# ATP-ase activity in the odontoblastic layer of rat incisor

## Determination with a radiochemical and a colorimetric method

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The ATP-splitting enzyme activity in odontoblasts isolated from rat incisors has been studied by means of a radiochemical and a colorimetric micromethod. The results with the two methods were virtually identical. The reaction was linear with time for at least 45 min. The pH optimum was found to be 9.8 independently of the ATP concentration. Maximal substrate saturation occurred at a total ATP concentration of 3 mM. $Ca^{2+}$ and $Mg^{2+}$ ions activated ATP degradation. $F^-$ ions did not affect the activity at low concentrations, whereas higher concentrations were inhibitory. $Na^+$ and $K^+$ ions had no influence on ATP splitting enzyme activity, while $PO_4^{3-}$ ions were slightly inhibitory. Urea inhibited the enzyme activity at concentrations above 1.5 M, while EDTA and EGTA were strong inhibitors at very low concentrations. When incubating in the presence of low concentrations of specific inhibitors for nonspecific alkaline phosphatase, levamisole and R 8231, about 20% ATP degrading enzyme activity remained. In conclusion it is suggested that there are at least two ATP degrading phosphatases active at alkaline pH.

*Key-words:* Biochemistry; mineralization; dentinogenesis

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Several histochemical studies have demonstrated ATP-splitting enzyme activity in hard-tissue-forming cells. The recorded phosphatase activity exhibited essentially the same microscopic localization as nonspecific alkaline phosphatase (APase, EC 3.1.3.1). (*Burstone*,1960; *Magnusson, Heyden & Arwill* 1974). Inorganic pyrophosphatase (PPiase, EC 3.6.1.1) activity in hamster molars (*Wöltgens, Bonting & Bijvoet*, 1970, 1971), in the odontoblastic layer of rat incisor (*Granström & Linde*, 1975b) and in rat incisor odontoblasts and ameloblasts (*Fredén, Linde & Magnusson*, 1975) has been studied and all these authors agree that one single enzyme is responsible for both APase and PPiase activity in biological calcification. *Linde & Magnusson* (1975) using the specific APase inhibitors levamisole and R 8231 found strong evidence for the existence of a $Ca^{2+}$-ATPase in cells involved in hard tissue formation.

We thus decided to make a comparative study of ATP degrading enzyme activity and APase in the odontoblastic layer of rat incisors

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using both a radiochemical (*Cartier & Thuillier*, 1971) and a colorimetric (*Wöltgens & Ahsman*, 1970) technique, both methods originally designed for PPiase assay, using ATP as a substrate.

## MATERIALS AND METHODS

Odontoblasts were dissected out from maxillary incisors of male Sprague-Dawley strain rats (b.w. 200 g) as described by *Linde* (1972).

The *radiochemical assay* was modified after *Cartier & Thuillier* (1971). The odontoblasts were homogenized in a glass homogenizer containing 1 ml distilled water for 5 min. in the cold. The enzyme reaction was performed in microtubes containing 1.1 ml 0.1 M glycine-NaOH buffer with a total $Na_2$-ATP concentration of 3 mM. To this, 10 µl of 0.69 µM γ-[$^{32}$P]ATP (ammonium salt, 14.5 Ci/mmol; The Radiochemical Centre, Amersham, England) was added. The pH of the mixture was 9.8. When optimal pH was studied, the pH was varied between 7.4 and 11.8. The reaction was started by the addition of 20 µl cell homogenate and incubated at 37 °C for 30 min. Blanks consisted of the same reaction mixture with the homogenate exchanged for 20 µl distilled water. The reaction was stopped by rapid cooling in ice and adding 200 µl 40 mM ammoniummolybdate in 1.25 M $H_2SO_4$. By the subsequent addition of 100 µl 100 mM triethylamine, pH 4.0, a yellow precipitate containing the liberated orthophosphate was formed. The tubes were centrifuqed for 10 min at 1000 x g and the precipitates were washed twice. The pellets were dissolved in 100 µl acetone, transferred to scintillation vials and dissolved in 10 ml Instagel® (Packard) for radioactive counting. This was done with an efficieny of about 87%.

