Histochemical studies on β-glucuronidase activity in developing teeth and bone of rat and Macaque monkey

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The enzyme β-glucuronidase (E.C. 3.2.1.31.) has been studied in developing teeth and bone of Sprague-Dawley rats and Macaque monkeys (*Macaca Irus*) by means of histochemistry using naphthol-AS-BI-β-D-glucuronide as substrate and hexazotized pararosanilin as coupling reagent. Since β-glucuronidase is sensitive to fixatives a freeze-sectioning technique has been used which made it possible to section highly mineralized tissues without previous fixation and decalcification. In the bone enzyme activity was found in osteoclasts and osteoblasts. β-glucuronidase activity was found in teeth of both species in odontoblasts, ameloblasts, stratum intermedium, inner and outer enamel epithelium. When enamel matrix formation was completed as indicated by the reduction of ameloblast length the enzyme activity of the ameloblasts increased.

Key-words: Glucuronidase; bone and bones; histochemistry; monkeys, rats; odontogenesis, tooth calcification

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Studies of enzyme activities in developing tissues may reveal unique structure-activity relationship which might yield information on the functional roles of enzymes and the specific functions of certain cell types. Acid hydrolases have been demonstrated in developing and resorbing cells of teeth and bone (*Quintarelli*, 1961; *Katchburian, Katchburian & Pearse*, 1967; *Vaes*, 1969; *Hammarström, Hanker & Toverud*, 1971) with rats and mice being used in most of the investigations. More recent studies on acid phosphatase activity during tooth and bone formation in rats and monkeys have revealed changes during different stages of tooth formation. Some species differences have also been noted (*Hammarström et al.*, 1971; *Hammarström & Hasselgren*, 1974). The studies herein reported have been carried out to describe the developmental changes and the structure-activity relationship of another lysosomal enzyme, β-glucuronidase. Comparisons have been made among the different cell types of developing teeth and bone.

β-glucuronidase (β - D - Glucuronide-glucuronohydrolase, E.C. 3.2.1.31) is widespread in the mammalian body (*Fishman*, 1955; *Biochemists Handbook*, 1961; *Barka & Anderson*, 1963) but its function is still largely unknown. In teeth there is agreement that it is found in odontoblasts in

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high activity (*Yoshioka et al.*, 1960; *Quintarelli*, 1961; *Yoshiki*, 1962; *Fullmer*, 1965; *Katchburian et al.*, 1967; *Badran, Leonard & Provenza*, 1970; *Engström, Linde & Persliden*, 1972).

However, the literature contain conflicting results regarding its presence in other types of cells. For instance there have been reports (*Yoshiki*, 1962; *Badran et al.*, 1970) that the enzyme is not present in ameloblasts as indicated by *Yoshioka et al.* (1960), *Quintarelli* (1961), *Fullmer* (1965) and *Katchburian et al.* (1967).

All these investigations were carried out on mice and rats but β-glucuronidase has also been demonstrated in dental cells at the onset of human tooth ontogeny (*Larmas & Larmas*, 1974), and in bovine dental pulp by means of biochemical methods (*Graziano & Amici*, 1961). No histochemical study has been made on the late stage in tooth development.

In bone β-glucuronidase has been found in osteoblasts (*Quintarelli* 1961, *Cabrini*, 1961; *Gubisch & Schlager*, 1961), osteocytes (*Gubisch & Schlager*, 1961) and osteoclasts (*Cabrini*, 1961; *Gubisch & Schlager*, 1961; *Warner*, 1964; *Vaes*, 1969).

Since β-glucuronidase has been reported to be very sensitive to fixatives (*Nachlas, Prinn & Seligman*, 1956; *Barka & Anderson*, 1963), we have used the freeze-sectioning technique of *Ullberg* (1954, 1958) by which it is possible to section highly mineralized tissues without previous fixation and demineralization.

