemical methods have become steadily popular in dental research. Radio-active phosphorus and more recently,

radio-active calcium have been used in studies on the mineral metabolism of teeth and bones. After administration of P^{32} to animals, the radio-activity was found to be deposited in the bones and teeth which were undergoing calcification at the time. In the long bones, the epiphyseal contained more radio-activity than the diaphyseal portions (Leblond et al, '50; Lacroix, Devis, Schicks, '52) and in the tooth, the dentin contained more than the enamel (Hevesy and Armstrong, '40; Volker and Sognnaes, '41; Sognnaes and Volker, '41). Circum-pulpal dentin was observed to be more radio-active than areas more distant from the pulp (Bevelander and Amler, '45; McCauley and Gilda, '43). In the continuously growing incisor of the rat, the radio-activity was found to increase with time after administration of P^{32} (Manly and Bale,'39; Leblond et al, '50). Subsequent experiments with Ca^{45} have revealed similar trends in the incorporation of the radio-element into the calcifying structures (Armstrong, '45; Armstrong and Barnum, '48; Harrison and Harrison, '50; D'Iorio and Lussier, '51; Carlsson, '51; '52; Singer et al, '52; Minder, '52).

In the foregoing experiments, the degree of calcification and permeability of the structures limited the amount of the radio-activity present. Thus, young animals were found to show a greater uptake of the radio-isotope (Volker and Sognnaes, '41). The purely physical aspects as distinguished from the metabolic phases of the incorporation of the radio-isotope were studied. Experimental removal of the pulp of dogs before

administration of the isotope have revealed that some isotope is found in such teeth, indicating that the process does operate. However, the amount of the isotope found in the pulpless tooth was much less than in the normal tooth (McCauley and Gilda, '43). The uptake of radio-activity from the surface of erupted teeth was also investigated (Bartlestone, '51). Studies conducted in vitro exposing dentin and enamel to solutions containing radio-activity resulted in the uptake of the radio-activity by these structures (Bevelander and Amler, '45; Falkenheim et al, '47; Underwood and Hodge, '52; Belanger, '53) as has been found in the case of the bones (Neuman and Mulryan, '50; '51).

An adaptation of the radiochemical methods to locate the isotope histologically has been attempted in radio-autographic methods. The results of the chemical analysis involving counting of the radio-activity were confirmed. Moreover, the radio-autographic method allows the investigation of the deposition of the mineral elements following apposition in detail. In this way previous studies on the apposition and calcification of the teeth by injection of substances such as alizarin (Schour and Hoffman, '39b; Hoffman and Schour, '40), sodium fluoride (Schour and Smith, '34; Schour and Hoffman, '39b) and strontium chloride (Weinmann, '42; Irving and Weinmann, '48) could be extended. In the systematic investigations of the deposition of P^{32} in the dentin of very young animal teeth, it was found that the radiophosphorus was deposited as layers

which showed in the autographs as a band after early time intervals following its administration (Belanger, '51; '52). In the present study, it was decided to confirm his experiments with P^{32} and extend the observations using Ca^{45}. The procedures involved will be elaborated elsewhere.

METHODS

Introductory remark

In the present study, which is an attempt to follow the process of calcification of the teeth, the radio-autographic method of analysis of undecalcified tissues is utilized. The manner of deposition of the mineral salts and their fate in the teeth was examined after administration of tracer doses of radio-active forms of calcium, Ca^{45}, and phosphorus, P^{32}. The process is best studied in the young, rapidly growing teeth; such material was obtained from young rats. Another reason for using very young rats was that the teeth, one of the hardest structures in the body, soon became too hard to prepare for histological examination in the undecalcified state. The necessity for using undecalcified sections, since decalcification would remove the administered radio-element, raised the problem of obtaining good histological section of the teeth when the amount of the mineral elements increased. Several methods were tried, of which the successful ones are described in detail.

Experimental animals

Selection of young rats

For reasons stated in the preceding paragraph, young rats were used in the present study. The black & white hooded rats obtained from the Royal Victoria Hospital animal room were used. Pregnant rats, which were nearly at term were put into

separate cages, where the young were born. These mothers were fed on Purina Fox Chow and water while the young were nursed by their mother. In order to obtain tissues of normal, healthy and growing rats, the litters were inspected daily and only those which were well nursed were used for the experiment.

Determination of age

The age of the young rats were determined from the day when they were first seen. Thus rats in the newborn group were injected on the day they were born. However, before starting the experiment of the newborn group, sufficent time was allowed, 3 - 4 hours, to observe whether the mother was nursing and caring for her young properly. The three day old group did not receive the radioelement until the third day of life.

Dose of radio-activity

The dose which gave suitable amounts of radio-activity to be deposited in the teeth for the radio-autographic procedure was found by trial. Sections of teeth obtained from a rat given a dose of 1 uc per gram of body weight in the case of Ca^{45} were, after the routine preparation for radio-autography, coated with photographic emulsion and stored for exposure. The optimal exposure was determined by developing test slides at frequent intervals, for example, one day after coating or even after a few hours if an underexposed autograph is preferred. This system allowed a limited range

in the dose of radio-activity to produce a radio-autograph as can be seen in the following sections. The same method was applied to obtain radio-autographs from P^{32} containing tissues.

Radio-active phosphorus

Phosphorus^{32} which decays much more rapidly, (14.3 days) than calcium (180 days) was injected in greater doses, namely 5 µc per gram of body weight. Also phosphorus is an element found as chemical component of soft tissues and body fluids. Therefore, it is inevitable that some of the injected P^{32} becomes incorporated into these components. The amounts diverted to them were negligible radio-autographically in the present study. The rats which averaged 5 grams received 25 uc (5.1 mg. P) in a volume of 0.04 cc (newborn group), while those weighing 7 grams each received 30 µc (90.9 mg. P) in a volume of 0.154 cc (3 day old group). The isotope, received as $H_3P^{32}O_4$ from Chalk River, Ontario, Canada was injected after dilution (see volumes used) with physiological saline (0.9% NaCl) solution when the volume was too small to measure in the tuberculin syringe.

Radio-active calcium

Calcium^{45} injections were in much smaller quantities than P32. The amount of calcium which a young rat could withstand in an injection is very small, excessive amounts

causing death of the animal. Ca^{45} received from the Clinton Laboratory, Oak Ridge, Tennes. as $CaCl_2$ was diluted with physiological saline solution to contain 1 uc per 0.01 cc (0.01 cc = 0.016 - 0.001 mg. Ca). One microcurie per gram of body weight gave ample radio-activity in the teeth. In some cases, 0.4 uc per gram of body weight was given and still good autographs were obtained. None of the doses used in the experiments gave any radiation damage or any other abnormality, as could be found by examination of the site of injection, (no necrosis, no loss of fur), or at autopsy, and the animals gained weight steadily.

Administration of radio-isotope

Groups of animals

Single injections were given to two age groups of rats; one, hereafter referred to as the newborn group, received the radio-element on the day they were born, while the other referred to as the 3 day old group, received it at three days of age. Two rats were injected twice with radiocalcium. These two rats received the first of the two injections on the third day of life and the second dose at six days of age.

Route of injection

All rats were given the solution of radio-element by the subcutaneous route; the volume was measured in a 1 cc tuberculin syringe graduated into 0.01 cc. The rat was held in the left hand; the skin on the back of the neck of the rat

was held between the thumb and index finger, taking care not to squash the neck and its contents including the trachea while the rest of the trunk, feet and the tail was held under control by means of the remaining three fingers and the palm, all cupped to surround the rat. The syringe, fitted snugly with a 26 gauge injection needle of 1 inch length was inserted under the skin between the thumb and index finger, and passed caudally and the solution deposited over the rump. Such a method allowed injections to remain at the site of deposition, since the hole left by the needle at the site of entry into the skin is not over the area containing the solution. The deposited solution was absorbed in a matter of about 15 minutes. There were no unfavorable reactions after the injections, and the animals behaved normally, suckling, sleeping or just playing about in their usual ways.

Sacrifice

Time intervals between injection and sacrifice

The time interval between injection and sacrifice were selected in such a manner as to be comparable among the litters used. Thus, rats were sacrificed at 1 - 4 hours, 1, 3, 6, 9, 12, 15, and in one instance 18 days after injection. In one of the series on the three day old group however, the animals were sacrificed on the 7th and 8th days after injection. In the foregoing experiments each rat received a single subcutaneous injection. However, the two rats which

were injected twice with radio-calcium, were sacrificed three and five days after the second dose.

Method of sacrifice and extrication of teeth

After the required length of time following the administration of the radio-element, the rats were sacrificed under anesthesia and their jaws removed and immediately fixed in a slightly alkalinized (pH 7 - 8) alcoholic solution of 10% formalin. Anesthesia was carried out in a glass container, lined with cotton wool at the bottom which was moistened with chloroform or ether. The bottle was closed and the analgesic allowed to saturate the air in the bottle. The rat was put into this bottle, and in a matter of minutes, the rat was anesthetized.

Immediately after anesthesia had set in, (the movement of the limbs ceased, the head did not move when the mouth was touched, or in the case of rats 12 days and older, the eyes were no longer responsive to touch), the head was decapitated. With the left hand a fine pair of forceps was used to hold the head; one arm of the forceps was inserted into the trachea while the other was over the base of the head at the back or over the ventral side of the neck. The cheek muscles were cut with a pair of fine scissors. The blade was passed between the rami of the mandible and the coronoid, condyloid and angular processes separated from the pterygoid processes and any remaining muscles to the head. This process was carried out on both sides of the jaw and the lower jaw freed. This was then cut in the

middle, between the two incisor teeth, (jaws are separated) and the accompanying flesh was quickly removed including the tongue and immediately fixed. The region of the diastema of the maxilla was held by means of forceps while with a sharp scalpel, the whole snout was cut transversely just anterior to the first upper molars. The two portions were put into the fixative. Two days later, when the soft tissues had somewhat hardened from fixation, the various pieces were trimmed to shapes convenient for embedding. The mandible was examined and any remaining soft tissues were removed. Care was taken not to disturb the area over the molars and incisors. In some cases, the mandible was cut at the base of the first molar to separate the molars from the incisors, and to obtain a more longitudinal section. However, not much difference was found whether the last measure was preformed or not. The upper molars needed much careful preparation. By using fine forceps to hold the specimen, in such a way as not to injure the region about the molars, which could be seen as a pair of ridges, the ridge containing the molars were separated by slicing with a sharp scalpel on either side of it. The slicing was carried from the palate through the cerebrum and skull and any tissue not belonging to the molar ridge on the posterior border was also removed. The resulting slab of tissue with the molars was such that it permitted easy embedding, and there was not much tissue to be removed to reach the molars. In the case

of the upper incisors, bones about the snout were removed carefully excepting the alveolar bone surrounding each incisor which was permitted to remain.

The fixative made of the following composition, 3 parts 95% alcohol, to 1 part 40% formalin saturated with $MgCO_3$ was slightly alkalinized with NaOH. Such a fluid decreased the possibility of dissolution of mineral elements of the jaw and its contents. Moreover, the fact that a predominantly organic solution was used, decreased the solubility of the inorganic salts. Another important aspect in selection of the fixative is that it should not interfere in the process of analysis employed, in this case, radio-autography. It should not cause artifacts from the spontaneous reduction of silver ions due to chemicals used in the fixation process. This has been overcome with the use of alcoholic 10% formaldehyde fixative.

Histological techniques

Embedding media and sectioning

After fixation, the tissues were dehydrated in dioxane, and embedded in preparation for sectioning. With increasing hardness of the teeth they required embedding material of a harder and cohesive nature. Teeth of rats 6 days of age and under were embedded in paraffin, those up to 12 days of age in celloidin and those including some of 12 days of age and older in an acrylic plastic, methyl methacrylate.

Paraffin embedded material

Paraffin embedded specimens were sectioned at 6 μ on the Spencer Rotary Microtome, in which the specimen, mounted on a wooden block was held in a clamp. It may be recalled that with this microtome, each turn of the wheel initiates a forward motion of the block by a given distance, 6 μ in this instance, while the knife remained stationary. The sections obtained by this method were floated on a drop of water on a microscope slide coated with egg albumin-glycerin (1:1) mixture. This was then placed on a warming plate (40 - 50° C.) which removes the wrinkles and dries the sections. Following this, the tissues were processed for radio-autography, of which the important steps will be elaborated.

Celloidin embedded material

In the celloidin technique, the specimens, after dehydration in dioxane, were immersed in a 1:1 mixture of absolute alcohol and ether for at least 24 hours, and then immersed into a solution of celloidin. The tissues were passed through increasing concentrations of celloidin solution, starting from about 7 1/2% solution to a 30% solution, with the time of immersion in each solution being at least 48 hours, more often, longer. When the tissues were saturated with the 30% solution, they were then arranged in an enamel tray containing 30% solution of celloidin, and placed in an air tight container and left to dry slowly. Once or twice a day, the lid was removed for a brief moment to allow the solvent which had evaporated into the space in the container to escape.

This was continued for several days until the celloidin block became jelly-like at which time they were removed from the tray by cutting blocks of partially hardened celloidin containing the tissues. They were cut into convenient shapes for sectioning later. The blocks of tissue were then placed in an 80% solution of alcohol and allowed to harden to a point where the finger nail no longer cut deeply into the block (however, it may still be marked by the nail). These blocks, now at the stage to be sectioned were kept moist with a 70 - 80% solution of alcohol at all times. The sectioning was carried out on a Reichert Sliding Microtome, in which the block advanced up an inclined plane with each stroke of the knife over the block. The block and the knife was kept constantly flooded with alcohol. Sections of the tissue were preserved in an alcoholic solution until mounted onto microscope slides. The egg albumin-glycerin mixture was smeared over a clean slide, and the tissue carefully placed on it. After the excess alcohol was drained by tilting the slide, absolute alcohol was flooded over the tissue twice, each time being drained in the manner described. Then it was flooded with ether - absolute alcohol mixture and also drained. The latter solution has a tendency to dissolve the celloidin so that extreme care must be exercised so as not to lose the orientation of the tissues. When the alcohol-ether solution has almost dried, and the tissues seem to have adhered to the slide, it is then placed in a solution of 80% alcohol and left until the celloidin has firmed. Following staining

they are dehydrated and coated with a 1% celloidin solution, and then the slides are ready to be radio-autographed.

Methyl methacrylate embedded material

The plastic in the monomer form contains a quinone inhibitor which must be removed before use. This was accomplished by washing a volume of the methyl methacrylate with a 1 N NaOh in a separatory funnel. Three washings have proven adequate removal of the inhibitor. The plastic was then dried over sodium sulphate and filtered; the process was usually repeated 2 - 3 times. To speed the polymerization process a catalyst, benzoyl peroxide, up to 5% was added to the liquid and again filtered (Atkinson, '49; '50; '52). This mixture keeps in the fluid form for some time if kept in the refrigerator.

The tissues to be embedded were dehydrated through alcohols and brought to soak in chloroform. It was then immersed in the monomer for 8 - 12 hours after which it was transferred into a glass jar with an air tight lid and after arranging the tissues in convenient directions for sectioning later, covered with fresh monomer. A layer of polymerized plastic on the bottom of the jar made beforehand proved useful in providing a base on which to rest the tissues. Bits of polymerized plastic cut into small blocks were used to stand the tissues wherever needed. It was then polymerized at 37° C; a higher temperature tended to cause foaming of the plastic. After the plastic had polymerized (in 2 days or so) the jar was broken by knocking it against

a hard surface. Only the jar broke and the block of plastic with the tissues remained intact. These blocks were then shaped by sawing them into convenient blocks for sectioning.