For the *colorimetric determination* a method modified after *Wöltgens & Ahsman* (1970) was used. The odontoblasts were transferred to a teflon-glass homogenizer and homogenized in 500 µl 0.1 M glycine-NaOH buffer pH 9.8. The enzyme reaction was performed in microtubes containing 105 µl 0.1 M glycine-NaOH buffer. The pH of the reaction mixture was 9.8 except when optimal pH was studied. In this case, pH was varied between 7.4 and 11.8. The total concentration of $Na_2$-ATP was 3 mM. The reaction mixture was preincubated at 37 °C for 5 min., and the enzymatic reaction was started by adding 20 µl of the homogenate. The incubation was performed at 37 °C for 30 min. in a water bath. In the blanks, the homogenate was exchanged for 20 µl 0.1 M glycine-NaOH buffer. The reaction was stopped by cooling in ice and adding 200 µl 10% trichloroacetic acid containing 5 mM $CuSO_4$, followed by the addition of 300 µl 1% ammoniummolybdate in 0.575 M $H_2SO_4$ containing 80 mg/ml $FeSO_4$. The liberated orthophosphate was measured after 30 min. at 700 nm in a standard spectrophotometer. The optical absorption of 20 µl odontoblast homogenate was subtracted from the measurements.

The enzyme activity was related to cell amount in the homogenate assayed as DNA by the method of *Kissane & Robins* (1958). The pH-optimum was determined as described above. The effect on enzyme activity of increasing substrate concentrations up to 100 mM was studied. Linearity of the assay system with increasing amounts of homogenate and with time (up to 5 hrs) was investigated, and optimal incubation temperature was studied. Odontoblast homogenates were incubated in a water bath at 56 °C for time intervals up to 2 h to study the heat stability of the ATP splitting enzyme activity, and remaining activity was measured. The influence of $Mg^{2+}$, $Ca^{2+}$, $Na^+$, $K^+$, $F^-$, and $PO_4^{3-}$ ions on the enzyme activity was studied. When $Na^+$ ions were studied, the glycine-NaOH buffer was exchanged for a 0.1 M Tris-HCl buffer pH 9.8. Different concentrations of urea, EDTA and EGTA were also tested.

## RESULTS

In this study, all tests were performed in at least duplicate with both the radioactive and colorimetric assay method. The results from these were virtually identical.

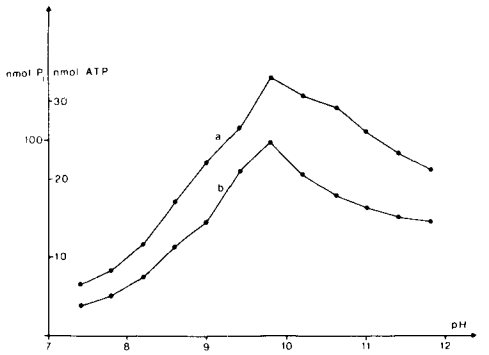


Fig. 1. Influence of pH on ATP-splitting enzyme activity. The enzyme was assayed in 0.1 M glycine-NaOH buffer. a) colorimetric method, b) radiochemical method.

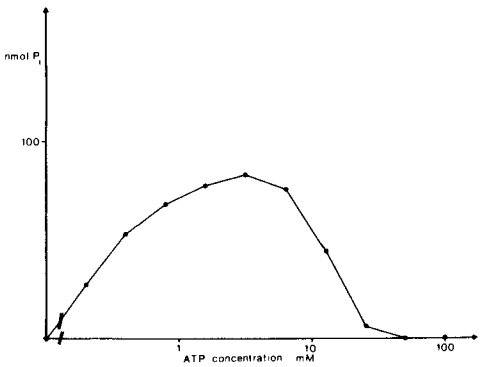


Fig. 2. Influence of different ATP concentrations on enzyme activity. Optimal activity was obtained with a 3 mM substrate concentration. Note that the abscissa is logarithmic. Assay made by the colorimetric method.

*pH optimum*. The pH optimum was found to be 9.8 (Fig. 1). All the following incubations were thus made at pH 9.8 using glycine-NaOH buffer. The total activity of the ATP-splitting enzyme activity measured per DNA was with the radiochemical method 1.83 ± 0.61 (n = 12) μmol ATP/min and mg DNA and with the colorimetric method without the addition of inhibitor 7.16 ± 3.3 (n = 9) μmol $P_i$/min and mg DNA.

*Effect of ATP concentration*. Low concentrations of ATP revealed a normal substrate saturation (Fig. 2). At higher concentrations of ATP (6–100 mM) the enzyme reaction was

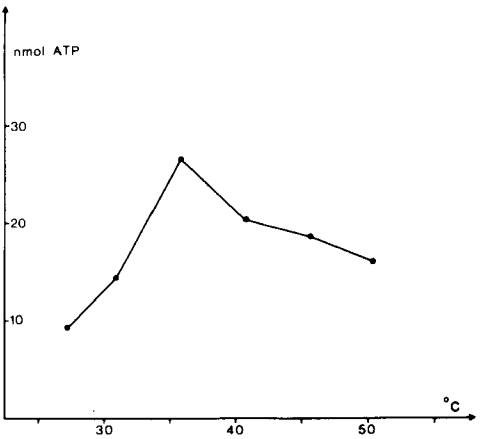


Fig. 3. ATP-degrading enzyme activity at different incubation temperature. The optimal activity was obtained at 37 °C. Assay made by the radiochemical method.

inhibited. Maximal ATP hydrolysis occurred at 3 mM ATP which was used in the following studies.

