

Inorganic pyrophosphatase in isolated enamel organ and odontoblasts from the rat incisor

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The inorganic pyrophosphatase (PP_iase) activity was determined by a colorimetric method in the odontoblasts and the parts of the enamel organ related to enamel matrix formation and enamel maturation. The effects on PP_i hydrolysis by EDTA, R 8231, urea and heat treatment were found to be almost identical to those reported for nonspecific alkaline phosphatase (APase) in the same tissues. The Mg²⁺ activation curve for PP_iase was also similar. Like those of APase, these characteristics of PP_iase activity were identical in the three locations studied. It is suggested that the close similarity in the properties of PP_iase and APase is due to activity of the same enzyme, a concept which is in agreement with recent biochemical and histochemical studies of calcification.

Key-words: Pyrophosphatase; odontoblasts; amelogenesis

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Inorganic pyrophosphate (PP_i) has been suggested to be an inhibitor of epitactic nucleation in tissues, and may be specifically broken down in mineralizing areas by a locally active pyrophosphatase (Fleisch & Neuman, 1961; Fleisch, 1964; Russell & Fleisch, 1970). Evidence that inorganic pyrophosphatase (PP_iase; EC 3.6.1) is identical with nonspecific alkaline phosphatase (APase; EC 3.1.3.1) has been reported in bone (Eaton & Moss, 1968), in homogenates of the stratum intermedium-ameloblastic cell layers of hamster molars (Wöltgens, Bonting & Bijvet, 1970) and in odontoblasts from the rat incisor (Linde & Magnusson, 1974; Granström & Linde, 1974 b).

Histochemical staining reactions indicating PP_iase activity have been recorded in the stratum intermedium and subodontoblastic cells of rat molar germs (Larsson, 1974 b). The deposits indicating PP_iase activity had the same ultrastructural localization as had those indicating APase activity (Larsson, 1974 a). The close involvement of APase in mineralization might thus partly be explained by its presumed identity with PP_iase and consequently its ability to degrade PP_i. Recently, the nonspecific APase (assayed as p-nitrophenylphosphatase, pNPPase) present in bone, odontoblasts, enamel organ and calcifying cartilage has been shown to have several properties in

common (*Granström & Linde, 1974 a*).

The aims of the present study were to record the activity of PP_iase in the enamel organ and the odontoblasts of the rat incisor and to compare some characteristics of PP_iase with those reported for APase in the same tissues. Since the parts of the enamel organ related to enamel matrix formation and enamel maturation may have different functions, these parts were studied separately.

MATERIAL AND METHODS

Fifty male rats (Sprague-Dawley strain, b.w. 200 g) were used. The rats were killed by decapitation. From the maxillary incisors specimens of the enamel organ representing the stage of enamel matrix formation (S-zone) and the stage of enamel maturation (M-zone) were dissected out as described by *Fredén (1973)*. From separate rats part of the odontoblastic layer was collected from the maxillary incisors using the technique of *Linde (1972)*.

The fresh tissue samples were transferred to motor-driven teflon-glass homogenizers containing 600 μ l 0.1 M glycine-NaOH buffer pH 8.7 and homogenized for 2 min. in ice. For the determination of PP_iase activity in the three different homogenates, 20 μ l homogenates were mixed with 75 μ l 5 mM tetrasodium pyrophosphate in 0.1 M glycine-NaOH buffer pH 8.7 and 25 μ l of the same buffer. To these 25 μ l, the different substances, described below, were added. After incubation for 90 min. in a water bath at 37°C the reaction was stopped by adding trichloroacetic acid, and the phosphate liberated was determined colorimetrically as described by *Wöltgens and Ahsmann (1970)*. As blanks, pyrophosphate was incubated without the addition

of homogenate. From the spectrophotometric readings the amount of phosphate in the homogenate, determined colorimetrically, was subtracted.

The pH-optimum of the PP_iase activity recorded was measured by studying the hydrolytic activity at various pH:s between pH 7.6 and 10.8.

The linearity of the assay system was investigated by using different amounts of homogenate and some incubations were followed for 3 hrs. Temperature-activity curves were made to determine at which temperature maximal hydrolysis was obtained. The heat lability was tested by incubating homogenates in a water bath at 56°C for varying times, and the remaining activity was measured. The influence of Mg²⁺ ion concentrations up to 10 mM, of urea up to 6 M, of Na₂-EDTA (Titriplex III; Merck) up to 1 mM and 0.1 mM R 8231 (generously donated by Dr. M. Borgers, Janssen Pharmaceutica, Belgium) on the PP_iase activity was also studied. To relate the PP_iase activity at optimal assay conditions to the amount of cells present, duplicate samples of the homogenates were assayed for DNA content according to the method of *Kissane and Robins (1958)*.

RESULTS

All experiments were made in triplicates unless otherwise indicated.

pH optimum. The maximal PP_iase activity was recorded at pH 8.7 (Fig. 1) in the enamel organ S-zone (matrix formation zone), in the enamel organ M-zone (maturation zone) and in the odontoblasts. The total activity of PP_iase in the different homogenates at this pH was related to the amount of DNA and the results are presented in Table I.