To section the plastic embedded jaws a specially adapted apparatus was rebuilt in the department with the help of Mr. Weiss. A Black and Decker Valve Resurfacing Machine was fitted with a fine, rotating carborundum wheel, (available as thin as 150 μ) made with a rubber base, which was advanced slowly through the block. The spindle of wheel was capable of 12,000 r.p.m. without causing any vibrations. To keep the temperature of the surface being cut within a reasonable temperature range, a constant stream of cold tap water was allowed to flow on the block. Guards made of clear plastic kept the spray of water from this stream under control. A micrometer feed was adjusted to the wheel and sections were of the order of 0.3 - 1.0 mm in thickness but with extreme care, sections of the order of 50 μ (0.05 mm) could be cut. After sectioning one side, a plastic coverslip was put on the cut surface, which had been moistened in the meantime, with a bit of acetone. This produced an adhesion of the coverslip which made it easier to catch the section as well as offer support to the section while it was being cut. The exposed surface of the jaw was then filed on a sharp, rust-free metal files of decreasing coarseness. Then the combination of plastic coverslip and methyl methacrylate embedded jaw was placed in xylene. When the attachment between the section and coverslip became loosened, it was removed from

the plastic, and glued with household cement onto microscope slides with the ground face down. During the process of soaking in xylene, dust from filing was washed away. After the cement had dried, the remaining surface was filed. It was then washed free of scrapings with tap water and a short flooding with distilled water and allowed to dry completely. This was then processed in the usual radio-autographic manipulations.

Grinding method

Another method was tried to obtain sections of hard teeth. In this method, whole jaws after fixation, were dehydrated and immersed in ethylene dichloride or ether-alcohol (1:1). The tissues were then glued onto microscopic slides with generous amounts of polymerized methyl methacrylate dissolved in ethylene dichloride or by clear Revlon nail polish in the latter case. After thoroughly drying the adhesive, the jaws were then ground on a revolving carborundum wheel, which was attached onto a rod, fitting into an Atlas Drilling Press. In this arrangement, the carborundum wheel was horizontal, so that a pan of water was placed below, in which the wheel revolved. In this way the radio-active bone and tooth powder removed by grinding was prevented from escaping into the air as dust and also the ground surface was kept cool by the water. After grinding the teeth to a desired place as determined by examination under the dissecting microscope or under low magnification on the light

microscope, it was washed in tap water to free it of all bone, tooth and plastic (or nail polish) powders, dried and removed from the slide. This was accomplished rather easily, in most cases, since the adhesion of the tissue to the glass slide was not too solid. If the adhesion was solid, a scalpel was used while a drop of water was allowed to creep underneath the tissue, in much the same way as collodion bag is separated from the wall of a test tube. The cleaned, dried tissue was then glued onto the slide, this time with the ground surface down over the glass. In order to prevent the formation of bubbles between the ground surface of the tissue, and the glass, the ground surface was covered with the gluing material for a few seconds before pressing it down onto the slide. The grinding was carried out again, and when the section appeared transparent under the 100x magnification of the light microscope, the sections were considered thin enough and the edges of the glue, which also served as the embedding material for the tissue, was sealed with more nail polish. These were then autographed.

Fixing fluids

In addition to the radio-autographic method of locating the constituents of thejaws and teeth, histological stains were utilized. Sections were stained withMallory's triple stain for connective tissues, hematoxylin and eosin, toluidine blue, alizarin stained according to Dahl's method, alizarin adapted for purposes of radio-autography of Ca^{45} and P^{32}

containing tissues, and the periodic acid Schiff stains.

Various fixatives were employed for these stains; the composition of these fluids will be listed, followed by precautions necessary to use them successfully.

Alcoholic 10% formaldehyde

95% alcohol---------- 3 parts

40% formalin saturated with $MgCO_3$---- 1 part

Alkalinized with NaOH until pH 7 - 8

Tissues are fixed for 48 hours and then dehydrated without washing in water.

Susa

$HgCl_2$---------- 9 g.

NaCl---------- 1 g.

Distilled H_2O---------- 140 cc.

Boil and cool, add to the following:

20% aqueous trichloroacetic acid----- 20 cc.

40% formaldehyde (formalin)---------- 40 cc.

Glacial acetic acid---------- 8 cc.

Fixation is carried on for 6 - 12 hours, after which the tissues are washed in several changes of 95% alcohol, then dehydrated. The tissues are not to be stored in this fluid since they tend to harden excessively.

Carnoy

95% alcohol---------- 60 cc.

Chloroform---------- 30 cc.

Glacial acetic acid---------- 10 cc.

Tissues are fixed over a period of 12 - 24 hours, and stored in 70% alcohol if necessary. Ninety-five percent alcohol is employed for washing which is done just before dehydration.

Orth

3% potassium bichromate---------- 90 cc.

40% formaldehyde---------- 10 cc.

These two fluids are mixed just before use and fixation is carried on for 24 - 48 hours in the dark at room temperature. After fixation, the tissues are washed in running water for 24 hours before dehydration. For storage, 4% formaldehyde solution is used.

Excepting for the first fixative in which the tissues remained undecalcified, the fluids used contained acidic substances (even formalin when not saturated with $MgCO_3$ is acidic), which removed the mineral salts to variable degrees. For histochemical studies, teeth of rats of not more than 9 days of age were utilized. Therefore, the teeth were sectioned after paraffin embedding.

Sections of tissues which were fixed in fluids containing $HgCl_2$ require an immersion into an 80% alcohol in which iodine is dissolved for 5 minutes followed by a 1 minute wash in water, then immersion into a 5% solution of sodium thiosulphate ($Na_2S_2O_3$) in 50% alcohol during the hydration process. If this step is not followed, artifacts occur in the sections during staining in the way of dark precipitates, presumably of mercury compounds.

Histological stains utilized in the present study

Basic fuchsin

After deparaffinization in xylene the sections are passed through decreasing concentrations of alcohols into distilled water (hydration process). The slides containing undecalcified teeth and bones are immersed into an aqueous 0.1% basic fuchsin solution for 1 - 2 minutes, rinsed rapidly in water, and dehydrated through increasing concentrations of alcohols, through absolute alcohol-ether 1:1 and dipped into 1% cellodin for 1 minute and allowed to stand dry. Celloidin coat is put on whenever radio-autographs are to be made.

Alizarin -- Dahl's method

This method by Dahl is claimed to be a sensitive stain for calcium. Thus it was decided to attempt a histochemical localization of calcium in the tissues using his method. The process consists of immersion of the section after being in 95% ethyl alcohol into an ammoniated aqueous 1% alizarin solution for 2 minutes, followed by rinsing in distilled water, until the background colouring of the tissue became a faint pink. Then it is dehydrated through alcohols and after clearing in xylene was to be mounted in cedar wood oil. In this experiment, mounting was done in Canada balsam, and this last step was the only alteration of Dahl's method.

Alizarin -- adapted for radio-autography

After hydration, the tissues are immersed for 2 - 4

minutes in an aqueous solution of 0.1% alizarin red S. Excess stain is washed out with 50% alcohol and dehydrated through alcohols, followed by absolute alcohol-ether, 1:1 and finally 1% coat of celloidin in preparation for the autographic procedure.

Mallory's triple stain for connective tissue

After hydration, the tissues are immersed into 0.1% aqueous acid fuchsin for 3 minutes, then rinsed rapidly in water and the stain fixed in 1% phosphomolybdic acid for 3 - 5 minutes. The slides are then rinsed again in water and put into a mixture of aqueous solution of aniline blue-orange G-oxalic acid for 2 minutes, rinsed in water, differentiated in 95% alcohol, dehydrated, cleared in xylene and mounted in Canada balsam.

Hematoxylin and eosin

Hydration of the tissues is followed by immersion in Harris' hematoxylin for 2 - 5 minutes, a rinse in water and differentiated in acid-alcohol. A wash in water is then succeeded by immersion into ammonia water or Li_2CO_3 solution, rinsed in tap water and counter stained with 1% eosin until the tissues are overstained (2 minutes or more). After staining with eosin, the tissues are dehydrated, cleared and mounted. Eosin tends to wash out in the alcohols and appear much lighter therefore, overstaining is allowed.

Toluidine blue

After deparaffinization and hydration, the slides are

immersed into 0.1% aqueous solution of toluidine blue for 2 - 3 minutes. The slides are then washed in n-butyl alcohol until the superficial stain is removed, and rapidly dehydrated by rinsing once in 95% alcohol, two changes of absolute alcohol, absolute alcohol-xylol and three changes of pure xylol and then immediately mounted in Canada Balsam.

Periodic acid-Schiff -- Hotchkiss' method

After hydration, hydrolysis of the tissues is carried for 10 minutes in periodic acid (0.8% solution) which is followed by a rinse in 70% alcohol and immediately after, put into fuchsin sulpurous acid (Schiff reagent). After 15 minutes the purple colour, indicating a positive reaction was observed. An additional 15 minutes in the fuchsin sulphurous acid did not appear to change the intensity of the colour of the tissues as observed with the naked eye. The fuchsin sulphurous acid is followed by three washings in sulfite solution containing potassium metabisulfite and hydrochloric acid and distilled water, dehydrated through alcohols and mounted in Canada balsam.

Semi-quantitative analysis of retention of radio-activity during staining

Safranin, the stain which had been previously used on tissues containing cartilage and calcified structures did not possess the qualities suitable for observing mineral salts to teeth and bones. There was a certain amount of loss of the mineral salts, which resulted in reduced radio-activity in the case of those tissues in which the radio-

element Ca^{45} or P^{32} was present, which in turn resulted in poor radio-autographs. Moreover, only those structures which contained much organic material such as cells, (osteocytes, osteoblasts, chondrocytes), organic matrix which had not fully calcified and cartilage stained. The mineral elements did not stain. It was already known that the routine histological stain, hematoxylin and eosin removed the mineral salts of teeth and bones.

The necessity of finding a stain which retained the radio-activity as well as staining the mineral salts became urgent, and as a result, a semi-quantitative analysis was preformed to determine the effect of alizarin and basic fuchsin staining on the retention of the Ca^{45} within the teeth and bones.

The sections of tissue containing the radio-activity were mounted onto microscope slides and the radio-activity measured by holding over the sections the tube of the Geiger Counter (Laboratory Monitor, Tracer Lab, Boston). The tissues were then stained either with alizarin or basic fuchsin, dehydrated through graded alcohols, ether-alcohol and finished by a coat of 1% celloidin which is used in protecting the section to be coated later with emulsion. Counts of radio-activity were then taken and these same tissues were then radio-autographed. In this manner, a double check was made on the retention of the Ca^{45}. Along with alizarin and basic fuchsin, safranin stain was also tried, however, with this stain, the radio-autographic intensity only was compared and

and no comparison counts were made.

Measurement of radio-activity before and after staining with either basic fuchsin or alizarin resulted in no significant loss in either case; however, only alizarin stained the calcium phosphate, the basic fuchsin staining only the organic substances. Moreover, basic fuchsin did not form a stable complex in the tissues, and consequently, while dehydrating according to routine histological methods, much of the colour was lost from the tissues. Subsequent autographs gave equal intensities for both alizarin and basic fuchsin.

The phosphorus^{32} containing tissues were not subjected to such an analysis but after staining with alizarin, phosphorus still gave comparable counts with unstained phosphorus containing sections and gave radio-autographs of equal intensity after identical time exposures, showing that the P^{32} was also retained.

It was decided then, to use alizarin as a routine stain for tissues containing radio-active elements in the teeth. Thus tissues subjected to the radio-autographic analysis consisted of alizarin stained and unstained sections.

Radio-autography

Methods of radio-autography

Radio-autography involves the use of a photographic emulsion to locate the site containing the radio-element in tissues. There are several autographic techniques in use, some of which are crude while others are more refined. The

methods will be enumerated here together with their relative merits and a brief statement of the procedures involved. In all the procedures excepting in the mounting method, the tissues are coated with celloidin by dipping the slide into a 1% ether-alcohol solution of celloidin.

The Contact Method

In this method, a slide mounted with tissues containing the radio-element is placed next to a photographic plate or film in the dark. It is stored for exposure in a light tight x-ray pressure cassette in a refrigerator. After exposure the film only is developed, washed and dried. The developing procedure will be explained later.

This method is simple but limited by the low resolution of the resulting autograph. Also, pressure which is applied for obtaining close contact with the tissue and emulsion may in some cases where the tissue is thick, e.g. ground bones and teeth, cause artifact autographic reactions.

The Mounting Method

In this method the section is mounted directly on a photographic plate. The sections are floated on a warm (40°C) water bath to remove wrinkles and then refloated onto distilled waterbath at 18 - 20°C and in the darkroom, under a red safelight a photographic plate is placed under the section. The excess water is drained off, and the tissue and plate allowed to dry; the tissue adheres to the plate when water is dried. After exposure it is developed and stained with hematoxylin or other suitable dyes not causing too much absorption

of the dye into the gelatin of the photographic emulsion. Then it is dried, cleared and mounted in Canada Balsam.

This method has the advantage over the contact method in that finer resolution is obtained. However, disadvantages such as the failure of the fluids used in developing and fixing to penetrate the tissue to the plate, and the staining of the gelatin with dyes and artifacts arising from the chemical action of the tissues are encountered.

The Coating Method with Strip Film Emulsion

The celloidin coated slides are covered with stripping film emulsion in this method. The stripping film is mounted on a glass support from which it must be removed before use. This is done by cutting the emulsion with a sharp razor along the edges of the glass support. Starting from a corner, the emulsion is slowly peeled off and put into a pan of distilled water (dupanol 1% optional) with the surface that was adhering onto the plate facing upwards and allowed to soak for 5 minutes. The slide with the tissues is passed underneath the emulsion and lifted out of the water with the emulsion over it. It is drained and the edges of the emulsion are folded underneath the slide. This is allowed to dry and is then exposed. After exposure, the slides are processed in the usual way for autographs. The excess emulsion which was folded under is removed after the mounting medium has completely dried.

This method allows intimate contact between tissue and

emulsion and the autographs offer fine resolution but the thickness of the emulsion cannot be controlled.

The Coating Method with Fluid Emulsion

Bulk emulsions of the fluid type or pellicles after soaking for 24 hours in water and dupanol solution are heated to 37°C over a waterbath in the dark. This melts the emulsion so that an even spreading over the tissues and glass slide is obtained. The area containing the tissues is demarcated by a diamond marker then placed over a warming table (37°C). After the slide has warmed, the demarcated area is covered with fluid emulsion from a medicine dropper and spread by a fine soft camel's hair brush. This is then allowed to dry completely and exposed in light tight plastic slide boxes and developed at the required times in the usual way. Two drops of undiluted emulsion spread over an area of one square inch dries to a thickness of about 20μ.

This method, the best yet devised, is the method used routinely in this laboratory. It allows fine resolution, control of the thickness of the emulsion over the tissues, and permits stained and unstained tissues to be autographed.

The Inverting Method

This method requires the use of a special emulsion, the Kodak matrix emulsion. This is coated on the tissues in the same manner as in the coated method. All procedures are the same except that after a washing and before dehydration, the slides are put under the water and the emulsion and tissue

peeled off the slide by means of a razor. The combination of emulsion-tissue is placed reversed on a fresh slide which had been coated with a layer of egg albumin-glycerol mixture. This was then air dried by a fan and when dry the edges of the emulsion was sealed with 1% celloidin. When the celloidin had dried the section which now face up are stained and the whole preparation mounted.

This method allows the staining of the tissues which would otherwise lose the radio-activity before autography. However the dye must be such that it does not colour the emulsion too deeply. Basic fuchsin and safranin dyes have been used successfully by Belanger. Extreme care must be exercised in the inverting process in order to avoid displacement of the emulsion and tissue for it would nullify the fine resolution otherwise possible with this method. All procedures in the darkroom are carried out under the series OA yellow-green filter, which is rather bright and allows ease of working.

Development and fixation

After exposure, the following manipulations are carried out under a photographic safelight. The boxes containing the slides with the emulsion are opened and placed in a steel slide holder with the emulsion side facing the same direction. This is then placed, emulsion side up, in a beaker containing Dektol developer (Kodak D-72) diluted with distilled water, 1 part developer to 2 parts water and developed for 1½ minutes.

This and the following procedures until the mounting process is done at a temperature of 18 - 20°C to prevent peeling off of the emulsion. After developing, the slides are quickly rinsed in distilled water and immersed into an acid fixer with hardener (Kodak) and allowed to fix until the emulsion has cleared (about 6 - 10 minutes). The slides are washed in running tap water and dehydrated in alcohols of increasing concentration and passed through absolute alcohol-xylol, and cleared in 3 changes of xylol. The time in each solution is two minutes. The slides may be immediately mounted or allowed to soak in Canada Balsam solution before mounting the glass cover slip. The slides are constantly watched for bubbles which form on evaporation of the solvent of the mounting medium. As soon as these appear, they are filled with more Canada balsam.

