



Effect of Glutaraldehyde and Decalcifying Agents on Acid Phosphomonoester Hydrolase Activity in the Enamel Organ of the Rat Incisor: A Biochemical Study Comparing Enamel Organ with Liver¹

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Received for publication November 16, 1979 and in revised form February 1, 1980 (MS 79-255)

Enamel organs were dissected from the labial surface of unfixed and glutaraldehyde-fixed rat incisors and assayed biochemically at pH 5.0 for acid phosphomonoester hydrolase activity using cytidine 5'-monophosphate (5'-CMP), β -glycerophosphate (β GP), phosphorylcholine (PC), and phosphoserine (PS) as substrates. Whole homogenates from unfixed enamel organs showed substantial enzyme activity toward 5'-CMP and β GP, but $1/4$ and $1/8$ less activity toward PC and PS, respectively. Perfusion fixation with 2% glutaraldehyde resulted in a net loss of 80% of the enzyme activity toward each substrate. Lineweaver-Burk plots revealed that the fixative depressed the rate of hydrolysis of substrate (decrease in V_{max}) and it also lowered the affinity of enzymes for substrate (increase in K_m). Hence, fixed tissue generally

required two or three times as much substrate to saturate the enzymes, but less substrate was hydrolyzed, as compared to unfixed tissue. Decalcification of fixed incisors with either formic citric acid, ethylenediaminetetraacetic acid (EDTA), or ethyleneglycoltetraacetic acid (EGTA) did not further alter enzyme activity in the enamel organ as determined by Lineweaver-Burk plots. However, EDTA and EGTA were found to increase the susceptibility of fixed enzymes to inhibition by lead ions. This chelator-enhanced lead inhibition was greatest following decalcification with EGTA and using PC as substrate. Similar results were obtained for liver. **KEY WORDS:** Enamel organ; Liver; Acid phosphatase; Spectrophotometry; Substrate specificity; Aldehydes; EDTA; Enzyme inhibitors; Rat.

Introduction

Acid phosphatase (E.C. 3.1.3.2) constitutes one of several phosphatase activities that have been identified within cells of the enamel organ (3, 18, 19, 22, 32, 33, 35, 52-55). The ameloblasts appear to account for most of the acid phosphatase activity in the enamel organ, especially during the stage when the cell forms and secretes the enamel matrix proteins (3, 22, 33, 35, 52-54).

Despite a seemingly extensive literature, there is presently little detailed biochemical information about the hydrolysis of phosphorylated molecules at acid pH within the enamel organ (3, 55). Hence, it is difficult to plan an ultrastructural study for the cytochemical localization of acid-dependent phosphatases,

since it is not clear if the amount or type of substrate used in conventional cytochemical media (14) is appropriate for use with the enamel organ (3, 18, 22, 53) or if enzymes in the enamel organ respond differently to aldehyde fixation than elsewhere (21, 23). Similarly, it remains unclear whether the decalcification of hard tissue with a chelating agent such as ethylenediaminetetraacetic acid (EDTA) alters the activity of acid-dependent phosphatases in fixed tissue (6, 11, 34, 38, 46, 61).

The purpose of this study was to assess "acid phosphomonoester hydrolase" (APH) activity in homogenates from the enamel organ using four structurally different molecules that contain a single phosphate-ester bond, and to identify any gross changes in the APH activity toward each of these molecules that may occur during the processing of enamel organ specimens for electron microscopic cytochemistry. The cytochemical technique under investigation is one which is used frequently to localize phosphatases in various soft (see refs. 14, 20, 23, 42, 57) and hard tissues (see refs. 33, 40, 52-54). It is based on capture of the enzyme-derived orthophosphate by lead ions (12, 29).

¹This study was supported by a grant from the Conseil de la Recherche en Santé du Québec and by Grant DE 05424-01 from the National Institute of Dental Research, National Institutes of Health, U.S.A.

Materials and Methods

A total of 134 male Sprague-Dawley rats weighing 150 g were used in this study.