*Linearity with time*. By following the enzyme reaction for 5 hrs it was revealed that the reaction was linear with time for 45 min., after which a decrease in activity was noted. (The incubation time of 30 min. used routinely is thus well within the linear part of the curve.)

*Linearity with homogenate amounts added*. The liberation of orthophosphate from ATP showed a linear increase with increasing odontoblast homogenate amounts up to 50 μl. (The 20 μl routinely used is thus well within the linear part of the curve.)

*Effect of temperature on enzyme activity*. ATP-splitting enzyme activity was determined at different incubation temperatures in the range 27–52 °C. Optimal condition was found at 37 °C (Fig. 3). All incubations were thus performed at 37 °C. When the homogenates were incubated at 56 °C for different times, a drastic reduction of the ATP-splitting activity was noticed, as recorded by a subsequent 30 min. incubation with substrate at 37 °C. After a 10 min. incubation at 56 °C only 10% of the activity remained and after 20 min. the enzyme activity was almost completely lost.

*Ion effects*. $Ca^{2+}$ or $Mg^{2+}$ ions alone activated

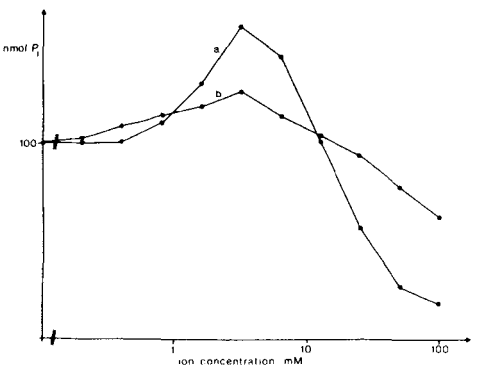


Fig. 4. Effect of different concentrations of divalent cations, measured by the colorimetric method. Note that the abscissa is logarithmic. The maximal activation with $Mg^{2+}$ (a) and $Ca^{2+}$ (b) ions was obtained at 3 mM concentrations, the activation by $Mg^{2+}$ ions being stronger than that by $Ca^{2+}$ ions.

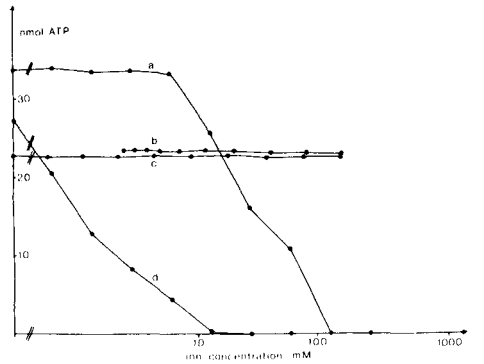


Fig. 5. Effect of different concentrations of $F^-$ (a), $Na^+$ (b), $K^+$ (c) and $PO_4^{3-}$ (d) ions on ATP-degrading enzyme activity measured by the radiochemical method. $F^-$ ions did not affect the ATP-splitting enzyme activity at concentrations below 8 mM. $Na^+$ and $K^+$ ions had no effect, whereas $PO_4^{3-}$-ions were inhibitory at all concentrations. Note that the abscissa is logarithmic. The effect of $Na^+$ ions (b) was investigated using a 0.1 M tris-HCl buffer. Due to the $Na^+$ content of the substrate, this curve starts at a 6 mM ion concentration.

the ATP-degradation. Maximal activity at an ATP concentration of 3 mM occurred at a divalent cation concentration of 3 mM (Fig. 4). If the ATP concentration was 2 mM or 5 mM it was found that maximal enzyme activity also in these cases occurred at a substrate-divalent cation ratio of 1:1. $Na^+$ or $K^+$ had no effect on the enzyme activity (Fig. 5). $F^-$ ions did not affect the enzyme activity at concentrations below 8 mM. At higher concentrations an inhibition was noticed, and at 250 mM the ATP degradation was totally inhibited (Fig. 5). $PO_4^{3-}$ ions up to 250 mM were also found to have an inhibitor effect (Fig. 5).