MATERIAL AND METHODS

Healthy Macaque monkeys (*Macaca irus*) one day, one month and one year old and rats of the Sprague-Dawley strain one to sixteen days old were used in this investigation. The monkeys were killed by an overdose of mebumalsodium and then decapitated. The heads were then frozen in hexane cooled with solid CO_2. At this temperature (—75°C) the jaws were separated from the scull and split in the midline with a saw. Erupted parts of the teeth were removed with a diamond disc cooled with solid CO_2. Each half of the jaw was then embedded in an aqueous solution of carboxymethylcellulose on a large microtome stage and frozen by immersion in hexane cooled with solid CO_2. The rats were anaesthetized with ether and then killed by freezing in hexane cooled with solid CO_2. Immediately before the freezing the animals were embedded in an aqueous solution of carboxymethylcellulose applied on a large microtome stage.

After the freeze-embedding the specimens were ready for sectioning which was performed in a freeze box (—18°C). Sectioning of the monkey jaws and rat bodies followed the method described by *Ullberg* (1954, 1958). 10—20 μm thick sections were taken from both animals and Scotch tape No. 688 (Minnesota Mining and Manufacturing Co.) was attached to the exposed surface of the frozen specimen before the cutting so that the sections would come off adhering to the tape. Sections from rats were taken at the level of the molar teeth. After sectioning the specimens were freeze-dried at —18°C for two days. To protect the sections from condensation of moisture they were brought to room temperature in an air-tight box.

Sections attached to the tape were incubated for histochemical demonstration of β-glucuronidase activity using naphthol-AS-BI-β-D-glucuronidase as substrate and hexazotized pararosanilin as coupler according to the method described by *Hayashi, Nakajima & Fishman* (1964). (The naphthol-AS-BIglucuronide and the parasosanilin were obtained from the

Sigma Chemical Company, St. Louis, USA). The incubations were carried out at pH 5.2 at a temperature of 37°C for one hour. In order to obtain a semi-quantitative estimation of the enzyme activity the time required for the stain to appear in the various cells was noted. Control incubations without substrate were made simultaneously. After incubation the sections were washed in distilled water and gas bubbles were removed in vacuo before embedding in glycerin jelly. Some sections were mounted in Euparal (GBI Labs Ltd, Manchester, England) after dehydration in alcohol. In some sections the cell nuclei were stained with methyl green.

RESULTS

β-glucuronidase activity was found in both dental and bone cells in the monkey as well as in the rat. In both species the highest activities were found in osteoclasts, odontoblasts and postsecretory ameloblasts. The osteoclastic activity appeared to be much higher in the rat than in the monkey while the activities in odontoblasts and ameloblasts were rather similar in the rat and monkey (Table I).

Table I. *Time required for the stain to appear in the various cells*

*)Time in minutes	Monkey	Rat
10	Sebaceous glands	
15		Osteoclasts
20	Odontoblasts Postsecretory ameloblasts	
25	Osteoclasts	Odontoblasts Postsecretory ameloblasts
50	Inner and outer enamel epithelium Secretory ameloblasts	Inner and outer enamel epithelium Secretory ameloblasts
60	Osteoblasts Stratum intermedium Endothelial cells Subodontoblastic cellayer	Osteoblasts Stratum intermedium Endothelial cells Subodontoblastic cellayer

*)Estimated at 5-minute intervals and shown for positive recordings only

In both species the enzyme activities in dental cells showed similar changes during development (Figs. 1—4).

During the bud, cap and early bell stages of tooth formation almost no β-glucuronidase activity could be demonstrated in the odontogenic cells. Only a weak enzymatic staining could be seen in endothelial cells of the dental papilla and some activity could also be demonstrated in the outer and inner enamel epithelium. Just prior to enamel formation a distinct staining appeared in the distal part of the differentiating ameloblasts. In the secretory stage of the ameloblasts the enzyme activity was lower than in differentiating ameloblasts. When enamel matrix formation was completed as indicated by the reduction of ameloblast length β-glucuronidase activity increased (Figs. 1, 2 & 4). The stain for β-glucuronidase activity in the short postsecretory ameloblasts remained unchanged during the eruptive phase of the tooth in the reduced enamel epithelium. At all stages the enzyme activity was localized to the distal part of the ameloblasts.

During all stages of enamel formation a weak staining could be seen in the cells of stratum intermedium. β-glucuronidase activity was also found in the outer enamel epithelium and the activity increased in the part that covered the secretory ameloblasts.