Specimen Preparation

Unfixed tissue. Rats were anesthetized with ether and killed by decapitation. The maxillary and mandibular jaws were dissected from the head, cleaned of adhering soft tissues, and placed in a solution of 0.1 M sodium cacodylate buffer and 0.15 M sucrose, pH 7.4 (washing buffer), kept at 4°C. The left median lobe of the liver was also removed and placed in the washing buffer. Enzyme assays for these unfixed specimens were generally in progress within 2 hr after decapitating the rats. In the course of this study it was found that the amount of enzyme activity in unfixed tissue treated with washing buffer was comparable to the amount detected in unfixed tissue treated with 0.15 M sucrose alone, and unfixed tissue could remain in the washing buffer at 4°C for up to 1 week without loss of enzyme activity (data not shown). Furthermore, perfusion of the rat with normal saline prior to decapitation was not found to alter markedly the amount of enzyme activity detectable in unfixed tissues (data not shown) (8, 58).

Fixed tissue. Rats were anesthetized by intraperitoneal injection of chloral hydrate (0.4 mg/g body weight) and perfused via the ascending aorta with a solution of 2% glutaraldehyde (v/v) in 0.1 M sodium cacodylate buffer and 5 mM calcium chloride, pH 7.3. The perfusion was done at room temperature for exactly 10 min at a drip rate of 25 ml fixative/min. The jaws and left median lobe of the liver were removed and immersed in fresh fixative for 1 hr at 4°C. After fixation, all specimens were rinsed for 5 hr at 4°C in washing buffer. Specimens that were to receive no further treatments were kept in the washing buffer for 24 hr prior to enzyme assay. Otherwise, the jaw and liver specimens were placed in gauze bags and suspended in one of the following solutions kept at 4°C with continuous agitation; 1) 4.13% disodium EDTA (w/v) adjusted to pH 7.2 with sodium

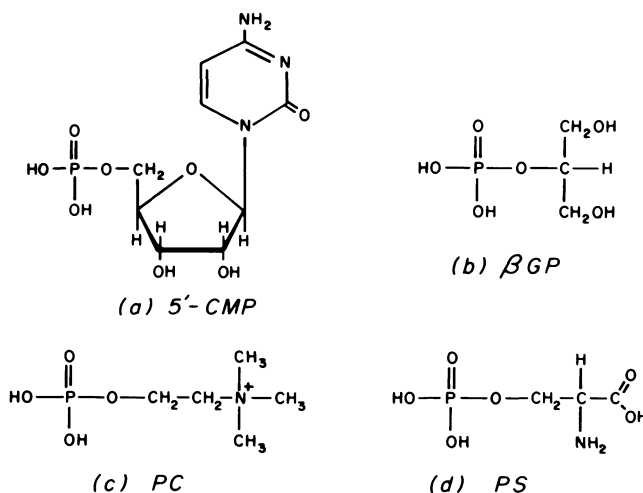


Figure 1. Structural formulas for the substrates used in this study.

hydroxide pellets (4.4 g/liter) (59), 2) 4.13% ethyleneglycoltetraacetic acid (EGTA) (w/v) (ethylene bis-oxetylenenitrilo-tetraacetic acid) solubilized and adjusted to pH 7.2 with sodium hydroxide pellets (9.2 g/liter), 3) 1% formic acid (v/v) and 10% sodium citrate (w/v) adjusted to pH 5.0 with concentrated hydrochloric acid (17), and 4) washing buffer, at pH 7.2, which served as the control to the decalcifying agents. Specimens were immersed in these solutions from 4 to 21 days. All solutions were changed every 5th day. After decalcification, the specimens were rinsed for 2 days at 4°C in washing buffer prior to enzyme assay.

Isolation of enamel organs (modified from Josephsen (31)). All dissections were done under a stereoscopic microscope at 4°C in a petri dish filled with washing buffer. The bony plate covering the labial side of each incisor was removed piecemeal, either by fracturing

Figure 2. Comparison of results expressing APH activity in enamel organ and liver (unfixed) relative to the wet weight of the tissue (left side) or to the protein content of the homogenate (right side); determined at 10 mM concentration for all substrates (5'-CMP, β GP, PC, and PS). The amount of enzyme activity present in fixed tissue is also compared at the left side of the figure (fixed). The height of each column represents the mean \pm SD for three or more separate experiments. In all experiments the amount of Pi released was proportional with time over the assay period.