*Effects of inhibitors*. Urea had little effect on ATP-splitting enzyme activity at concentrations below 1.5 M, but at a 6 M concentration a strong inactivation was found (Fig. 6). EDTA and the specific $Ca^{2+}$-chelator EGTA were shown to be very potent inhibitors (Fig. 6). Already at a concentration of 0.1 mM the enzyme activity was reduced by 70% compared to the normal value. Varying amounts of the APase inhibitors levamisole and R 8231 were added to the incubation medium together with 3 mM $Ca^{2+}$ or $Mg^{2+}$. The inhibition was not complete. Under the present assay conditions about 20% of the ATP degrading activity remained (Fig. 7). It was also found that at levamisole or R 8231 concentrations above 2.5 mM the colorimetric reaction was affected *per se* in that a precipitate was formed when adding the reagent. A precipitate was formed at all concentrations when adding levamisole and R 8231 using the radiochemical method.

## DISCUSSION

The present investigation confirms earlier histochemical findings of an ATP-splitting enzyme activity in the odontoblast layer of rat incisors. Of the two assay techniques used, the sensitivity of the radiochemical method was not found to be superior to that of the colorimetric method. However, by increasing the specific radioactivity of ATP in the incubation medium it may be possible to increase the sensitivity of the radiochemical method.

We have earlier demonstrated a close similarity between the APases present in odontoblasts, mineralizing cartilage and the two zones of the rat incisor enamel organ (*Granström & Linde*, 1975a). *Fredén, Linde & Magnusson* (1975) have shown, by using a colorimetric method, that PPiase activities in the odontoblast layer and in the two zones of the enamel organ from the rat incisor have several proper-

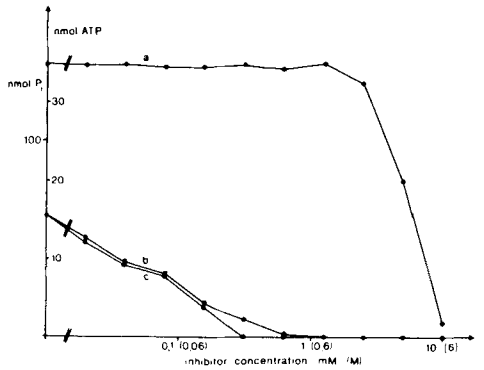


Fig. 6. Influence of urea (a) concentration up to 6 M on ATP-degrading enzyme activity assayed by the radiochemical method, and influence of EGTA (b) and EDTA (c) up to 10 mM on ATP-splitting enzyme activity measured by the colorimetric method. Urea had little effect on the activity until a 1.5 M concentrations. Note that the abscissa is logarithmic.

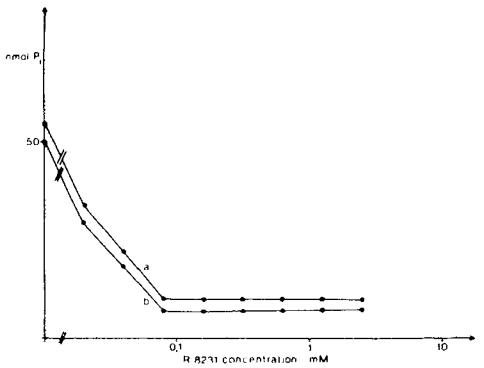


Fig. 7. Influence of R 8231 in the presence of 3 mM $Mg^{2+}$ (a) and 3 mM $Ca^{2+}$ (b) ions on ATP-degrading enzyme activity. The activity was strongly inhibited, but above a concentration of 0.08 mM R 8231 20% enzyme activity remained. Note that the abscissa is logarithmic. Assayed by the colorimetric method only.

ties in common. The properties demonstrated agree well with findings for odontoblast $PP_i$-ase using a radiochemical method (*Granström & Linde*, 1975b). It was concluded that PPiase and APase activities are due to one single enzyme in the calcification loci studied.

The present study has shown several similarities between ATP-splitting enzyme activity in odontoblasts, on one hand, and APase in different mineralizing tissues, on the other, in their response to different parameters. However, no activation by $Ca^{2+}$ ion of APase or PPiase was found in our earlier studies. Furthermore, the ATP degrading activity was not totally inhibited by levamisole and R 8231 as were APase and PPiase. These findings agree with the suggestion of *Linde & Magnusson* (1975) that more than one phosphatase is active at alkaline pH in biological mineralization. One is a nonspecific alkaline phosphatase, which is active against p-nitro-phenyl phosphate, β-glycerophosphate, $PP_i$ and ATP among other substrates. The other one is a specific ATP-ase, which is not inhibited by levamisole and R 8231. Further studies of this $Ca^{2+}$-ATPase are in progress.

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