Emulsion used in present study

In the present study on the teeth, the coated method has been used. NT4 from British Kodak, Ilford G5 from England, NTB3&NTB2 from Kodak, U.S.A. emulsions have been utilized. All of these emulsions are the bulk or fluid type. Of those listed, the NT4 emulsion grain size is the largest, while Ilford G5 is the smallest. The latter was also the most viscous and required dilution with water before coating.

Other pertinent remarks on the technique and resulting autographs have been made where they apply.

RESULT

The results of the present study, both histochemical and radio-autographic, will be presented in detail. The observations on radio-autographs fall into three groups of Ca^{45} and a section on P^{32} containing teeth. The three groups are the newborn and 3 day old groups, each of which received a single injection and the third group which received two injections of radiocalcium. Before commencing the observations, however, a brief outline of the histology of a typical section of a molar and incisor of a young rat will be presented. The results of the histological staining will precede the radio-autographic results.

General histology of the tooth with special reference to young rat teeth

The histology of the young rat teeth, such as those used in this investigation will be presented. The orientation of the various cells and the surrounding structures applicable to both incisor and molar teeth will be given; the peculiarities of each type of teeth will then follow.

The tooth is embedded in the alveolar socket which is a pocket formed in the maxilla or mandible. In the adult, it is lined with periodontal membrane, a tough fibrous structure joining the roots of the tooth to the walls of the alveolar socket. In young animals, such as those used in the present study, this periodontal membrane has not yet developed and

therefore, the tooth is surrounded by connective tissue only. Among the trabeculae of the alveolar bone are found osteoclasts, whose function is generally believed to be bone resorption. In regions other than those occupied by osteoclasts, osteoblasts, the cells which form bone matrix, are present.

The tooth which has not yet erupted is surrounded by a complete enamel organ, consisting of an inner enamel epithelium, stratum intermedium, stellate reticulum and outer enamel epithelium. The inner enamel epithelium, formed of cells known as ameloblasts or ganoblasts, elaborate the enamel matrix. Fine fiber-like structures from the ameloblasts are observed to pass into the enamel. These are known as Tomes' processes and are found in the region of the newly formed enamel referred to as pre-enamel. This area may be distinguished readily in stained sections, which usually take up a lesser amount of the common histological dyes than the enamel. The enamel which has not yet fully calcified appears homogeneous. The junction of the inner and outer enamel epithelium consists of undifferentiated cells and it is known as the cervical loop. The area bounded by the cervical loop consists of flattened cells, mostly of connective tissue elements and has been referred to as the hammock ligament (Sicher, '42).

The pulp cavity is occupied by mesenchymal cells which at the periphery are differentiated into odontoblasts, the dentin matrix forming cells. The orientation of the odontoblasts and the ameloblasts are then, such that the matrices

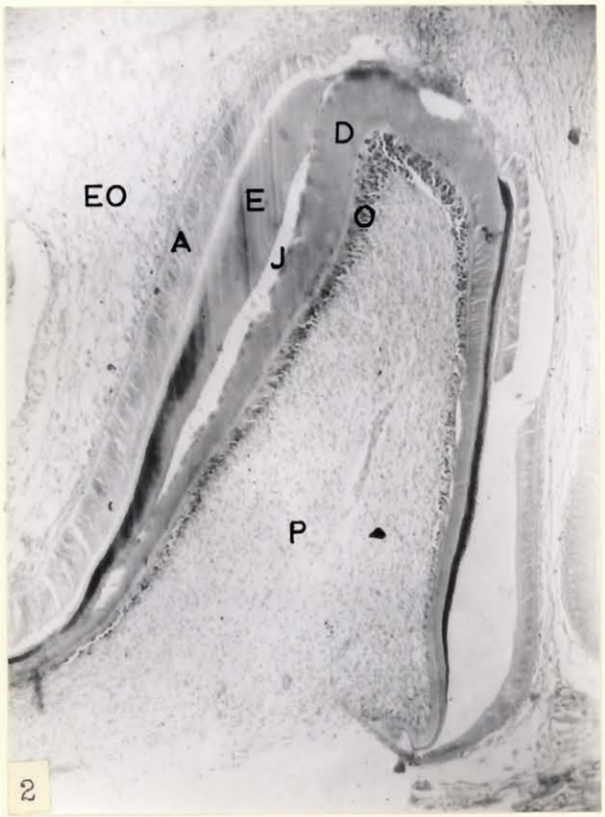
which they elaborate meet; the boundary is the sharply defined dentino-enamel junction. In the pulp are numerous blood vessels which ramify and penetrate among the odontoblasts. Nerve fibers are also present in the pulp; however, special staining is required before these can be observed. A zone between the odontoblasts and dentin, distinguished by a lighter uptake of histological dyes, is known as predentin. Passing through the predentin and dentin, to the dentino-enamel junction from the odontoblasts are the dentinal tubules. They are found to branch occasionally in the dentin but near the dentino-enamel junction, they form numerous branches. These dentinal tubules are occupied by odontoblastic processes known as Tomès' fibers or dentinal fibers. Their function has generally been thought to be a channel for nutrients to the dentin and also as a conveyer of impulses to the nerve endings in the pulp. Thus, although not true nerve elements, they help convey the stimuli reaching the dentin. The fluid occupying the remaining space in the dentinal tubules is referred to as dental lymph. The area of dentin around the dentinal tubules are known as the Sheath of Neumann and have been demonstrated to have staining characteristics which differ from the rest of the dentin. There is generally, a greater uptake of histological dyes so that viewed longitudinally in a histological preparation, the sheath appears as two parallel lines between which pass the dentinal tubules.

The incisor tooth grows continually throughout the life of the rat and is rootless. The enamel organ on the labial

and slightly overlapping onto the lateral surfaces of the incisors, is a continually functioning structure. It is at first large, but with growth of the incisor, decreases in size and becomes composed of cuboidal cells. In the adult, it persists on the convex surface up to the gingival margin. On the lingual or concave surface only a rudiment of it exists near the base. Thus, enamel is absent on the lingual surface but present on the convex or labial surface. A mixture of cells from the original enamel organ and those of the connective tissue elements cover the enamel free surface of the incisor, which differentiate into cementoblasts and elaborate cementum. The periodontal fibers connecting the incisor to the alveolar bone merge into the cementum. However, as noted earlier, the periodontal membrane is not yet developed in the rats selected in the present study.

The molar tooth, in contrast, develops roots commencing about the 12th day after birth and cementum is eventually formed over them. The periodontal membrane also becomes conspicuous at about the same time. Therefore, the majority of the molars examined in this study, which are from rats under 12 days of age, do not possess roots. In the molar, regions of discontinuity of the enamel about the summit of the cusps are found; these are known as the enamel free areas of the molars. During the developmental stages of the tooth, it is covered with columnar cells which are continuous with the ameloblasts.

PLATE 2



Cusp of a molar of a six day old rat. Mallory's triple stain. Note the uptake of the dyes over the enamel; blue from aniline blue appears light, red from acid fuchsin appears black in the photomicrographs. Magnification x 225. EO: enamel organ; A: ameloblasts; E: enamel; J: dentino-enamel junction; D: dentin; P: pulp.

Histochemical reactions

Mallory's triple stain for connective tissue

Mallory's triple stain for connective tissue contains three dyes, namely, acid fuchsin, orange G and aniline blue. With these stains, the cells excepting for the older odontoblasts were coloured a gray mauve, the nucleoli a bright red. The cytoplasm of the ameloblasts just before the pre-enamel appeared foamy. The enamel showed an interesting manner of uptake of the dye, depending on the stage of its development. During the very early stage, the surface nearest the ameloblasts stained blue and with time changed so that it became red in colour. Finally, it again stained blue in the region nearest the dentin (Fig. 2). The region of the young enamel, commonly referred to as pre-enamel, showed a palisaded arrangement of the organic content, with each unit appearing as if it were made of a row of soap bubbles compressed in a tube. Dentin, however, showed the uptake of only the blue component of Mallory's stains. The predentin stained a much lighter blue than dentin proper and a dark blue line marked the boundary between the two parts. The dentinal tubules appeared slightly lighter in colour than the dentin. The uptake of the dye among the odontoblasts differed. The younger newly formed cells are stained a grayish purple throughout but the older nuclei stained red, while the cytoplasm remained the same colour as in the young cells. The alveolar bone and cartilage model still remaining stained a blue colour, similar in shade to that observed in the dentin.

Hematoxylin and eosin

This useful combination of dyes, which stains basophilic substances blue and acidophilic substances red, showed the dentinal ends of the ameloblasts to be foamy and also more basophilic than the area just above the nucleus. The taller the ameloblasts, the foamier it appeared and when the foaminess reached a maximum, the basophilia at this end of the ameloblast disappeared. Beyond this area the ameloblasts, at first red and homogeneous changed with development, that is, appeared hollow and traversed by strands of cytoplasm from the enamel. The enamel looked foamy in the newly formed regions and when a quantity of the foamy enamel was observed, resembled the soap bubbles as described in the foregoing section. Beyond this area, the enamel matrix became homogeneous in texture. The dentin stained a mauve colour while the predentin displayed a pale acidophilia. The structural details appeared identical to that obtained after Mallory's stains. The bones of the surrounding structures stained a pinkish mauve.

Toluidine blue

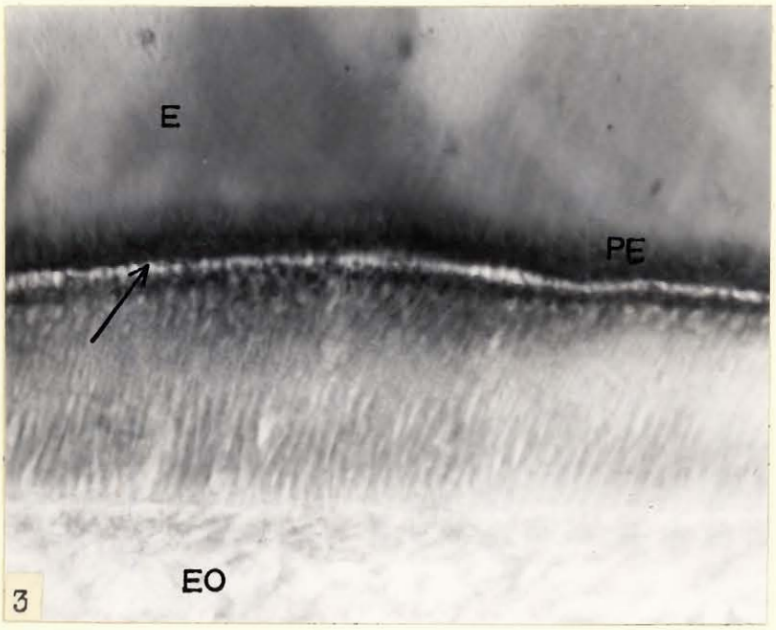
Toluidine blue stained all structures blue excepting those exhibiting metachromasia which was indicated by a mauve to a reddish mauve colour. This dye showed varying intensities of metachromasia with different fixing fluids. It was found that Susa or Carnoy fixed material gave a more positive response. Alcoholic 10% formaldehyde and Orth were found to be less suitable to demonstrate metachromasia. The enamel of an Orth fixed tooth appeared a greenish blue after staining

with toluidine blue while after Susa, Carnoy, or alcoholic 10% formaldehyde fixation, it was colourless. Dentin was intensely metachromatic while predentin displayed a weaker but definite metachromasia. It was greater in the occlusal than in the basal regions of the dentin. The ameloblasts and odontoblasts showed orthochromatic staining, that is, a blue colour. The matrix forming ends of these cells took up a greater quantity of the dye although neither exhibited metachromasia. Bone and cartilage also showed metachromasia. The ground substance of the pulp displayed a weak but distinct metachromasia as did the connective tissue and enamel organ, although the intensity was much less in the latter two.

Periodic acid-Schiff

This stain shows the sites containing chemical linkages of 1,2 glycols and alpha amino hydroxyl groups, indicating the presence of polysaccharides, glycoproteins and mucoproteins. The cytoplasm of the ameloblasts, showed an uptake of the colour slightly greater than the enamel and which increased in intensity towards the dentinal end of the cells. The enamel displayed a background tint of light mauve, excepting in the region of the pre-enamel, where a colourless region next to the ameloblasts was followed in turn by a narrow band which took up more of the dye. Tomes' processes which took up more of the dye were observed in these two differently staining areas. The dentin was the most deeply stained of the dental structures. Predentin, however, showed only the background

PLATE 3



Molar enamel and ameloblasts of a nine day old rat. Alizarin, Dahl's method. Note staining of cytoplasm of ameloblasts, Tomes' processes (arrow), and pre-enamel. Magnification x 600. EO: enamel organ; A: ameloblasts; PE: pre-enamel; E: enamel.

colouring of light mauve. The odontoblasts displayed a slightly greater response than the ameloblasts. The alveolar bone and cartilage showed positive reaction to the periodic acid-Schiff stain. Following incubation in a 0.1% solution of alpha amylase at 37°C., the intensity of the colour in the dentin, cartilage, and bone spicules decreased so that these components were of the same intensity as the enamel.

Alizarin--Dahl's method

Alizarin, a dye of the oxyquinone structure is known to combine with calcium to form a red colour. Therefore, tissues which contain calcified structures are revealed by this dye. The enamel and bone stained homogeneously red with alizarin red S. Pre-enamel hardly took up any of the dye. Dentin for some unknown reason stained erratically, more so from those of older animals and for the most part, stained lightly. The predentin if wide, showed two differently staining zones, a homogeneous one just before the dentin and the other close to the odontoblasts, which did not stain. The enamel-pre-enamel and dentin-predentin borders displayed a greater uptake of the dye than did any other area in the enamel and dentin respectively. Indeed, this outline mode of staining of the border was also seen around each bone spicule. On close observation, it became apparent that this manner of staining was the site of deposition of calcium salts. The dentinal end of the ameloblast was observed to be more darkly stained than the rest of the cell (Fig. 3) and the intensity of the colour was

identical to that of the outline staining between the enamel and pre-enamel. The odontoblasts, although not as distinctly, showed similar uptake of the dye. In a few instances, the colour at these ends of the cells could be distinctly observed to be derived from tiny globules of substances which stained intensely with alizarin. These stained globules were concentrated at the border of the pre-enamel and ameloblasts, but a few were seen some distance away. The parts containing these globules appeared foamy. The dentino-enamel junction also stained with alizarin.

Alizarin--adapted for radio-autography

In staining the sections to be radio-autographed with alizarin over-staining was avoided. Therefore, only the enamel, dentin and bone spicules were coloured; the cellular elements were faintly pink. The limits of these components were clearly visible. Over-staining was avoided because analysis of the autographs would be made difficult especially after short exposures, in which the concentration of the reduced silver grains are low.

Analysis of radio-autographs

The radio-autographic reactions obtained from tissues of animals following injection of radio-isotope has provided new information regarding the metabolism and in the case of growing tissues, the resulting morphology. The energy radiating from an area of tissue containing the radio-active isotope reduces the silver ions of the photographic emulsion overlying

it. This radiation from the isotope is not directed in any one plane; thus, the silver ions in the emulsion become reduced from many angles from the same source. It is inevitable, therefore, that the silver grains observed in a radio-autograph is the sum of all the radiations from the whole tissue containing the radio-activity. This is a disadvantage resulting in reduced resolution of the autographs. By judicious control of various factors increasing resolution such as, the use of fog free emulsion, minimal time of exposure, a thin layer of emulsion and cutting down of the space between the emulsion and the tissue (interspace), radio-autographs can be obtained which are true to the actual conditions existing in the tissue under study.