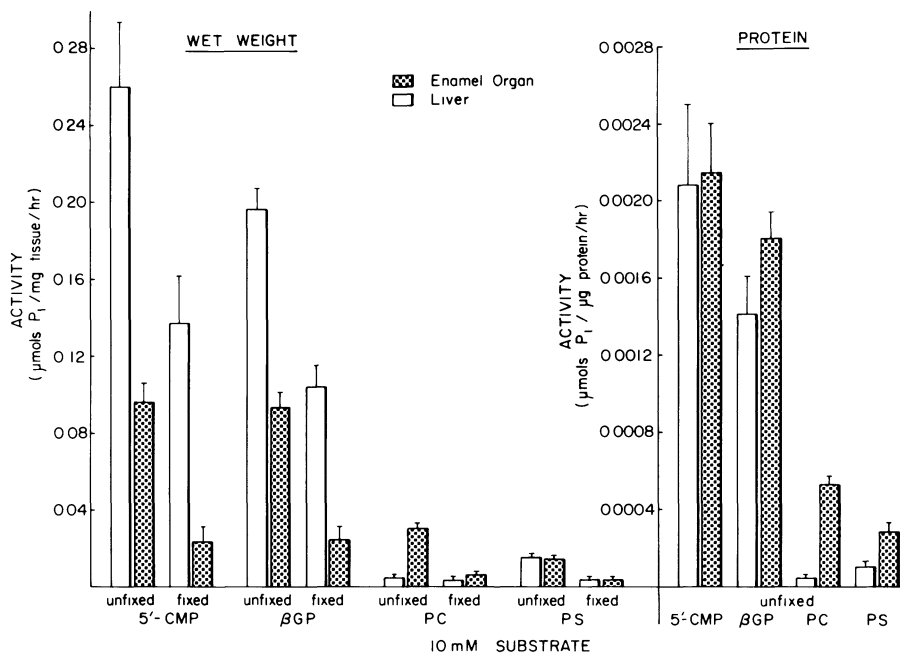
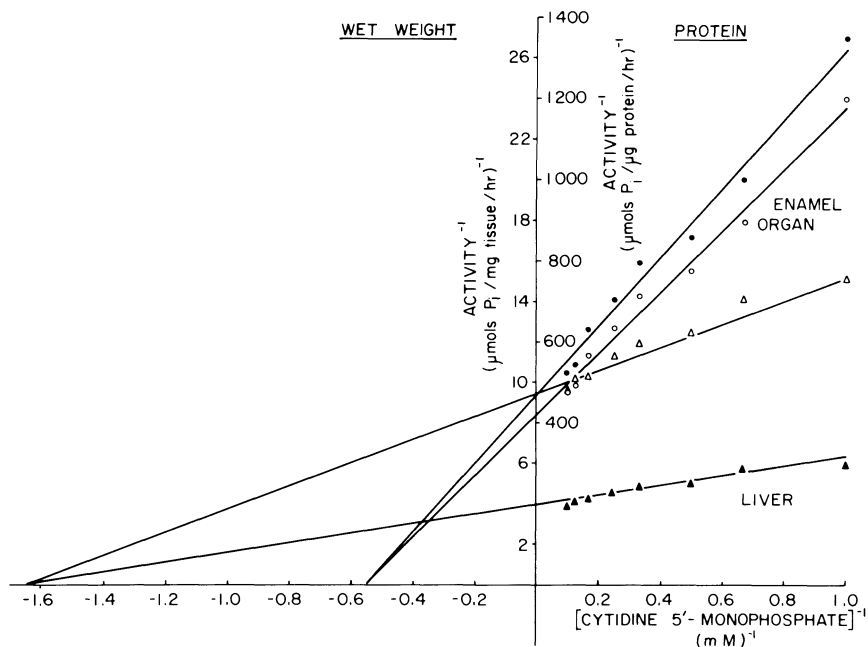