Prior to dentin formation a weak positive staining could be demonstrated

Fig. 1. β-glucuronidase in the mesial cusp of the first upper molar of a 9-day-old rat. Incubation at pH 5.2. Note the enzyme activity in secretory ameloblasts (SA), postsecretory ameloblasts (PS), odontoblasts (O) and osteoclasts at the bone (B) surface.
Note also that the short postsecretory ameloblasts showed a higher enzyme activity than the tall secretory ameloblasts.
OE = oral epithelium; E = enamel; D = dentin; P = pulp (× 100)
Fig. 2. Detail of Fig. 1 showing that the β-glucuronidase activity was localized to the distal part of the ameloblasts at some distance from the enamel (E) surface. (OC) osteoclast REE = reduced enamel epithelium. (× 250).

in the differentiating odontoblasts. The enzyme activity then increased and was rather high during dentin formation. A weak enzyme activity was noted in the sub-odontoblastic cellayer. After the completion of the main dentin formation odontoblastic β-glucuronidase activity seemed to be reduced as indicated by the weak activity in the fully formed deciduous teeth of the monkey. No staining could be seen in cementoblasts.

An intense β-glucuronidase activity could be demonstrated in osteoclasts of both monkey and rat, but was especially high in the rat. A much weaker staining was found in the osteoblasts and no enzyme activity could be demonstrated in the osteocytes. The control incubations without substrate gave no staining.

DISCUSSION

The freeze-sectioning method devised by *Ullberg* (1954, 1958) was well suited for the histochemical demonstration of β-glucuronidase. The staining for enzyme activity was distinct and there was no sign of diffusion of the enzyme or the naphthol after the enzymatic hydrolysis. The enzyme activity could be assayed during all stages

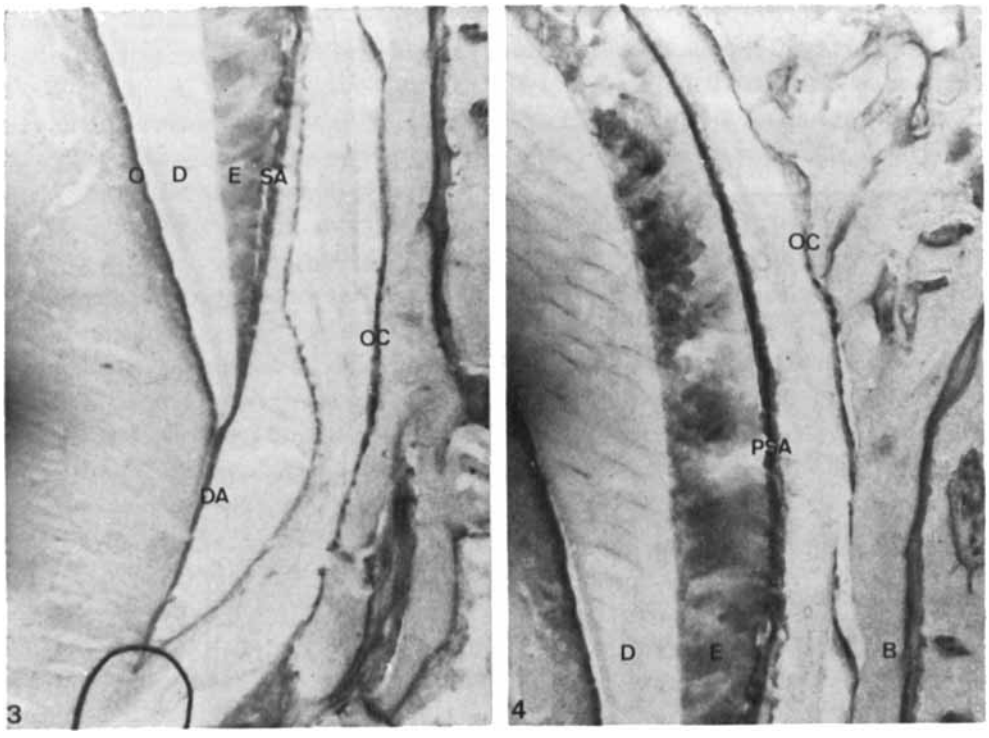

Fig. 3. β-glucuronidase activity in a developing incisor of a one year old monkey. There is a higher enzyme activity in the differentiating ameloblasts (DA) than in the secretory ameloblasts (SA). Note also the enzyme activity in odontoblasts (O) and osteoclasts (OC). D = dentin, E = enamel (× 50).
Fig. 4. β-glucuronidase activity in the same tooth as in Fig. 3. Note the high activity in the post-secretory ameloblasts (PSA) and osteoclasts (OC).
D = dentin, E = enamel, B = bone (× 50).