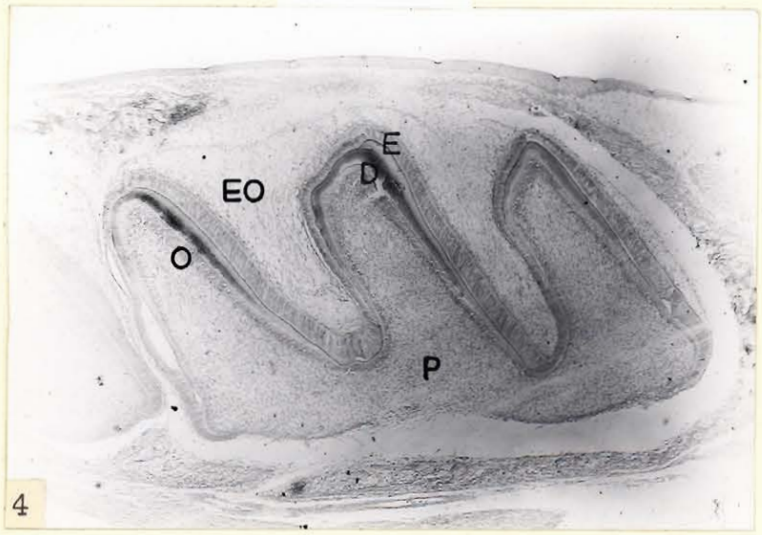
Radio-autographs of teeth of rats of newborn group injected with Ca^{45}

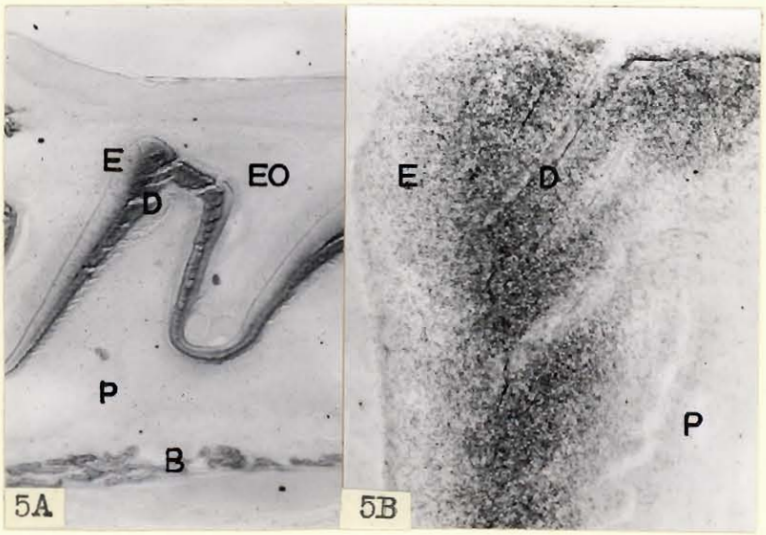
The uptake of injected radio-isotope is amazingly rapid and this phenomenon was demonstrated in the present investigation. Two hours after the administration of the radio-calcium by the subcutaneous route to newborn rats, sufficient amounts of the tracer element were incorporated into the alveolar bone, enamel and dentin matrices to produce radio-autographic reactions.

Molar teeth--enamel

The molars of newborn rats are very small, indeed. Therefore, attention was devoted to incisors instead and no attempt

PLATE 4





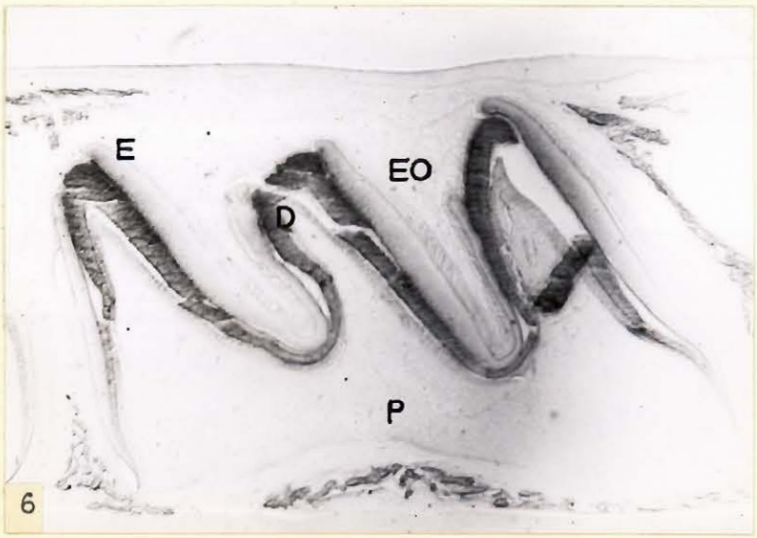


PLATE 4

Explanation of figures

Coated radio-autographs of molars of rats injected with Ca^{45} at birth and sacrificed at various intervals thereafter (Newborn group). Alizarin stained sections photographed using yellow red filter. The autographic reactions appears grey. Note that the reaction is greater over the dentin than over the enamel. EO: enamel organ; E: enamel; D: dentin; P; pulp; O: odontoblasts; B: alveolar bone. Magnification x 20 except Fig. 5B; x 145.

Fig. 4 3 days after injection.

Fig. 5A 6 days after injection.

Fig. 5B Higher magnification of centre cusp of molar Fig. 5A, showing the distribution of reduced silver grains of the autograph over the dentin and enamel.

Fig. 6 9 days after injection.

was made to obtain radio-autographs of molars at this age. One day after the injection of a tracer amount of Ca^{45}, the distribution in the molars was still quite difficult to resolve, the amounts of dentin and enamel being very small. Reduced silver grains of the photographic emulsion were found dispersed on both sides of the dentino-enamel junction, the greater proportion being present over the dentin. The autographic reaction over the enamel increased in intensity from the basal parts of the cusp to the incisal or occlusal portion, that is the basal-incisal or basal-occlusal gradient. Three days after the administration of radio-calcium to newborn rats (Fig. 4), the molar enamel showed radio-autographic reactions which were not much clearer; however, by the sixth day (Fig. 5), the concentration of the reduced silver grains of the autographic reaction showed gradients in two directions. There was observed in addition to the basal-occlusal gradient, a radial one consisting of a decreasing concentrations of the silver grains from the dentino-enamel junction to the outer surface of the enamel. The radio-autographic reaction over the enamel at first was very light, and increased somewhat with increase in time after injection and sacrifice. In none were there radio-autographic reactions over the enamel which were greater than that observed over the dentin of the molar in the age groups selected in this experiment.

The enamel organ showed no autographic reaction; the few scattered grains observed were that due to the inevitable fogging of the emulsion, which constituted the background reaction.

Molar teeth--dentin

One day after the injection of radio-calcium the dentin of the molars showed a reaction close to the dentino-enamel junction. The autographs obtained at later time intervals were more distinct. Two gradients were observed; one, as in the case of the enamel, was the basal-occlusal or basal-incisal gradient in which the reaction increased from the base to the tip of the cusps. The second, a radial gradient, was reversed in direction to that of the enamel; it increased from the odontoblastic margin to the dentino-enamel junction. This pattern was observed at all the time intervals selected (Figs. 4,5 and 6). Predentin showed a minimal reaction, which was not a significant autographic reaction. The pulp exhibited no reaction, those few grains observed being merely the background fog of the emulsion.

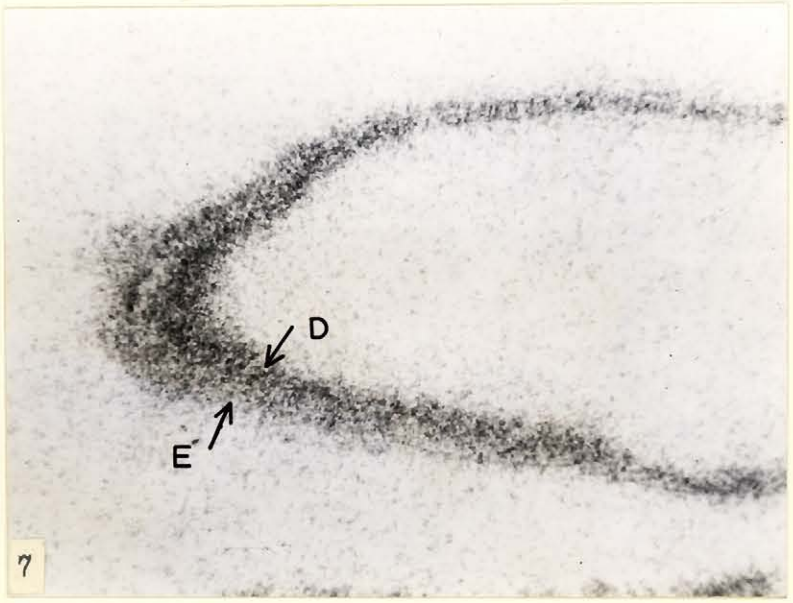
Alveolar bone

Radio-autographs of the alveolar bone, which appears in the form of spicules in a histological section, showed concentration of silver grains in the core of each spicule. The radio-autographic reaction observed over the trabeculae of the alveolar bone was identical in the mandible and maxilla.

Lower incisor teeth--enamel

Radio-autographs obtained from the incisor showed reactions which were similar to that of the molar teeth over the enamel, but different over the dentin. In the case of the enamel, two gradients were clearly visible as soon as

PLATE 5



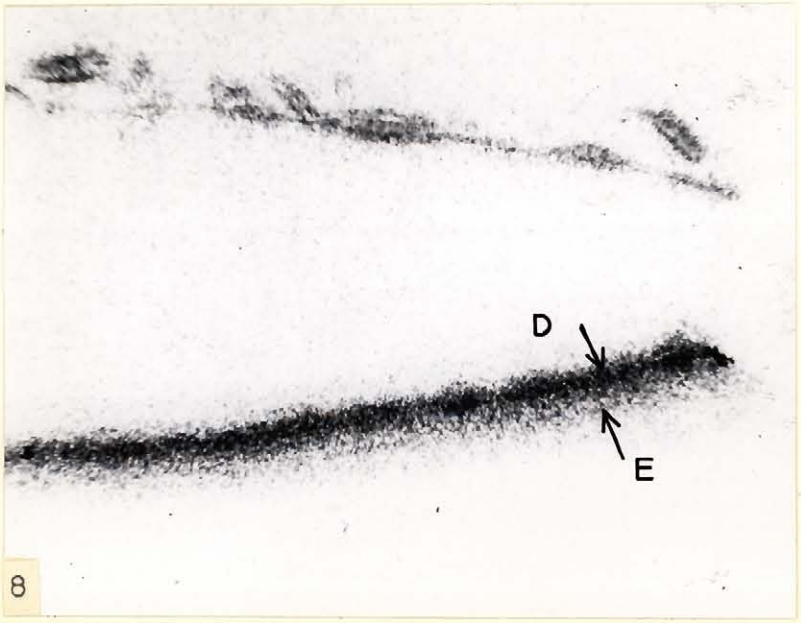


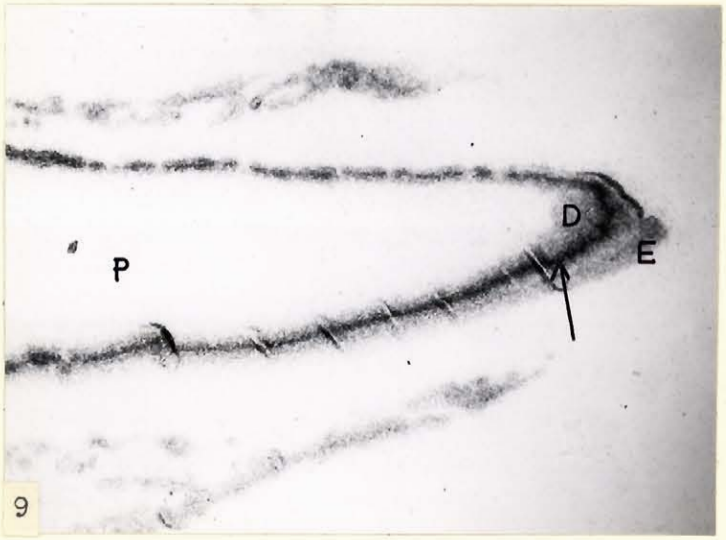
PLATE 5

Explanation of figures

Fig. 7 Coated unstained radio-autograph of a lower incisor of a newborn rat sacrificed 2 hours after administration of Ca^{45}. Note reaction over the dentin which is greater than that over the enamel. Magnification x 145. E: enamel; D: dentin.

Fig. 8 Coated unstained radio-autograph of a lower incisor of a rat injected with Ca^{45} at birth and sacrificed one day later (Newborn group). Note the reaction over the dentin which is greater than that over the enamel. Magnification x 145.

PLATE 6



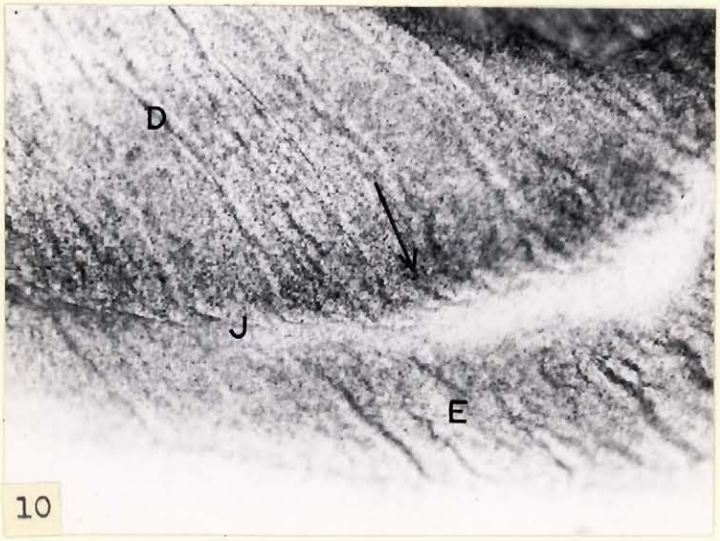


PLATE 6

Explanation of figures

Coated radio-autographs of lower incisors of rats injected with Ca^{45} at birth and sacrificed at various intervals thereafter (Newborn group). E: enamel; J; dentino-enamel junction; D: dentin; P: pulp.

Fig. 9 6 days after injection. Note the band of reaction (arrow) which is cone-shaped about the tip of the incisor. Magnification x 52.

Fig. 10 9 days after injection. Note the band of reaction (arrow) is lesssharply delineated.

there was enough matrix present to make these gradients visible. These two gradients as in the enamel of the molar are the basal-incisal gradient, increasing from the base to the incisal portion and the radial gradient, decreasing from the dentino-enamel junction to the outer surface (Figs. 7 - 10 inclusive). In all the age groups studied, the radioautographic intensity never exceeded that of the dentin. As in the case of the molar, there were no reactions due to the incorporation of Ca^{45} in the tissues of the enamel organ of the incisor tooth.

Lower incisor teeth--dentin

The dentin of the incisor, in contrast to that of the molar, is much thicker; consequently, better localization of the reduced silver grains was possible. Two and four hours after injection of the tracer element, it was found distributed in the dentin of the incisor with the maximum concentration in the region of the newly formed dentin (Fig. 7). Predentin contained very little if any reaction. One day later (Fig. 8), the autograph over the dentin was observed to have become distinct. The zone of maximum reaction was followed by one of lesser intensity bordering the predentin. Towards the base, where the thickness of the dentin matrix decreased progressively, the maximum reaction was observed to originate from an area closer to the predentin. On the lingual surface of the incisor dentin, which is thinner than on the labial, the intersection of the autographic reaction into the predentin

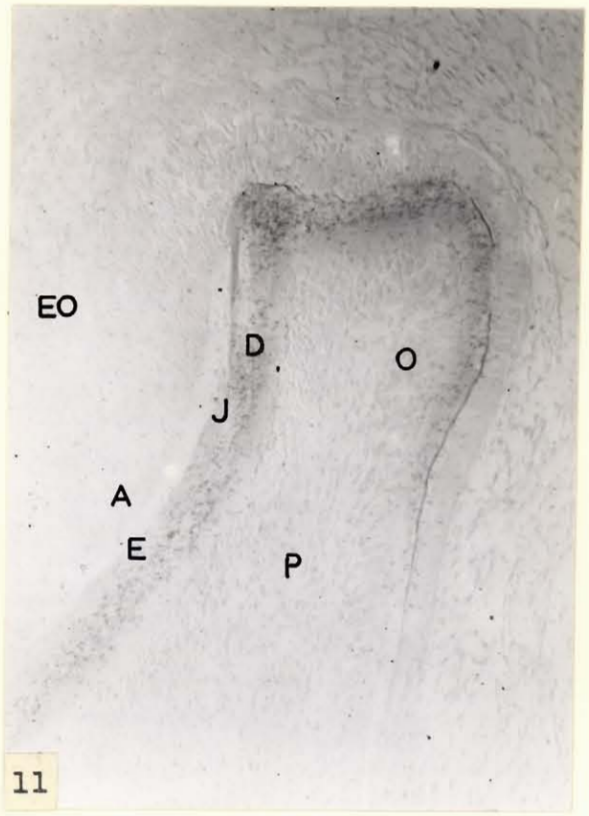
occured anterior to that on the labial surface. This cone-shaped autographic reaction persisted at later time intervals (Fig. 9). However, with time the sharpness of the band reaction diminished (Fig. 10). Thus, much of the reaction in the incisor dentin became absent about the 12th day; radio-autographs obtained at 15th and 18th day after injection showed very vague indications of a band, if at all. When no band was observed, an autographic pattern consisting of two gradients, as in the molar dentin were observed, that is, basal-incisal and radial gradients.