Figure 3. Effect of expressing enzyme activity in enamel organ (circles) and liver (triangles) relative to wet weight (closed symbols) or protein content (open symbols) on the determination of K_M (X-intercept) by double reciprocal plots of enzyme activity versus substrate concentration (5'-CMP); unfixed tissue. Note that in a given tissue the same value for K_M is derived by either method.



it with dental hand instruments (calcified or partially decalcified specimens) or by cutting it into sagittal strips with a #11 scalpel blade (decalcified specimens), which were then lifted from the jaw by blunt forceps. Any of the large venous channels underneath the bony plate that were not displaced during this initial dissection were teased from the labial connective tissue with fine forceps. All of the dental tissues forming the apical 1 mm of the incisor, and those forming the gingival margin, were discarded. The remainder of the enamel organ, comprising the secretory and maturation zones of amelogenesis (60) and adhering labial connective tissue layer, was then separated from the enamel surface, blotted gently on #50 Whatman filter paper, and placed in a small plastic beaker containing a 0.25 M sucrose solution at 4°C. In the case of unfixed specimens, the enamel organ was first grasped with blunt forceps near its midpoint along the sagittal length

of the tooth and pulled slowly in a posterior direction. A spoon excavator, with dulled edges, was then used to form a roll of tissue from the remainder of the enamel organ by stroking the excavator along the enamel surface in an anterior direction. Gouging or scraping of the immature enamel surface was avoided. In the case of fixed specimens, a dulled spoon excavator was first pushed through the enamel organ to contact the enamel surface (or enamel space in decalcified specimens) near the midpoint along the sagittal length of the tooth. The excavator was then worked slowly between the enamel organ and the enamel surface, in the posterior direction and in the anterior direction, thereby forming two rolls of tissue that could be grasped with forceps.

Enamel organs from an equivalent number of maxillary and mandibular incisors were pooled together. The amount of tissue used

Table 1. Comparison of K_M and V_{max} for substrate hydrolysis by APH activity in enamel organ and liver under unfixed and fixed conditions^a

Substrates	K_M		V_{max}				
			By protein		By wet weight		
			(nM P _i /μg protein/hr)		(nM P _i /mg tissue/hr)		
	unfixed (mM)	fixed (mM)	unfixed	fixed ^b	unfixed	fixed	
Enamel organ	5'-CMP	1.8 ^c	1.8 ^d	2.4	0.48	105.3	21.1
	βGP	2.5	6.7	2.2	0.66	114.9	34.7
	PC	2.3	6.7	0.67	0.18	35.7	9.6
	PS	3.1	10.5	0.34	0.12	17.0	6.1
Liver	5'-CMP	0.6	1.4	2.1	1.6	250.0	198.0
	βGP	2.5	3.6	1.7	1.0	232.6	138.9
	PC	6.7	20.0	0.04	0.03	7.8	6.1
	PS	2.2	10.5	0.13	0.03	19.6	5.0

^aEstimated from the double reciprocal plots in Figures 5-8.

^bEstimated from the data in the other three columns.

^cThe same value was obtained in two other experiments.

^dThe same value was obtained in the five experiments shown in Figure 9.

in the enzyme assay was generally 1 (unfixed) or 2 (fixed) enamel organs per milliliter of the 0.25 M sucrose solution. Pooled enamel organs were weighed in the beaker of sucrose.

Liver. Following dissection of the enamel organs, the left median lobe of the liver was removed from the washing buffer and cut into small wedge-shaped pieces. These were blotted gently on #50 Whatman filter paper, weighed, and placed in a separate plastic beaker containing a 0.25 M sucrose solution at 4°C. The amount of tissue used in the enzyme assay was generally 15 mg (unfixed) or 30 mg (fixed) liver per milliliter of the sucrose solution.