of tooth formation without the interference of fixation or demineralizing agents. To study the effect of formalin some sections were pretreated with 10% formalin at +4°C. After 30 minutes in formalin no β-glucuronidase activity was demonstrable in bone and teeth of the monkey or the rat. This finding is in agreement with *Nachlas et al.* (1956) who noted 77% reduction of β-glucuronidase activity after treatment with formalin for 30 minutes. *Badran et al.* (1970) who used glyoxal fixed frozen sections found so little β-glucuronidase activity in the secretory ameloblasts that its actual presence was considered doubtful. In the present investigation on nonfixed tissues a distinct enzyme activity was noted in ameloblasts. This is in agreement with previous studies by *Yoshioka et al.* (1960), *Quintarelli* (1961), *Fullmer* (1965) and *Katchburian et al.* (1967). In addition it was found that β-glucuronidase activity was increased in the short postsecretory ameloblasts as compared with the activity of secretory ameloblasts. The observation that the activity in the differentiating ameloblasts was higher than in the secretory ameloblasts is in agreement with studies on early stages of human tooth development *Larmas & Larmas* (1974). The finding of enzyme activity in stratum intermedium

confirms the results of *Yoshioka et al.* (1960), *Quintarelli* (1961), *Yoshiki* (1962), *Katchburian et al.* (1967) and *Badran et al.* (1970). The finding of β-glucuronidase activity in the outer and inner enamel epithelia is in agreement with studies by *Larmas & Larmas* (1974) on early stages of tooth formation in man. The previous reports on β-glucuronidase activity in stellate reticulum (*Quintarelli*, 1961, *Katchburian et al.*, 1967) could not be confirmed in this study. All investigators are in agreement on the odontoblastic β-glucuronidase activity (*Yoshioka et al.*, 1960, *Quintarelli*, 1961; *Yoshiki*, 1962, *Fullmer*, 1965; *Katchburian et al.*, 1967; *Badran et al.*, 1970; *Engström et al.*, 1972).

An interesting finding was that the enzyme activity in odontoblasts was highest during the most active metabolic stage of dentin formation and subsided when the main dentin formation was completed.

In bone the demonstration of β-glucuronidase in osteoclasts is in agreement with *Cabrini* (1961), *Gubisch & Schlager* (1961), Warner (1964) and *Vaes* (1969). The fact that the activity in osteoclasts of the rat was much higher than in the monkey may be due to a more rapid remodelling of the growing rat jaws. The considerably lower enzyme activity in osteoblasts has also been noted by previous investigators (*Quintarelli*, 1961, *Cabrini* 1961; *Gubisch & Schlager*, 1961).

We could not demonstrate any osteocytic β-glucuronidase activity but *Gubisch & Schlager* (1961) found a positive reaction in »intralacunary active osteocytes».

There is a group of enzymes with specifity for the β-glucoside linkage and they are commonly known as β-glucuronidases but there is no histochemical method described for their separation (*Pearse*, 1972).

β-glucuronidase is primarily a lysomal enzyme but there is also extralysosomal activity. According to *Fishman, Ide & Rufo* (1969) the histochemical technique for β-glucuronidase devised by *Hayashi et al.* (1964) which has been used in the present study mainly demonstrates the intralysosomal enzyme.

It may be of interest to compare the distribution of β-glucuronidase and acid phosphatase. In the monkey the enzymes are distributed in the same manner in the cells of bone and teeth. However, in the rat, there are distinct differences in the activities of the two acid hydrolases. Acid phosphatase activity was high during the secretory stage of the ameloblasts after which there was a marked decrease (*Hammarström et al.*, 1971). β-glucuronidase on the other hand was low during the secretory stage and increased during the post-secretory stage.

Thus far no function has been ascribed to the lysosomal enzymes in the formative cells of the hard tissues. In osteoclasts, however, β-glucuronidase has been associated with the degradation of components, in bone undergoing resorption (*Vaes*, 1969). In short postsecretory ameloblasts it may have a similar action in the resorption processes at the maturation of enamel.

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