During the 18 days in which the radio-element was being metabolized, the animals grew to some five times their weight at birth. The teeth and most notably their incisors grew much (2.8 mm. per week in young rats, Schour, '42). The original dentino-enamel junction at the tip is maintained while the continued deposition of dentin decreased the size of the pulp cavity. This process eventually resulted in completely filled tip and in fact this was observed in the incisors of 18 day old rats. The substance filling the tip of the pulp cavity also gave a slight radio-autographic reaction indicating the nature of its composition.

Radio-autographs of teeth of rats of 3 day old group injected with Ca^{45}

It was observed that the amount of dentin and enamel matrices present at the time of administration of the radio-isotope to newborn rats were rather small. Hence, radio-autographs

PLATE 7



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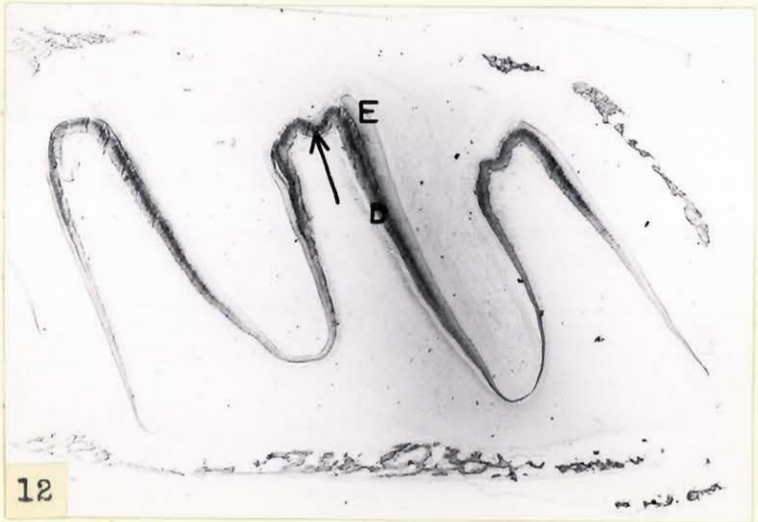

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PLATE 7

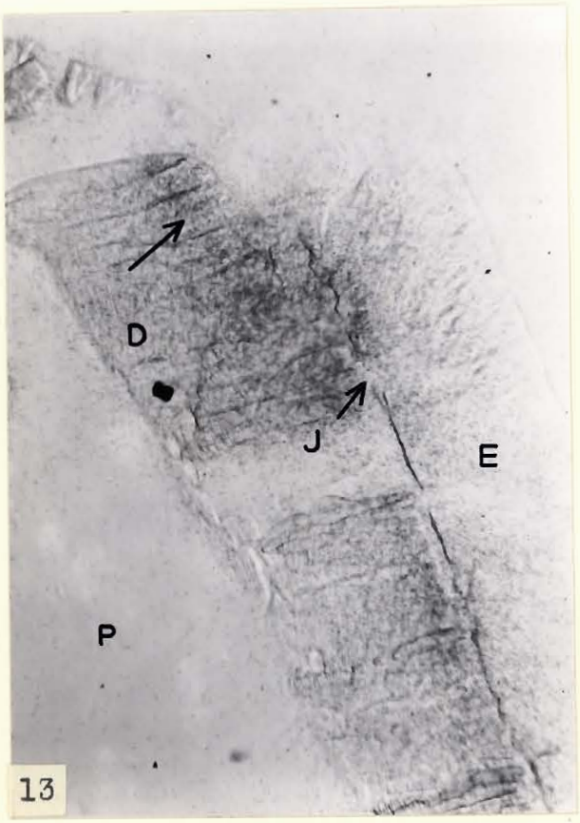
Explanation of figures

Coated radio-autographs of molars of 3 day old rats injected with Ca^{45} and sacrificed at various time intervals thereafter (3 day old group). Alizarin stained sections. EO: enamel organ; A: ameloblasts; E: enamel; J: dentino-enamel junction; D: dentin; O: odontoblasts; P: pulp.

Fig. 11 Molar cusp 1 hour after injection. Note reaction, which is mostly over the dentin, is diffuse. Magnification x 145.

Fig. 12 Molar 3 days after injection. Note the reaction now appears as a band (arrow) which is located in the dentin. Magnification x 20

PLATE 8



Coated unstained radio-autograph of a molar cusp of a rat injected with Ca^{45} at 3 days of age and sacrificed 9 days later (3 day old group). Note band of reaction still present (arrow) has lost its sharp delineation. Breaks in continuity of enamel and dentin due to fragmentation during preparation of section. Magnification x 145. E: enamel; J: dentino-enamel junction; D: dentin; P: pulp.

obtained from this group displayed the reaction in the dentin close to the dentino-enamel junction in the case of the incisors and no band in the dentin of the molar. Therefore, to observe the autographic pattern when the amount of matrices was greater, 3 day old rats were injected with tracer doses of Ca^{45}.

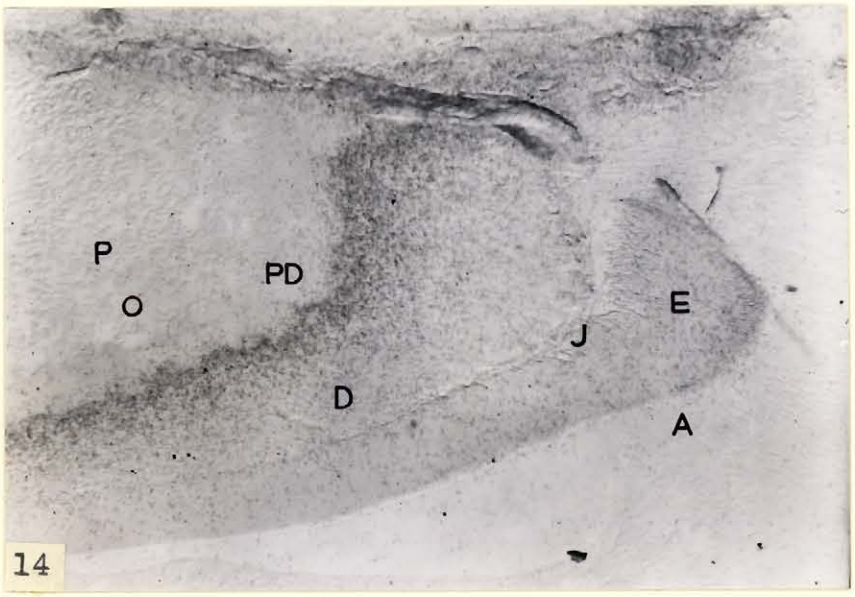
Molar teeth--enamel

In a 3 day old rat sacrificed as early as one hour after injection of Ca^{45} it was found to produce visible radio-autographs over the enamel of the molar tooth. At all time intervals, the radio-activity was deposited along two gradients, one, the basal-occlusal increasing from the basal to the occlusal parts and the other, radial, decreasing from the dentino-enamel junction to the outer surface of the enamel (Figs. 11, 12 and 13).

Molar teeth--dentin

Radio-autographic reactions observed in the dentin of molars after injection of radio-calcium to 3 day old rats, however, were different from those obtained after injection to the newborn group. At the end of the first hour (Fig. 11), the reaction in the dentin was diffuse. However, at one day the autographic pattern was seen to have an area of maximum intensity between the odontoblastic margin and the dentino-enamel junction in the shape of a cone around the cusps of the molars. Three days later the reaction was observed midway in the thickness of the dentin (Fig. 12) which had grown by

PLATE 9



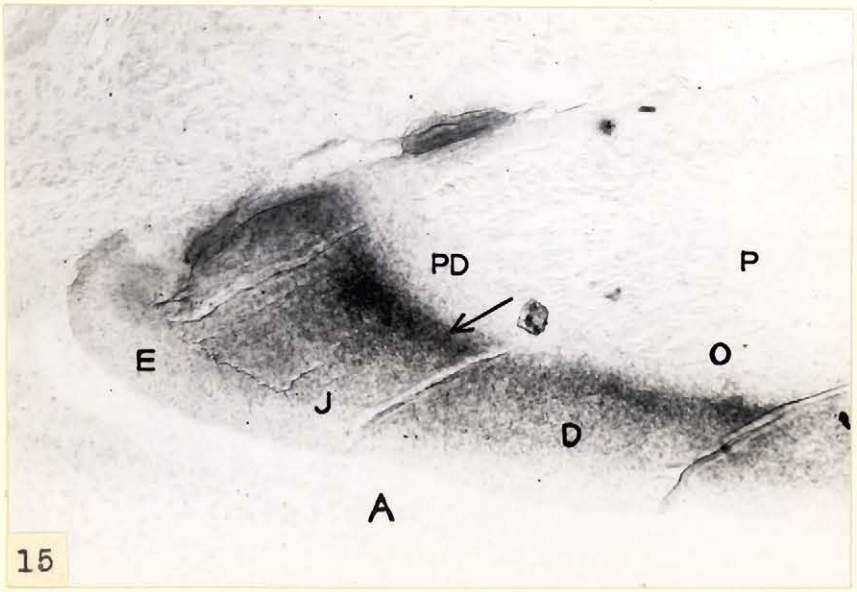


PLATE 9

Explanation of figures

Coated radio-autographs of lower incisor of 3 day old rats injected with Ca^{45} and sacrificed at various times thereafter (3 day old group). Alizarin stained sections. A: ameloblasts; E: enamel; J: dentino-enamel junction; D: dentin; PD: predentin; O: odontoblasts; P: pulp. Magnification x 145.

Fig. 14 1 hour after injection. Note the deposition of Ca^{45} in the layer of dentin adjacent to the predentin.

Fig. 15 1 day after injection. Note the autographic band (arrow) in the dentin.

apposition of matrix and mineral salts on the pulpal surface during the time interval. A similar band of reaction was observed throughout until the 9th day after injection of Ca^{45} (Fig. 13) when it was noted that the sharp delineation of the autographic reaction in the form of a band had decreased. The distribution of the autographic reaction obtained about this time and later show two gradients, the basal-occlusal and the radial as previously described. Thus, while much dentin was deposited on the pulpal surface since the time of administration of the tracer element, only a slight reaction was observed over this area. The amount of reduced silver grains over the predentin was insignificant at all times.

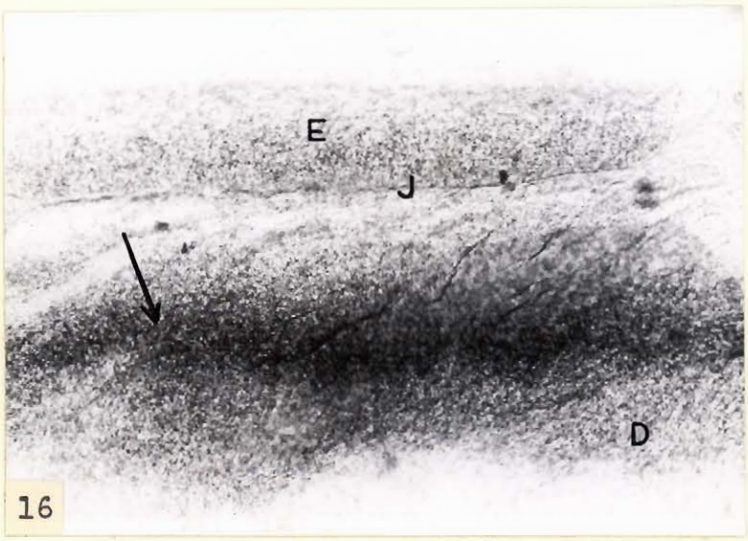
Lower incisor teeth--enamel

The autographic patterns in the incisor enamel from rats injected with Ca^{45} at the age of 3 days, were the same as those obtained from enamel of other teeth examined in the present study. At no time did the autographic reaction over the enamel exceed that of the dentin immediately adjacent to it.

Lower incisor teeth--dentin

One hour after the administration of the radio-isotope, it was deposited in the region of the newly formed dentin, while a lesser amount was found in the dentin which had already formed and a still lesser amount in the predentin (Fig. 14). One day later (Fig. 15), the autographic reaction was observed in the form of a band, followed by a layer of more recently

PLATE 10



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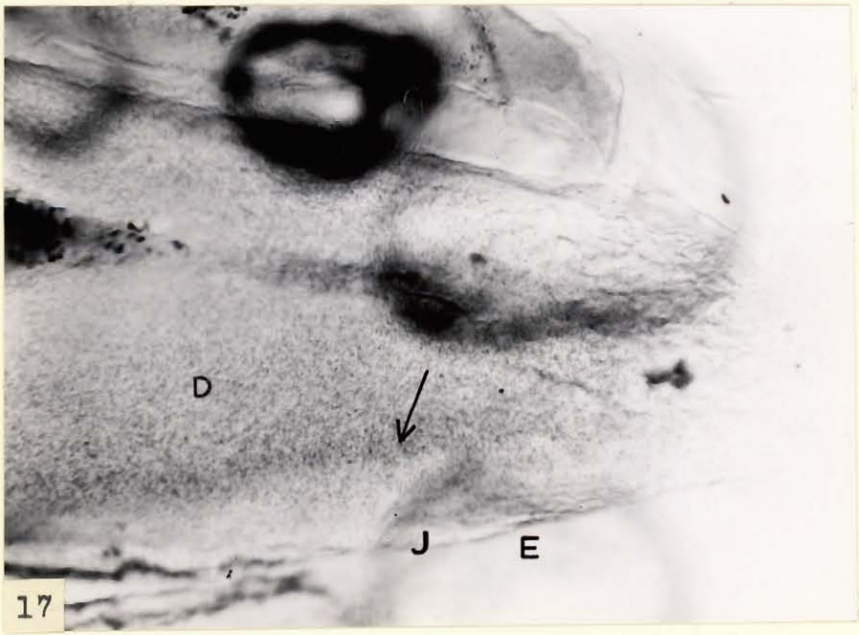

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PLATE 10

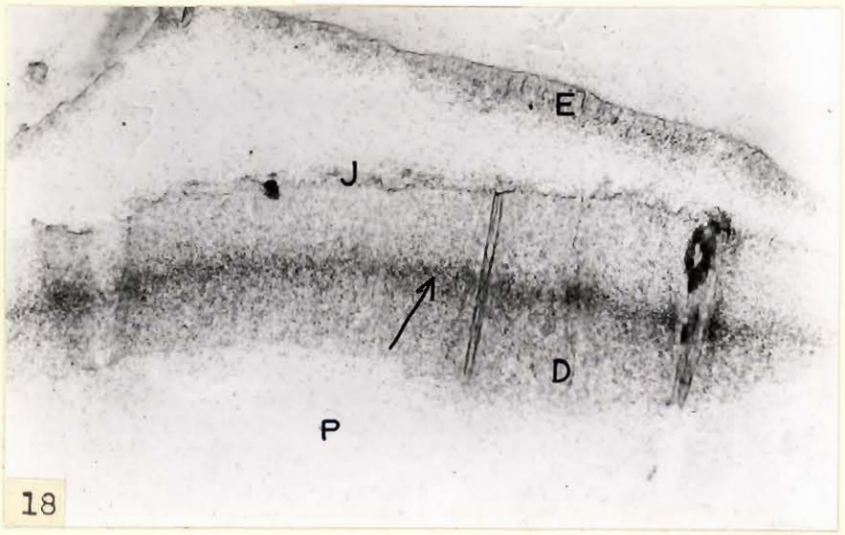
Explanation of figures

Coated radio-autographs of lower incisors of 3 day old rats injected with Ca^{45} and sacrificed at various intervals thereafter (3 day old group). E: enamel; J: dentino-enamel junction; D: dentin.