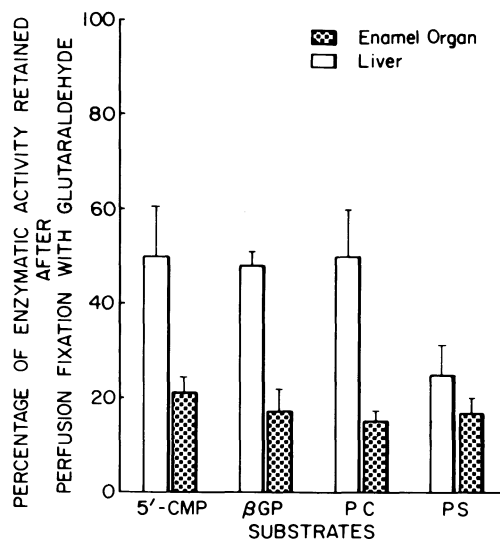
Homogenation of Tissue

Enamel organ and liver specimens were homogenized by hand at 4°C in 0.25 M sucrose using Dounce tissue grinders. The specimens were disrupted by making 20 strokes with the handle having a loose fit, and a fine homogenate was produced by making 40 strokes with the handle having a tight fit. Homogenates were kept at 4°C for 1 hr, then warmed to room temperature for the enzyme assay. Treatment of the homogenate with freeze/thawing and/or with Triton X-100 (2, 10, 27, 30) was not used in this study, since neither procedure appeared to increase the total amount of enzyme activity detectable in fixed tissue (data not shown) (4).

Enzyme Assay

APH activity was determined at pH 5.0 and at 37°C in plastic culture tubes containing the following components; 1) 0.1 ml stock acetate buffer (0.25 M sodium acetate buffer, pH 5.0), 2) 0.1 to 0.3 ml homogenate, and 3) 0.1 ml substrate. The final volume of the reaction mixture was 0.5 ml. Substrates used were a) cytidine 5'-monophosphate (5'-CMP), sodium salt (41); b) β -glycerophosphate (β GP), sodium salt (16); c) phosphorylcholine chloride (PC), calcium salt (50); and d) O-phospho-DL-serine (PS), sodium salt. Structural formulas for these substrates are shown in Figure 1. All substrates

Figure 4. Effect of 2% glutaraldehyde on enzyme activity in enamel organ and liver. The height of each column represents the mean \pm SD for five or more separate experiments; determined at 20 mM concentration for all substrates (5'-CMP, β GP, PC, and PS).



were dissolved and diluted in the stock acetate buffer. In cases where 5'-CMP was used as substrate, 5 mM manganese chloride (final concentration) was included in the reaction mixture (39). The results were essentially the same with or without this cation in the reaction mixture (data not shown).

Experiments consisted of varying either the amount of homogenate (0.1 to 0.3 ml) or the amount of substrate (0.5 to 20 mM, final concentration) in the reaction mixture, and in varying the length of the incubation time (30 to 90 min) at fixed concentrations of substrate and homogenate. In some experiments, 10 mM sodium fluoride or 4 mM lead acetate (final concentration) was included in the reaction mixture.

Controls consisted of incubating homogenate in the absence of substrate, of incubating substrate in the absence of homogenate, or of incubating both homogenate and substrate in the presence of 5% trichloroacetic acid (TCA) (w/v). These controls were necessary, since the homogenates and substrates contained detectable amounts of free orthophosphate (P_i) (data not shown). All results were corrected for this background contamination by free P_i .

Following incubation, the tubes were removed from the reciprocal shaker water bath and placed in an ice bath. The reaction was stopped by adding 0.5 ml of 10% TCA (w/v) to each tube. The tubes were centrifuged for 10 min at 1000 \times g and at 4°C. The tubes were warmed to room temperature and the concentration of P_i in the supernate was determined by the procedure of Ames and Dubin (1) using sodium phosphate monobasic ($NaHPO_4 \cdot H_2O$) as standard. The amount of protein in the homogenates was estimated by the procedure of Lowry et al. (37) or by means of the Bio-Rad protein assay kit using bovine serum albumin as standard (both techniques). Reproducible estimates of the protein content in homogenates from fixed tissue could not be obtained by either technique (8, 10, 27, 30). Hence, enzyme activity in unfixed and fixed tissue was generally expressed as micromoles of P_i released per milligram wet weight of tissue per hour (μ mol P_i /mg tissue/hr). Enzyme activity in unfixed tissue was also expressed as micromoles of P_i released per microgram protein in the homogenate per hour (μ mol P_i / μ g protein/hr) (these methods are compared in Figures 2 and 3). In Table 1, V_{max} was expressed in nanomoles (nM) of P_i to facilitate presentation of the data.