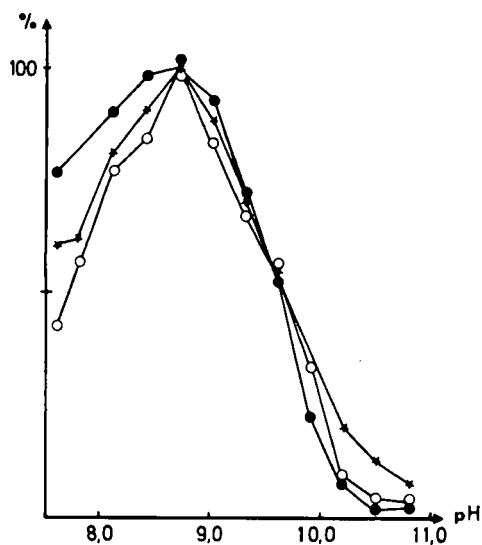


Fig. 1. Effect of different pH on PP_iase activity in odontoblasts (●), enamel organ S-zone (★) and enamel organ M-zone (○). The maximal activity (100%), corresponding to the values given in Table 1, was obtained at pH 8.7 for all the three tissues.

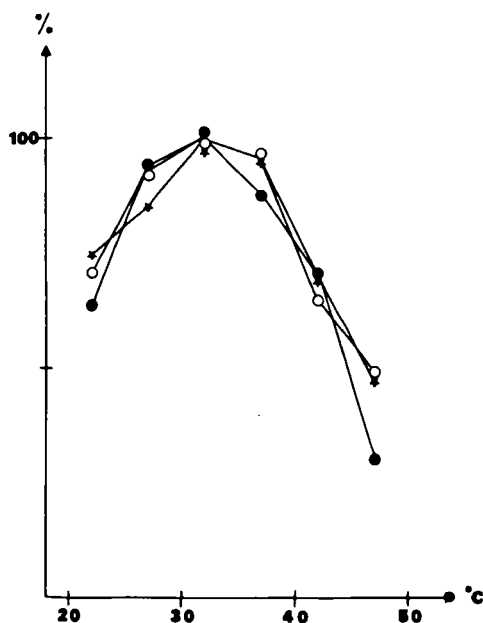


Fig. 2. PP_iase activity at different temperatures. The optimal activity (100%) for all the three tissues was obtained at 32°C.

Homogenate concentration and linearity with time. The assay system was found to be virtually linear with increasing homogenate amounts up to 50 μ l. A slight decrease in activity was found with time and after 2 hrs. no activity was left.

Temperature-activity curves and heat lability. Closely similar temperature-activity curves were obtained when homogenates from the three separate locations were tested, and a maximum around 32°C was recorded (Fig. 2). At 56°C the PP_iase was inactivated at an identical rate in each site and after 15 min. only 10% of the enzyme activity remained (Fig. 3).

Mg²⁺-activation. The Mg²⁺-activation curves for the PP_iase activities in the different homogenates at a substrate concentration of 3 mM were found to be similar. Low concentrations of Mg²⁺, near 1.25 mM, activated the PP_i hydrolysis while higher concentrations decreased

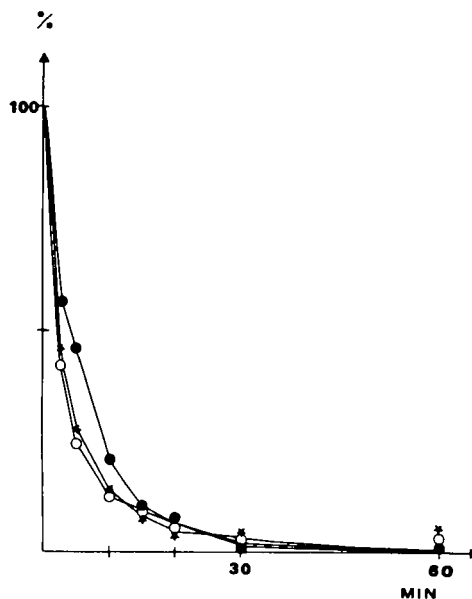


Fig. 3. Heat lability of PP_iase activity at 56°C. 90% of the original enzyme activity had been lost after 15 mins. of incubation.

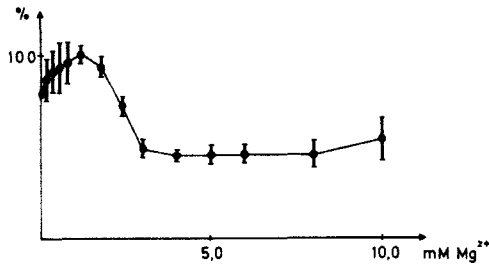


Fig. 4. Effect of different Mg^{2+} ion concentrations in the incubation media on the odontoblastic PP_i ase activity. Mean values \pm SD ($n = 5$) as a percentage of the activity recorded at the optimal Mg^{2+} concentration.

the enzyme activity considerably. This is shown for the odontoblasts in Fig. 4.