Fig. 16 3 days after injection. Note the autographic band (arrow) midway through the thickness of the dentin. Alizarin stained section.

Fig. 17 8 days after injection. Ground unstained section. Note the autographic band still present in the dentin (arrow). E marked where enamel normally present. In this preparation, it was accidentally lost during the grinding process. Magnification x 145.

PLATE 11



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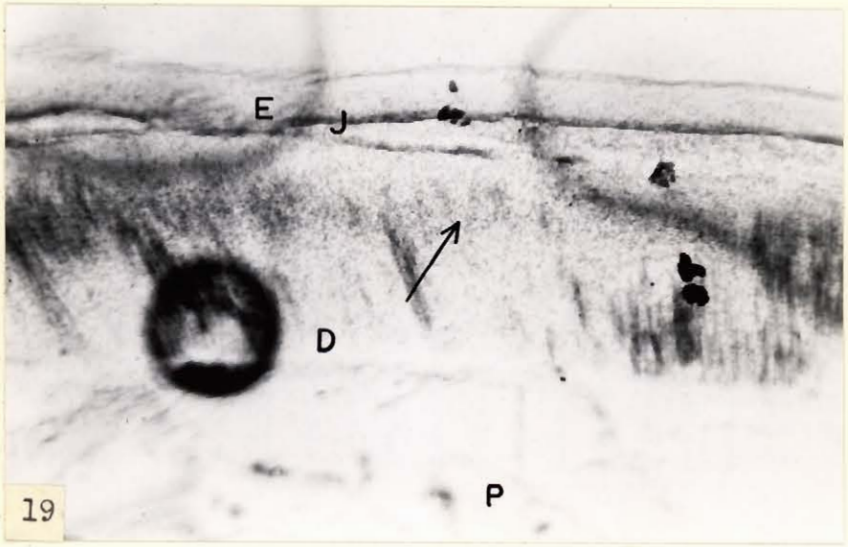

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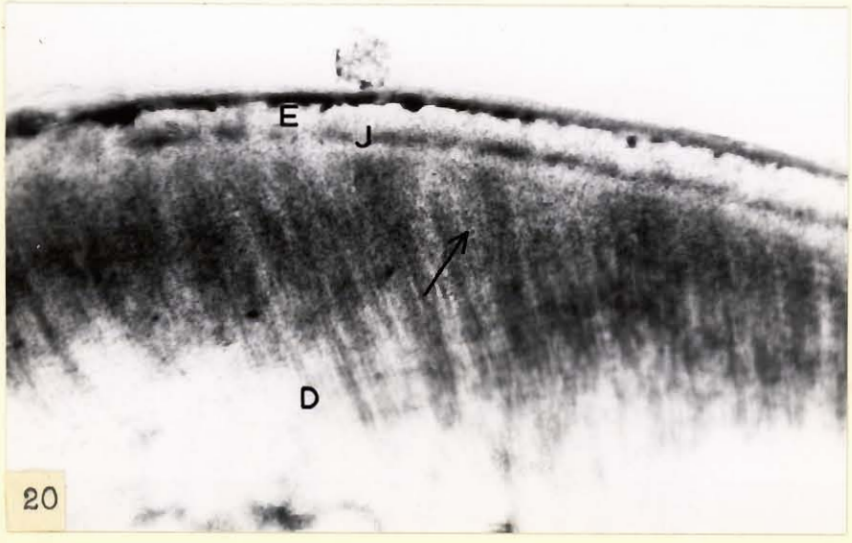

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PLATE 11

Explanation of figures

Coated radio-autographs of upper incisors of 3 day old rats injected with Ca^{45} and sacrificed at various intervals. These photomicrographs are from comparable areas of incisors in Fig. 21. Note the band of autographic reaction (arrow) and its distance from the dentino-enamel junction at each of the time intervals. E: enamel; J: dentino-enamel junction; D: dentin; P: pulp. Magnification x 145.

Fig. 18 3 days after injection. Alizarin stained section.

Fig. 19 6 days after injection. Ground unstained section.

Fig. 20 12 days after injection. Ground unstained section.

formed dentin, which was not nearly as reactive autographically. Three days later the band was seen midway in the thickness of the dentin (Fig. 16) and at 8 days it was still to be found in about the same site as in the earlier intervals (Fig. 17). At subsequent times, not much of the band reaction was observed which appeared replaced by reactions in the gradient pattern.

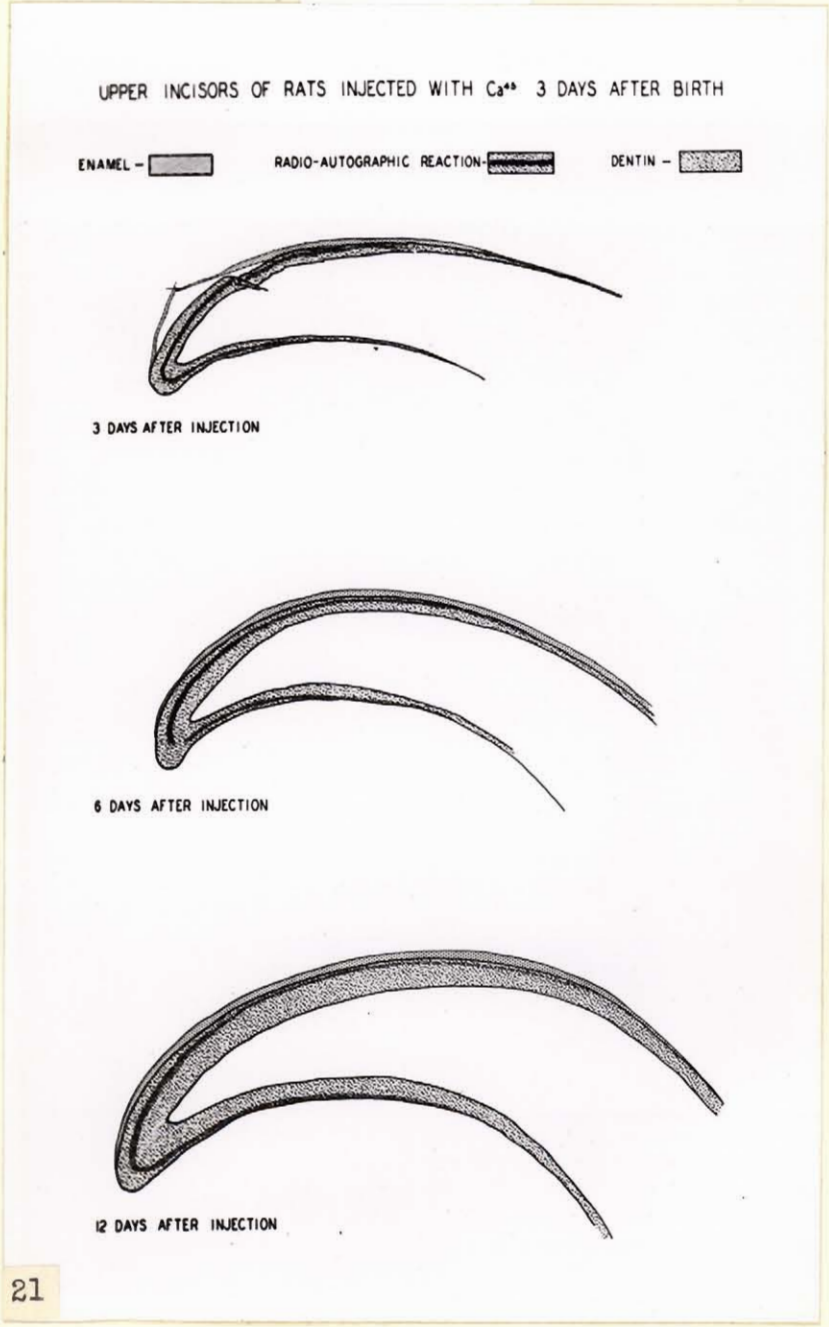
Upper incisor teeth--enamel and dentin

Although there may be no difference in the over-all pattern of growth between the lower and upper incisors, they will be examined separately.

The upper incisor enamel presented identical autographic reactions as in the enamel of the lower incisor. However, the dentin showed a distinct reaction in the form of a band until the 12th day, but much of the sharp delineation was lost with increasing time after injection (Fig. 18, 19 and 20).

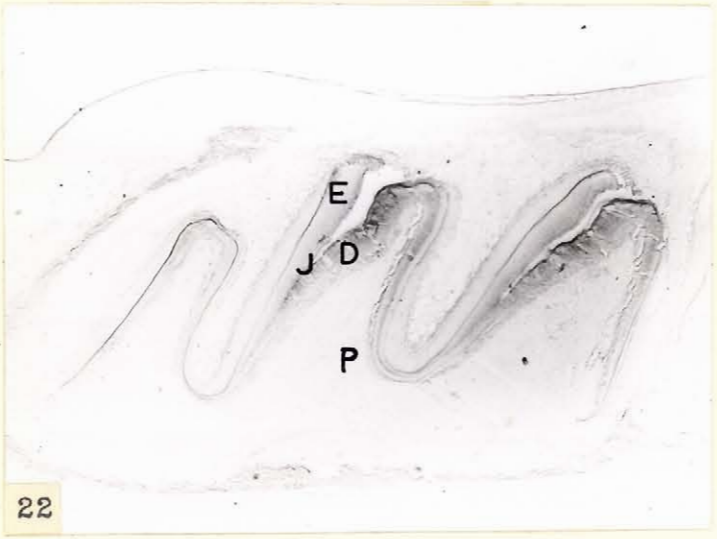
To determine the site of the radio-autographic band in the dentin of rats sacrificed at different times after Ca^{45} injection, measurements were carried out with an occular micrometer. Autographs of the upper incisors obtained after paraffin embedding (1 hour, 1 day and 3 days after injection) were utilized. Those from older rats were not available since the sections obtained after celloidin embedding were too fragmented to allow accurate measurement. Three positions of the dentin were measured; the distance between the site of maximum reaction of the band and the dentino-enamel junction were

PLATE 12



Camera lucida drawings showing relationships of dentin, enamel and site of autographic band reaction in the 3 day old group.

PLATE 13



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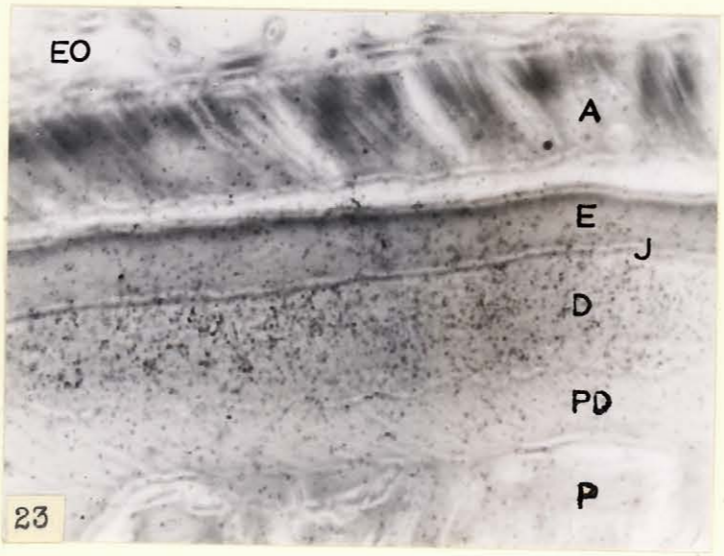

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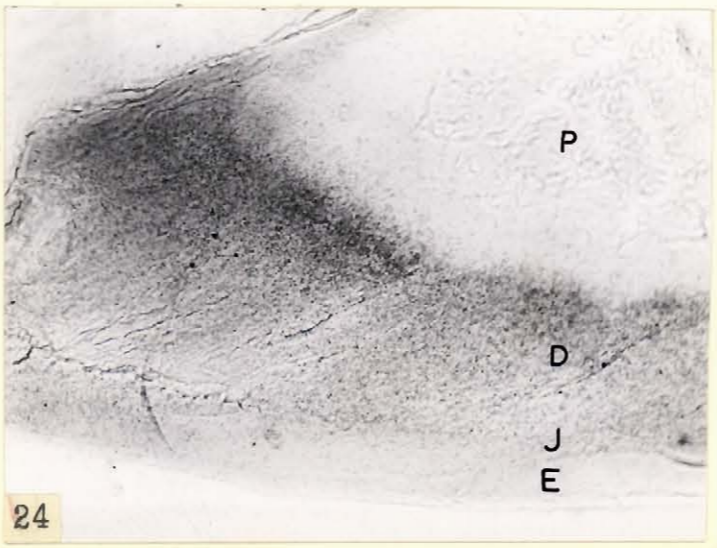

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PLATE 13

Explanation of figures

Coated radio-autographs of teeth of rats injected with P^{32}. Note that the autographic reactions over both enamel and dentin are identical to that obtained after Ca^{45} injection. EO: enamel organ; A: ameloblasts; E: enamel; J: dentino-enamel junction; D: dentin; PD: predentin; O: odontoblasts; P: pulp; B: alveolar bone.

Fig. 22 Molar tooth 6 days after injection at birth (Newborn group). Alizarin stained section. Magnification x 20

Fig. 23 Higher magnification of same molar as above. Note predentin hardly shows any autographic reaction while dentin shows the most.

Fig. 24 Lower incisor of 3 day old rat 3 hours after injection (3 day old group). Alizarin stained section. Magnification x 160

noted at the tip and at a constant distance, 7 units of the occular micrometer, away from the tip on the labial side. The third site was 3 units away from the tip on the lingual surface where the distance between the band reaction and the lingual surface of the dentin was measured. It was found that during the first three days after injection to 3 day old rats, there were no significant changes in the location of the autographic band.

Camera lucida drawings were made of ground preparations of the upper incisors of 9 and 15 day old rats as well as that of a 6 day old rat obtained after paraffin embedding (Fig. 21). When similar areas were compared, the site of the band reaction was found to be located in about the same distance from the dentino-enamel junction (Figs. 18, 19 and 20).

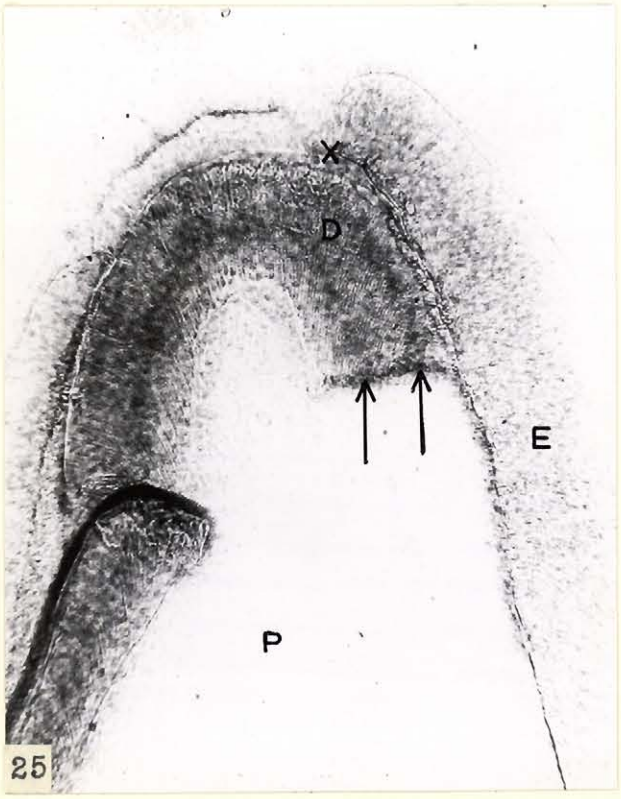
Radio-autographs of teeth of newborn and 3 day old rats injected with P^{32}

Radio-autographs obtained after the injection of P^{32} to newborn rats and two rats of 3 days of age, were identical to those obtained after the administration of Ca^{45}. The doses used in these experiments did not give any autographic reaction in the nuclei or cytoplasm which have phosphate containing components. (Figs. 22, 23 and 24).