Materials

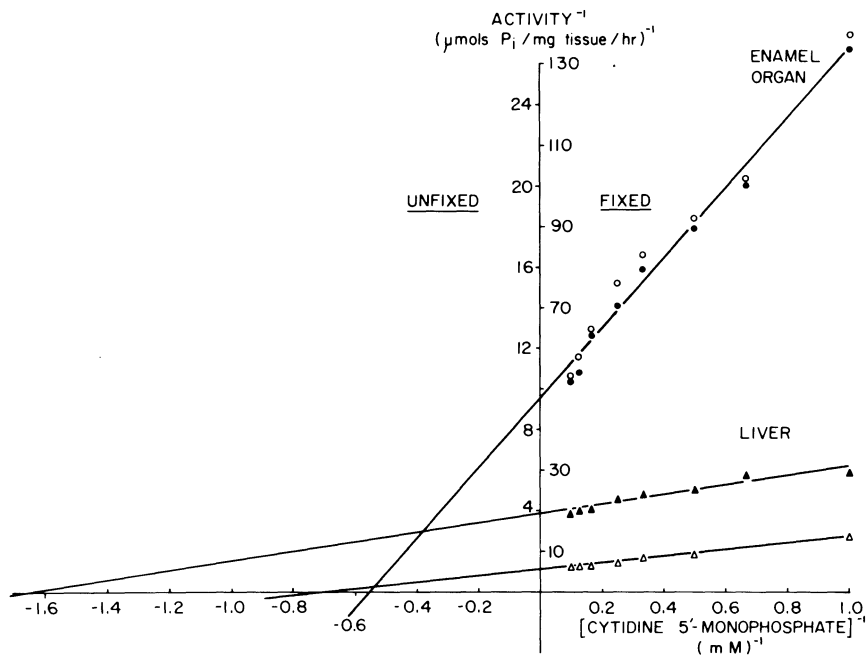
Glutaraldehyde was obtained from J.B.EM Services Inc., Montreal, Canada; sodium cacodylate from Electron Microscopy Sciences, Fort Washington, U.S.A.; the substrates (5'-CMP, β GP, PC, PS) and bovine serum albumin from the Sigma Chemical Company, St. Louis, U.S.A.; lead acetate ("suprapur") from BDH Chemicals, Toronto, Canada; the Bio-Rad protein assay kit from Bio-Rad Laboratories, Mississauga, Canada. All other chemicals were obtained from Fisher Scientific Company Ltd., Montreal, Canada.

Results

APH Activity in Unfixed Tissue

The phosphate-ester bond in 5'-CMP, β GP, PC, and PS (Figure 1) was hydrolyzed enzymatically at pH 5.0 in homogenates of enamel organ and liver (Figure 2). In both tissues APH activity appeared high using either 5'-CMP or β GP as substrate, but low using either PC or PS as substrate (Figure 2). Double reciprocal (Lineweaver-Burk) plots (34) of APH activity versus substrate concentration (Figure 3) showed that the release of P_i by enzymes in the homogenate was a function

Figure 5. Comparison by double reciprocal plots of enzyme activity toward 5'-CMP in enamel organ (circles) and liver (triangles) under unfixed (closed symbols) and fixed (open symbols) conditions.



of substrate concentration up to saturation levels, and hence, produced classical straight lines (Figure 3).