Effects of urea, EDTA and R 8231. The PP_i hydrolysis in the three separate homogenates were almost identically affected by the inhibitory compounds tested. At low concentrations urea had virtually no effect on the PP_i ase activity. Concentrations above 1 M, on the other hand, rapidly inactivated the enzyme. EDTA caused a strong inhibition and at a concentration of 0.06 mM the enzyme activity was decreased to about 10% of the control value. The addition of 0.1 mM R 8231 (Table I) to the incubation medium strongly inhibited the PP_i ase activity. Thus, in the R 8231 containing incubation medium only about 5% of the normal activity could be recorded.

Table I. Total PP_i ase activity (μ mol. liberated phosphate per min and mg DNA) and the effect of the presence of 0.1 mM R 8231 on PP_i ase activity in the tissues assayed

	PP_i ase		R 8231	
	\bar{x}	S.D.	\bar{x}	S.D.
Odontoblasts	11.5	0.3	0.7	0.1
Enamel organ S-zone	4.4	0.1	0.3	0.0
» » M-zone	4.2	0.2	0.3	0.0

$n = 5$

DISCUSSION

Considerable pyrophosphatase activities were found in the odontoblast samples as well as in the two different parts of the enamel organ. The odontoblast samples are contaminated by pulpal connective tissue only to a small degree (Linde, 1972, Hansson, Linde & Nygren, 1974). Thus, it seems likely to attribute a PP_i -hydrolyzing capacity to the odontoblasts. This is in contrast to the histochemical studies by Larsson (1974 b), which indicated that PP_i ase activity was to be found in the subodontoblastic zone only.

The present results confirm the biochemically demonstrated PP_i hydrolysis in the stratum intermedium-ameloblast area of the enamel organ (Wöltgens *et al.*, 1970) and in the odontoblasts (Linde & Magnusson, 1974; Granström & Linde, 1974 b). The values obtained by the micro dissection technique used here revealed no significant difference in PP_i ase activity associated with enamel matrix formation and enamel maturation. No conclusion on the cellular localization in the enamel organ can be drawn since the results refer to the sum of enzyme activity in the ameloblasts, the adjacent parts of the enamel organ and minor amounts of surrounding connective tissue (Fredén, 1973).

The PP_i ase present in several calcification areas is believed to be identical with nonspecific APase (EC 3.1.3.1; Eaton & Moss, 1968; Wöltgens *et al.*, 1970). The optimal pH for PP_i ase from the odontoblasts and the enamel organ was found to be 8.7. In the same tissues the pH optimum demonstrated for APase (assayed as p-NPPase) was near pH 10 (Granström & Linde, 1972; Fredén, Granström & Linde, 1973). However, this might be explained by the dissimilar binding pro-

properties for the two structurally different substrates rather than by degradation by separate enzymes. Thus, the pH optimum for calf intestinal APase varied when different substrates were used (Fernley & Walker, 1967) as did the Mg^{2+} -activation curve. The enzymic PP_i hydrolysis recorded in this study was inhibited by EDTA, urea and heat in the same way as p-NPPase in the same tissues (Granström & Linde, 1972; Fredén, Granström & Linde, 1973). Furthermore, R 8231 which has been shown to be a specific and very potent inhibitor of APase (van Belle, 1972; Borgers, 1973) drastically decreased the PP_i ase recorded in the present study. The magnitude of the inhibition was identical to that of p-NPPase in odontoblasts (Linde & Magnusson, 1974). The Mg^{2+} -activation curve obtained for PP_i ase is also similar to that for p-NPPase. Wöltgens *et al.* (1970) found that Mg^{2+} ions activated the PP_i ase activity optimally at an Mg^{2+}/PP_i ratio of 1:1. The current study gave another figure but this might be explained by dissimilarities in the assay conditions, e.g. the different pH:s used (8.7 and 9.5, respectively).

The present finding that the PP_i ase activities in the odontoblast layer and in the two zones of the enamel organ from the rat incisor have several properties in common indicates that this activity is due to the same enzymes. The properties demonstrated agree well with findings for odontoblast PP_i ase using a radiochemical assay method (Granström & Linde, 1974 b). Similarly, we have earlier demonstrated that the nonspecific APase is the same in odontoblasts, the two enamel organ zones and calcifying cartilage (Granström & Linde, 1974 a). Furthermore, our present findings indicate from the similar characteristics found, that the PP_i ase and APase activities present in the odontoblast

layer and in the enamel organ are due to one single enzyme.

Thus, nonspecific APase with a PP_i ase hydrolyzing capacity may be found in connection with formation of all mammalian mineralized tissues, calcifying cartilage (Ali, Sajdera & Anderson, 1970), bone (Eaton & Moss, 1967) enamel (Wöltgens *et al.*, 1970) and dentine. Inorganic pyrophosphate is released in several metabolic pathways and it has been suggested that it may prevent soft tissue calcification (Fleisch, 1964; Fleisch & Russell, 1970). A breakdown of PP_i by a locally active PP_i ase would thus be a necessity for mineralization to proceed.

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