Radio-autographs of teeth of rats injected twice with Ca^{45}

The persistence of the autographic reaction in the form

PLATE 14



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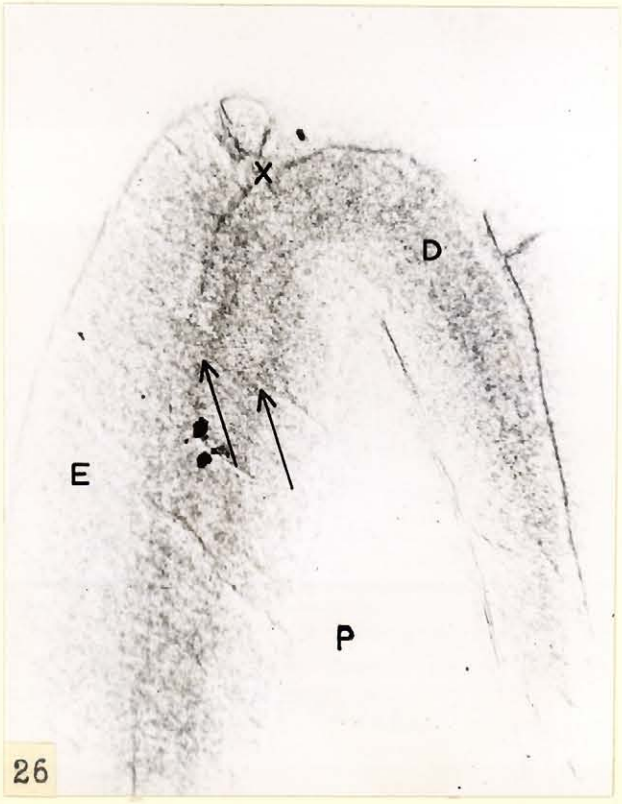

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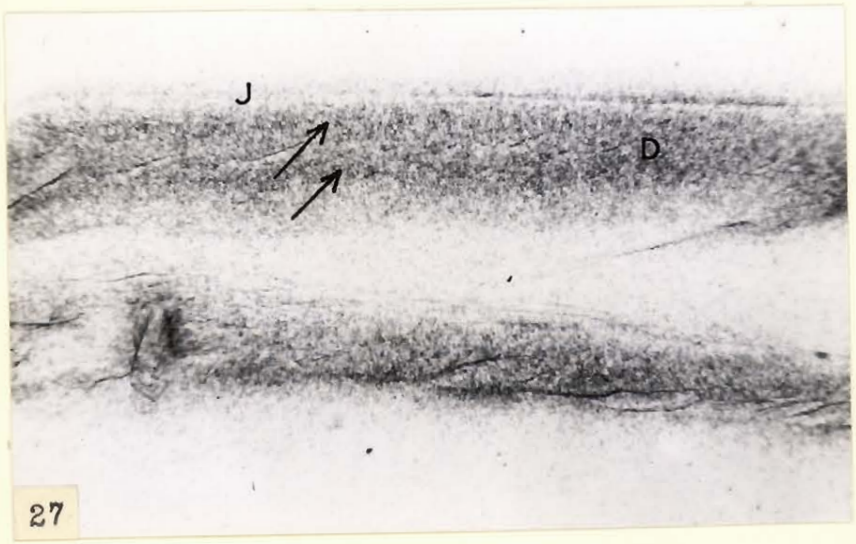
PLATE 14

Explanation of figures

Coated unstained radio-autographs of the third cusp of the upper first molar of a rat injected with Ca^{45} at 3 and 6 days of age and sacrificed at 9 days of age, Fig. 25, and 11 days of age, Fig. 26. Note the two lines of autographic reaction over the dentin (arrows) indicating the two injections, while over the enamel, the reaction consists of two gradients, basal-occlusal and radial. Magnification x 145. E: enamel; J: dentino-enamel junction; D: dentin; P: pulp; X: see text, p.

PLATE 15



Coated unstained radio-autograph of an upper incisor of a rat injected with Ca^{45} at 3 and 6 days of age and sacrificed at 9 days of age. Note the dentin possesses two lines of maximum autographic reactions (arrows), indicating the deposition of minerals which took place immediately after each injection. Lingual dentin here does not show bands of autographic reaction. Enamel is torn away. Magnification x 145. J: dentino-enamel junction; D: dentin.

of a band at early time intervals after administration of Ca^{45} led to the attempt to prove radio-autographically that the deposition of the mineral elements in the teeth was incremental in nature. The rats in this experiment, therefore, received a second injection by the same route on the third day after the first injection, which was given when they were 3 days of age.

Enamel of molar and incisor teeth

The radio-autographic reaction over the enamel 3 days after the second dose (9 day old rat) and 5 days after the second dose (11 day old rat) were identical. In spite of the two injections, the enamel showed only two gradients in the autographic pattern, the basal-occlusal gradient, increasing from the base to the occlusal parts and the radial gradient, decreasing from the dentino-enamel junction to the outer surface of the enamel (Figs. 25 and 26).

Dentin of molar and incisor teeth

The dentin, in contrast to the enamel, showed two bands of autographic reaction. These double bands or reactions were observed only near the tips of the molar cusps (Figs. 25 and 26) or near the incisal end of the incisor (Fig. 27), where there was a greater amount of dentin. The dentin of the second molar teeth, which in the singly dosed rats showed only a diffuse reaction consisting of a basal-occlusal and a radial gradients, now showed a reaction in the form of a band. In addition to this band reaction, there was a distribution of

autographic reaction over the dentin in the form of two gradients as in the first molar of a singly dosed rat.

The distance between the two autographic reactions in the dentin were measured in the 9 and 11 day old rat molars. These measurements were made on the photomicrographs of the autographs (Figs. 25 and 26). Both specimens are from the third cusp of the upper first molar. The magnification from the original sample tooth had been carefully noted by recording the number of times a unit of the stage micrometer was magnified on the frosted glass plate on which the specimens are focused, using the same combination of objective (nosepiece) and occular as for the photomicrograph. This was found to be 145 times. The distance between the bands measured 8 mm., 3 cm. from the site where the enamel terminates at the tip of the cusp (see X Figs. 25 and 26). The time interval between the two injections was three days. Therefore, the amount of growth per day was calculated by multiplying 8 mm by 1000 for conversion into μ and dividing by 3 days and 145, the magnification, that is, $\frac{(8)(1000)}{(3)(145)}$ or 18.4 micra per day.

Alveolar bone

The alveolar bone displayed an autographic pattern which was essentially the same as in those after a single injection.

DISCUSSION

Similarity of Ca^{45} and P^{32} radio-autographs

The radio-autographic method of analysis has been utilized by many investigators (Bélanger and Leblond, '46; Gross and Leblond, '47; Bélanger, '50; Gross et al, '51; Ceccaldi, '51; Fitzgerald et al, '53). It is indeed one of the most sensitive methods available for localizing tissue components on a histological section.

For radio-autographic investigations on the mineral metabolism of the calcified structures, P^{32} and Ca^{45} has often been used but other radio-isotopes which become deposited in the bones and teeth have also been utilized. In the present study, radio-autographs of teeth obtained after Ca^{45} and P^{32} administration to rats were found to be deposited in the same areas of dentin and enamel at all times selected (Figs. 5, 14, 22,and 24). Thus, in the very young rat teeth, the fate of these two elements are identical.

In radio-autography, resolution is the term applied to the degree of separation possible between adjacent points or areas of autographic reaction. It depends on the geometrical relationship between the radio-active source and the emulsion, the characteristics of the photographic emulsion and the energy of radiation of the isotope. In the coated method, all factors are constant except the last. Resolution has been found to parallel the space rate of energy loss of the radiating

isotope (Gross et al, '51).

Radiophosphorus is a convenient isotope with which to conduct radio-autographic investigations. However, it has the disadvantage of producing autographs of lower resolution because of energy of disintegration (1.69 mev.). Radiocalcium which has a lower radiation energy (0.25 mev.), produces autographs of better resolution.

Thus, with advantages to be had in increased resolution and its long half life, 180 days, compared to that of P^{32}, 14.3 days, the bulk of the present study has been carried out with Ca^{45}.

Deposition of Ca^{45} and P^{32}

Following administration of tracer dose of radio-element P^{32}, it has been found to be immediately deposited on the calcifying structures of the body (Manly et al, '39; '40; Sognnaes and Volker, '41; Percival and Leblond, '48; Leblond et al, '50; Belanger, '52; Comar et al, '52; Engfeldt et al, '52). Calcium^{45} likewise undergoes specific binding into calcifying structures (Carlsson, '51; D'Iorio and Lussier, '51; Minder, '52; Singer et al, '52).

The rate of deposition of the isotope is rapid. After intravenous injection of Ca^{45} into the rabbit, 66% had been absorbed in serum or blood proteins in 3.6 minutes and that by 10 minutes, maximum absorption had taken place in the blood (Minder, '52). The deposition has been visualized in

radio-autographs as early as 2½ minutes after the administration of Ca^{45}, P^{32} and also Sr^{90} (Comar et al, '52). Much of the radio-element deposited in bones soon after injection has been found to be removed by some mechanism, presumably ion exchange although the possibility of release in a hypothetical process described as recrystallization may also be considered.

Classical views hold that the dental structures, that is the enamel and dentin do not contain any unstable forms of mineral salts in the normal condition. Pathological conditions such as caries may, however, produce removal of salts; the dissolution is due in many instances to external forces. In vitro experiments of absorption and exchange of mineral elements of teeth have, however, proven the presence of a labile fraction which is higher in the dentin than in the enamel (Bevelander and Amler, '45; Underwood and Hodge, '52; Belanger, '53). The present study although not directed quantitatively to prove whether such ions in flux exist at all, do indicate the presence of a stable moiety in the mineral elements in the growing teeth. Analysis of the radio-autographs at various time intervals after injection of the radio-isotope to young rats revealed that the intensity of the autographic reaction did not decrease under similar lengths of exposure. This is in a way a proof that the mineral salts once deposited in the teeth do not leave the structure as has been found in the case of bones. With

the help of radiochemical method on older rats, there have been demonstrated in the continually growing incisor, an increase in radio-activity with time after administration of the isotope (Leblond et al, '50; Carlsson, '51; '52). Leblond et al, '50, using whole jaws of newborn rats, showed the specific activity to increase and then fall by the third day. However, in 50 gram and 240 - 280 gram rats, the specific activity increased over a period lasting 8 days. The specific activity of blood in the mean time decreased markedly, while bones showed variations in the drop according to different regions. In Carlsson's, '51, observation of the incisor there was no decrease in the uptake of Ca^{45} between rats of 70 (168 grams) and 210 (280 grams) days and that in 43 day old rats, the activity per unit weight of ash became higher in incisors than in the bones (that is, had reversed) and there was no evidence of a loss of Ca^{45} from the incisor over an 11 day period. Carlsson, '52, observed that in a 63 day old rat (150 grams) given Ca^{45} as lactate by stomach tube, the uptake in the incisors, femurs, tibia-fibula ends and tibia-fibula shafts increased at a fairly high rate during the 18 hours over which the experiment was carried. It was found that the uptake per hour decreased rapidly for the femur, while for the incisors, the uptake remained almost unchanged. However, he made no attempt to separate the dentin from the enamel. The content of fluorine in the rat incisor also increased during the intake of this element in the diet and

even after the cessation of fluorine intake (Savchuk and Armstrong, '51).

Shape of the teeth and the sections obtained from them

Lower Incisor

In the course of the analysis of radio-autographs with the aim of elucidating the manner of development of the enamel and dentin, it became extremely important to know exactly the site where the section was obtained in the incisor or the molar. To follow the morphology in a curved structure as the incisor, which not only has a crescentic shape as viewed laterally, but also another although less sharp curve when viewed from above, is indeed a difficult problem. This is particularly marked in the lower incisors, which, for this disadvantage, has the point of merit--namely its length and size, which is greater than the upper incisors. The nature of the tissues which even at the age groups selected are hard, made the use of serial sections impossible. Only incisors considered long enough to include the areas in the incisal end have been considered. Even with this precaution, there remains the possibility that the sections are not thoroughly comparable. The difficulty of knowing the site of section in the teeth prompted the return to the more tedious grinding method. In this method, the tip of the teeth was constantly under observation, and the grinding was carried out in such a manner that the tip of the incisors (in the case of the molars, the tips of the cusps) were preserved.

Upper incisor

The upper incisors are broad and flat in comparison to other teeth of the rat and this was found to be convenient in grinding. However, they are much shorter than the lower teeth, and they undergo growth changes in compressed form, that is to say for instance, that the autographic reactions were found over a shorter distance. This presented the disadvantage that the tips of the upper incisors where the changes were observed were lost more readily than the tips of the lower incisors in the grinding process or in functional attrition, a paradoxical situation indeed.

Molar

The molars, fortunately, did not pose as much difficulty as did the incisors. Of the three molars in a row in each jaw of the rat, all of which develop similarly, but at different times, stress will be laid on the first molar which is the largest. This molar possesses at least three large cusps in a sagittal section and has a small area on the summit of the cusps which is free of enamel. Areas on different sections were compared by reference to this site, since these parts are small and thus ensured that relatively similar areas were being studied.

The increase in the thickness and length of the dentin and enamel matrices is very marked in the course of this experiment lasting 15 to 18 days. During this interval the pulp cavity is observed to decrease at the incisal or occlusal end of both types of teeth. The incisors after eruption about

the 10th day after birth, are from this time, increasingly exposed to conditions conducive to attrition. This has been noticed on ground sections of incisors of 15 day old rats, although they were still observed to be nursing from their mother.

Autographic pattern of the enamel

The enamel at all times exhibited an autographic pattern which hardly differed in the rats injected at different ages or in those which were injected twice with Ca^{45}. The reaction showed at all times the basal-incisal or basal-occlusal gradient, which increased as the tip of the tooth or cusp was neared. Although the mature or calcified enamel of the adult rat contains in the neighbourhood of 95% inorganic matter, these autographs did not show any indication of such a composition; the most intense of the autographic reaction in the enamel was not greater than that in the adjacent dentin. Such a result may at first appear puzzling. However, it has been shown that enamel formation occurs in two steps, namely, enamel matrix formation and enamel matrix maturation which involves the process of calcification. The majority of the teeth of rats which were used in these experiments may be said to be only at the matrix formation stage. The fact that the enamel remained intact more often after histological sectioning showed that the mineral content was less than that of dentin. Therefore, the light autographic reaction observed over the enamel matrix would most probably be that due to the

calcium deposited before the commencement of the maturation phase. The increase in the amount of silver grains observed at the tip of the molar cusp in older rats, for instance, figure 26, 11 day old rat molar, may be interpreted as indicating that the maturation phase had begun.

Maturation of the enamel commences from the incisal or occlusal portion towards the base and from the dentino-enamel junction outwards to the ameloblastic margin. Of the histochemical stains used in this study, only Mallory's triple stain showed the beginning of matrix maturation phase by a change from acidophilia to basophilia of the enamel matrix. This process is a gradual one and has also been shown by the fact that only one type of autographic reaction was obtained after giving two separate injections.

Acid insolubility in weak acids, a characteristic of young enamel and its translucency to Grenz rays (soft x-rays) (Applebaum, '38; '43), are other evidences of the early stage of enamel formation. Chemical analysis has established that enamel of this type contains only about 25% of the amount of mineral salts of the fully calcified enamel (Weinman et al, '42). Sognnaes, '52, has shown by electron microscopy the changes which accompany enamel matrix development. The changes are gradual; he has been able to demonstrate the presence of organic matter in mature enamel in which the organic matter was in finer dispersion than in the earlier period of its development. The changes observed at calcification is

the accumulation of calcium salts and the removal of water (Wassermann, '44; Marsland, '52), and there is little decrease in the organic content of the enamel (Deakins, '42).