Effect of Glutaraldehyde on APH Activity

APH activity in enamel organ and liver was noticeably reduced following whole body perfusion with 2% glutaraldehyde (Figure 2, fixed). The relative amount of APH activity inhibited by fixation appeared similar for each substrate in the same tissue (Figure 4), but the enamel organ appeared to retain less APH activity toward each substrate after fixation than liver (Figure 4). Double reciprocal plots (Figures 5-8) revealed that fixation altered the characteristics of substrate hydrolysis within each tissue (summarized in Table 1). These changes included an increase in the amount of substrate required to saturate the enzymes (K_M elevated) and a decrease in the rate of hydrolysis of substrate by enzymes (V_{max} depressed) (Table 1). No alteration in K_M for 5'-CMP was observed, however, for enamel organ (Table 1; Figure 5).

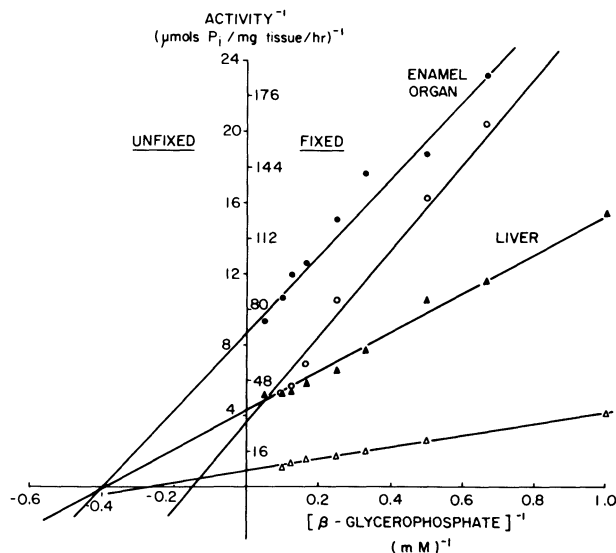
Effect of Treatment with Formic Citric Acid (FA), EDTA, or EGTA on APH Activity in Fixed Tissue

APH activity in enamel organ and liver did not seem altered following treatment of fixed tissues for up to 8 days with FA or 14 days in EDTA or EGTA (these are the usual times required to remove most of the mineral from the enamel in rats weighing between 100 and 150 g). This was confirmed by double reciprocal plots (Figures 9, 10), which showed no change in K_M and V_{max} for fixed tissues treated with these decalcifying agents (data not shown for β GP and PS, but the results were identical).

Effect of Fluoride and Lead on APH Activity

APH activity toward 5'-CMP, β GP, and PS was almost totally inhibited by 10 mM sodium fluoride, but it was only partially inhibited when PC was used as substrate (Figure 11). Fluoride produced the same relative amount of inhibition in unfixed tissue, fixed tissue, and fixed tissue treated with any of the decalcifying agents (Figure 11). Liver appeared slightly more

Figure 6. Comparison by double reciprocal plots of enzyme activity toward β GP in enamel organ (circles) and liver (triangles) under unfixed (closed symbols) and fixed (open symbols) conditions.



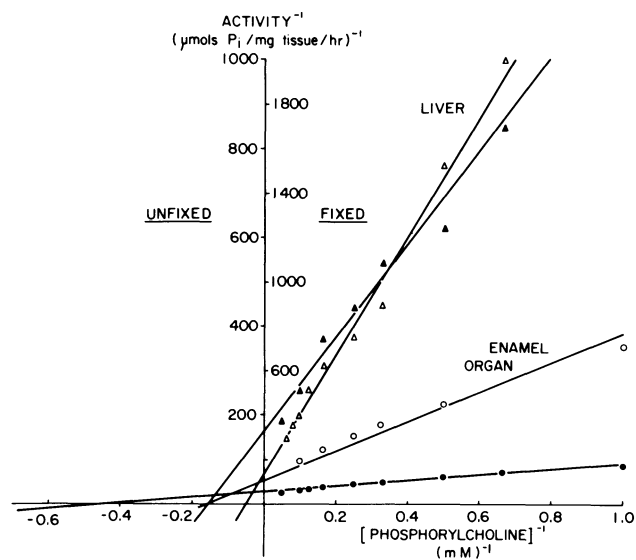


Figure 7. Comparison by double reciprocal plots of enzyme activity toward PC in enamel organ (circles) and liver (triangles) under unfixed (closed symbols) and fixed (open symbols) conditions.

sensitive to fluoride than enamel organ (Figure 11, 5'-CMP and β GP).