The intense red staining of the ameloblasts in the region adjacent to the pre-enamel after alizarin staining by Dahl's method is of interest. Alizarin dye reacts with calcium ions to produce a red colour. In the pH range used in this technique, only calcium is coloured and magnesium which also has a similar colour with this dye does not interfere (Dahl, '52). By micro-incineration technique the presence of Ca and Mg (micro-incineration does not distinguish between Ca and Mg, both have white residues) was demonstrated. In differentiated ameloblasts and odontoblasts these two elements were present in greater amounts than in the cells which were not concerned with enamel or dentin formation (Hamp, '40). The red colour obtained after alizarin staining may well be an indication of the presence of calcium in the ameloblasts.

The pathway by which the minerals enter the enamel matrix may not be obvious, but studies on permeability have shown that small ions and molecules do penetrate the enamel from both inner or dentinal surface and outer surface (Sognnaes and Volker, '41; Wasserman, '44; Jansen and Visser, '50; Wainwright and Belgorod, '51; Sognnaes and Shaw, '52; Wainwright, '53). Experimental removal of the pulp, papillectomy, (Lefkowitz et al, '44), on the cat resulted in uncalcified enamel matrix formation, while removal of the enamel organ including the ameloblasts, amelectomy, (Lefkowitz et al, '47), conducted on

dogs revealed that the calcification of enamel proceeded in the ususal manner. The experiments on papillectomy and amelectomy, however, has been a subject of controversy.

Autographic pattern of the dentin

In the dentin, the deposition of the mineral salts following apposition of the matrix is in the form of layers which is manifested radio-autographically as a band of reaction. This incremental deposition is made in the dentin adjacent to the predentin. The staining reaction obtained after the five stains used in this series were more or less similar in that the uptake of the dyes by the dentin was always greater than predentin, except for the dye alizarin, which did not stain dentin evenly. The change in the staining at the border of the two types of dentin is distinct and sudden. Results of the radio-autographs showed that predentin consistently did not show a deposition of Ca^{45} and P^{32} in appreciable quantities. Thus, the presence of some change in the predentin is indicated before it becomes dentin with the concomittant ability to incorporate calcium salts.

The incremental nature of the deposition of mineral elements was conclusively demonstrated by radio-autographs obtained after double injection of Ca^{45} to 3 day old rats. The two injections were recorded separately in the dentin. It was found that a certain amount of matrix was necessary to show the incremental pattern. In the molars of the newborn

group, the radio-isotope was incorporated in the dentin as soon as dentin matrix was formed. However, in the 3 day old group, there was enough matrix at the stage of incorporating the mineral salts at the time of injection, that a band was formed in the autographs. This fact was shown in the second molar of the rats which were dosed twice with radiocalcium. It showed only one band, obviously the response to the second injection of Ca^{45}, over a diffuse reaction in the form of two gradients. The diffuse reaction was identical to that seen in the first molar after injection of the isotope to the newborn rats.

General remarks on the incremental deposition of mineral salts

Previous observations of teeth have already shown lines of growth, known as the incremental lines of growth. The extreme sensitivity of the dental structures to physiological and pathological environment has been observed and has given rise to such structures as the neonatal lines. This fact was taken advantage of in the experimental investigations on the manner and rate of apposition of the organic matrix and calcification.

Alizarin

The quantitative aspects of the growth pattern of the rat molar were studied (Hoffman and Schour, '40). Ground sections of molars were obtained from rats which received multiple intraperitoneal or subcutaneous injections of 2%

solution of alizarin red S. Teeth of rats between 0 and 500 days of age were studied. Each injection of alizarin was found recorded separately in both primary and secondary dentin. The enamel, however, was diffusely coloured or not at all in the older rats. Thus, alizarin served as a marker by which growth of the dentin could be determined. It was found that there were gradients in the appositional growth; in decelerating order they are from the tip of the cusps to the apices of the roots, and called the locus gradient. The other, from the periphery of the tooth toward the center was called the radial gradient. An antero-posterior gradient was observed also, in which the gradient decreased from the first to the third molar. The teeth of older rats were found not to be growing as rapidly as in the younger rats and this was termed the age gradient. Atthe cuspal tip where the maximum rate was noted, the daily rate of apposition of primary dentin was 16 μ per day while at the apex of the root of the tooth, the rate was 4.3 μ in the young rats. With age the rate decreased until about 135 days of age, when primary dentin formation ceased. Secondary dentin which is first noticed between 35 - 45 days of age is found in the pulpal floor, horns and roof of the molar tooth. Here, a rate of 15.73 μ per day was observed at the beginning and decreased with age. These values were obtained by ocular micrometer measurements between the successive red lines from alizarin injections in the dentin.

Sodium fluoride

Experiments conducted on the incisor of the adult rats with sodium fluoride (Schour and Smith, '34) showed that the dentin and enamel were marked. In this case, however, they showed a pair of light and dark lines for each marker injection of NaF. The light layers were hypocalcified while the dark layer were normal or hypercalcifified. The distance between the pair of lines was measured to be 16 μ per day.

Strontium chloride

Similar studies by injection of strontium chloride as the marker substance also revealed the incremental pattern of growth (Weinmann, '42). The dentin was marked but the enamel was not. The daily rate of growth was found to be slightly greater for the rats which received strontium injections than alizarin.

Daily rate of deposition of mineral salts

The rhythmic manner of deposition of the matrix and mineral salts was demonstrated. The rate of 16 μ per day was found for many species of animals except for the monkey and human teeth in which the rate was 4 μ per day (Schour and Hoffman, '39a; '39b).

In the present study, the daily deposition of Ca^{45} was found to be 18.4 μ per day by measurement of the distance between the lines of autographic reactions in figures 25 and 26. This was in fairly close agreement with the values obtained by Schour and his coworkers who obtained the value of

16 μ per day. The rate obtained after radiocalcium injection is in much closer agreement with those obtained by Weinmann after strontium chloride injection. To reach a definite conclusion after measuring two cases is impossible but the similarity of the results is very interesting.

C^{14} radio-autographs

Radio-autographs of decalcified teeth obtained after injection of C^{14}-bicarbonate to young rats have revealed that the C^{14} was deposited in the predentinal matrix adjacent to the odontoblasts soon after injection and with subsequent deposition of matrix, this band was seen to be within the dentin at 72 hours (Greulich & Leblond, '53).

Thus, the incremental nature of matrix formation which had been long suspected was conclusively proven. The deposition of this isotope was proven to be stable since after double injections the bands were seen separately in the dentin (Greulich, '53 PhD. thesis).

Loss of sharpness of autographic band

With time the radio-autographic reaction obtained with Ca^{45} or with P^{32} which was at first a distinct band, became less sharply delineated. There are several possible explanations for this phenomenon.

Dissolution due to physico-chemical forces

One reason may be the local dissolution and precipitation of the mineral salts of the teeth; this dissolution may be

caused by the changes in physico-chemical forces of pressure and tension in the course of growth of the tooth. This would result in the diffuse reaction, since the dissolved ions would hardly likely be deposited in the same relationships as before. However, the determination, morphologically, of a physical entity in a biological system, which is not even in a state of equilibrium but of rapid growth, is indeed a difficult problem, especially when the magnitude of such a force is unknown. For the present then, this remains as a plausible explanation.

Ionic exchange

Another very probable explanation, especially in the light of recent experiments in vitro, is the phenomenon of ionic exchange (Underwood and Hodge, '52; Belanger, '53). The former group exposed powdered fresh and glycol-ashed human dentin and powdered dried human enamel to aqueous solutions containing radiocalcium. Dentin showed greater absorption than enamel; in the latter only 2% of the calcium atoms appeared to exchange. Glycol ashed dentin was found to exchange rapidly at first, involving as much as 1/5 of the total calcium atoms while fresh dentin showed slower but more extensive exchange. The observations were carried out for 12 days on glycol ashed dentin and dried enamel and 30 days for fresh dentin. Belanger, '53, immersed histological sections of teeth of hamsters, rats and human into buffered solutions containing P^{32} or Ca^{45} for periods from 4 to 24 hours at room

temperature. After this treatment, the sections were autographed. Cementum was found to exchange more than dentin, and dentin more than enamel. He also observed that young tissues exchanged at a greater rate than older tissues. Both of these investigators have conducted their experiments *in vitro*, and conditions *in vivo* may be altogether different. That the specific activity measurements of the jaw and incisor should show increases with time after administration in both growing and adult rats is indeed conflicting evidence to the *in vitro* experiments (Leblond et al, '50; Carlsson, '51; '52). Moreover, the radioautographs obtained in this study did not show decreases in intensity with time after administration. That the older parts of the dentin are accessible to the body fluids is known for there are present the dentinal tubules, which contain the dental lymph surrounding the odontoblastic process in them.

Interstitial calcification

Yet another factor which can contribute to the disappearance of the sharp band reaction is the further accumulation of calcium salts in the dentin. There can be no doubt of this for with time the teeth are found to increase in hardness; in fact it becomes increasingly hard to obtain good histological sections. Radio-active ions of calcium or phosphorus released in the process of remodelling of bone (for instance the funnel or metaphyseal region of long bones, Leblond et al, '50) might constitute the source of the radio-element since the amount in the blood decreases rapidly after administration. The further

accumulation of salts can be surmised without difficulty for it involves only the interstitial addition of calcium and phosphorus. With aging, the dentinal tubules decrease in diameter while in transparent dentin, the tubules are obliterated with mineral salts.

An attempt to determine the amount of deposition of calcium was made by Carlsson, '52. The uptake in mg. per hour in the incisors of a 63 day old rat was found to be of the order of 0.121 - 0.07 mg. Ca per hour, the range being due to diurnal variation.

The "movement" of the band reaction

Summary of P^{32} autographs obtained by Belanger

Previous experiments on teeth of rats and hamsters with radio-active phosphorus have been published (Belanger, '51; '52). These experiments, conducted radio-autographically, were claimed to show the displacement of the band of reaction due to P^{32} through the dentin.

Essentially, Belanger found that with time after administration of P^{32} the sharpness of the autographic reaction, which first appeared as a line or band, decreased. He found also that soon after injection, the P^{32} was deposited in the dentin near the odontoblasts, but at later time intervals found that the band had moved so that it was close to the dentino-enamel junction and eventually the band disappeared. The reaction in the enamel was not in the form of a sharp band.

In the dentin of rats which were injected twice at an interval of 10 days, he found only one band. Thus, he concluded that after the initial physical exchange reaction, the band ascends towards the dentino-enamel junction from the pulp and disappears, leaving in its wake a diffuse distribution of radio-activity. The rate of displacement was concluded to be inversely proportional to the age of the dentin and had ceased in the adult tissue.

Precautions necessary to interpret autographic results

Belanger's contention of the dynamic aspect of dentin is provoking indeed. His first argument is based on the fact that with time the band seems to have moved closer to the dentino-enamel junction. To assess the distance between the band and the dentino-enamel junction, it is necessary that all sections be taken from the same region, for example the apices of the cusps, or else this distance will vary. In Belanger's article, it was noted that the photomicrographs of dentin 8 days after P^{32} injection (Fig. 9, p. 547, Anat. Rec. 114, 529-554, 1952) is thinner instead of thicker as expected than that of 6 days after injection (Fig. 8, p. 547, ibid.). Presumably the sections of the two molars were not taken from the same areas. These observations cast some doubt on the accuracy of his conclusions.

Belanger's second argument is that the band of reaction eventually disappears and is replaced by a diffuse line of reaction near the dentino-enamel junction as shown in one of his photomicrographs taken 10 days after injection (Fig. 11,

p. 549, idib.).

In the present work, none of the phenomena observed by Belanger was present in the animals injected at 3 days of age but in those injected at birth, a loss of the band reaction with time was observed in the incisor. However, it might be added that the ground sections of these incisors were not of especially high quality and autographs obtained from these ought to be interpreted with some reservation. It is concluded that "Belanger phenomenon" may have occured in the newborn, but did not exist in our animals injected at 3 days of age.

Persisting band reactions of the 3 day old group

Incisor and molar teeth from 3 day old rats given Ca^{45} were found with persisting band reactions 8 and 9 days after injection (Fig. 13 and 17). Moreover, in the hand ground sections of upper incisors, the autographic band reaction still persisted 12 days after injection to 3 day old rats (Fig. 20 and 21). Also, it was present in about the same area 6 days (Fig. 19), as 12 days (Fig. 20) after injection. Also by ocular micrometer measurements, it was found that between 1 hour and three days after injection to 3 day old rats, there were no significant movements of the autographic band in the upper incisors. Should there have been "movement" or "ascent" of the band towards the dentino-enamel junction it is most likely that the changes would be faster in younger than in the older rats as indeed Belanger, '52, has stated. It appears, therefore, that the conclusion the band moves is open to some doubt.

SUMMARY AND CONCLUSIONS

Growing teeth of young rats were studied by the coated radio-autographic method after subcutaneous injection of a tracer dose of $Ca^{45}Cl_2$ or $H_3P^{32}O_4$. For the preparation of histological sections, the embedding media used were paraffin, celloidin, and plastic. Ground sections were also used. While the sections were examined mainly by the radio-autographic technique, histochemical tests were also used.

Radio-autographs obtained under the present experimental conditions with Ca^{45} and P^{32} revealed similar patterns. Notwithstanding the differences in the shape of the molar and incisor teeth, the radio-autographic images were similar in both.

Predentin never showed any significant uptake of radio-activity. The radio-elements were incorporated into the layer of dentin adjacent to the predentin. Since the dentin displayed a greater uptake of histological dyes than predentin, it is concluded from these staining reactions that at the predentin-dentin border there is an addition of a substance which enables the incorporation of mineral salts.

The deposited radio-element showed as a band of autographic reaction. The dentin salts formed after the level of radio-element in the circulation had fallen are no longer radio-active. Therefore, the band becomes separated from the predentin by a non radio-active area. With time the band was seen farther and farther away from the predentin and became

located well inside the dentin. However, its distance from the dentino-enamel junction was not significantly changed. Thus, in the molars and incisors of the 3 day old group, the band reaction was found present 12 days after injection of Ca^{45} at about the same distance from the junction as at earlier time intervals. The dentin of rats given two injections of Ca^{45} three days apart recorded them separately as two bands of autographic reaction.

The autographic reaction in the form of a band confirms that dentin forms by successive deposition of layers in an incremental pattern. The rate of deposition of mineral salts found in the present study compared well with the values obtained by other methods.

With time, there was a loss in the sharpness of delineation of the autographic band reaction. Interstitial calcification has been suggested as a plausible explanation for the observation. Some degree of rearrangement of the mineral salts under the influence of a mechanical factor such as pressure and tension exerted in the course of development or ionic exchange of calcium and phosphorus may also play a role.

The newborn and 3 day old groups presented somewhat different autographic reactions. The molars of the newborn group did not show a band reaction but instead, displayed a distribution of the autographic reaction over two gradients, the basal-occlusal increasing from the basal to the occlusal portions and the radial, increasing from the pulpal to the

dentino-enamel junction. The incisors of this group at first showed the radio-activity taken up in the dentin as a band. With time, however, this band was not observed. However, the interpretation of these autographs require some reservation. Factors mentioned in the previous paragraph may account for the low stability of the mineral salts of teeth of newborn rats.

The enamel at all times showed a more diffuse autographic reaction than dentin. The deposition of minerals occurred over two gradients. One the basal-incisal or basal occlusal gradient increased from the basal to the incisal or occlusal portions of the tooth. The other, a radial gradient, decreased from the dentino-enamel junction to the outer surface of the enamel.

It is concluded, therefore, that the mode of deposition of minerals in the dentin and enamel are different. In the dentin, it is incremental while it is not so in the enamel. This incremental manner of deposition of minerals is manifested in the autographs as a band which has been found to persist at least in 3 day old rats. Predentin does not have the ability to incorporate mineral salts and it is concluded that some change takes place at the predentin-dentin border so that minerals may be deposited there.

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