Lead was also found to inhibit up to 40% of the total APH activity in fixed tissue at a 4 mM concentration (Figure 12, control). APH activity toward 5'-CMP, β GP, and PS was similarly inhibited in fixed tissue and fixed tissue treated with either FA, EDTA, or EGTA (Figure 12, data shown for 5'-CMP only). However, APH activity toward PC was markedly inhibited by lead following treatment of fixed tissue with chelating agents (EDTA and EGTA) (Figure 12, PC). Enamel organ appeared generally more sensitive to lead than liver (Figure 12).

Discussion

Although it is commonly accepted that glutaraldehyde in concentrations above 1% lowers the activity of most phosphatases (2, 14, 23, 27, 28), it has not been evident that this inhibition is caused in part by a reduction in the affinity of enzymes for their substrate (increase in K_M) as well as by a reduction in the capacity of enzymes to hydrolyze P_i from the substrate (decrease in V_{max}). To the knowledge of the author, the only prior evidence for a fixative-induced alteration in K_M was presented by Berteloot and Hugon (8), who reported that glutaraldehyde elevates the K_M for substrate hydrolysis by hepatic glucose-6-phosphatase. It is not clear from the results of the present study, however, if glutaraldehyde affected equally the entire spectrum of enzymes responsible for the specific and/or nonspecific hydrolysis of each substrate (Figure 1), or if there was a more selective action of the glutaraldehyde upon only those enzymes that possessed the highest natural affinity for the substrate. It is also unclear why the K_M for hydrolysis of 5'-CMP in enamel organ remained unaltered

while other values rose 2- or 3-fold following fixation (K_M , Table 1).

The general findings of this study are consistent with observations reported by other investigators. These include: 1) the loss of greater than 50% of enzyme activity following relatively gentle fixation with glutaraldehyde (Figure 4) (2, 4, 27); 2) the higher affinity of certain enzymes for 5'-CMP compared to β GP, but the comparable amount of enzyme activity toward both molecules at saturation levels (Table 1, K_M and V_{max}) (13, 26, 41, 56); 3) the requirement of between 2.4 and 2.8 mM 5'-CMP and between 8.6 and 9.3 mM β GP to saturate enzymes in fixed liver (Table 1, double K_M values) (7, 16, 41, 43); 4) the inhibition of 90% (teeth) to 100% (other tissues) of enzyme activity toward β GP and 5'-CMP by 10 mM sodium fluoride (Figure 11) (reviewed in ref. 26) (55); 5) marked differences in the amount of enzyme activity toward PC compared to β GP or 5'-CMP within a given tissue (Figure 2) (45, 50); 6) limited inhibition of enzyme activity toward PC by 10 mM sodium fluoride (Figure 11) (47, 50); and 7) a general sensitivity of phosphatases to partial inhibition by low concentrations of lead ions (Figure 12) (reviewed in ref. 8) (62). Furthermore, the V_{max} for hydrolysis of β GP in enamel organ estimated in this study (Table 1) is remarkably similar to a value reported by Anderson and Toverud (3) using more purified enzyme preparations, that is, 2.2 units/g tissue or 132 nM P_i/mg tissue/hr, which they determined at pH 5.0 in acetate buffer with 50 mM β GP. Biochemical data about the hydrolysis of 5'-CMP, PC, and PS within the enamel organ are otherwise unavailable.

FA, EDTA, and EGTA have each been used as decalcifying agents for light and electron microscopic studies of hard tissues, although they are known to cause some physical or chemical damage in tissues (9, 25, 34, 39, 49). No allowances

Figure 8. Comparison by double reciprocal plots of enzyme activity toward PS in enamel organ (circles) and liver (triangles) under unfixed (closed symbols) and fixed (open symbols) conditions.

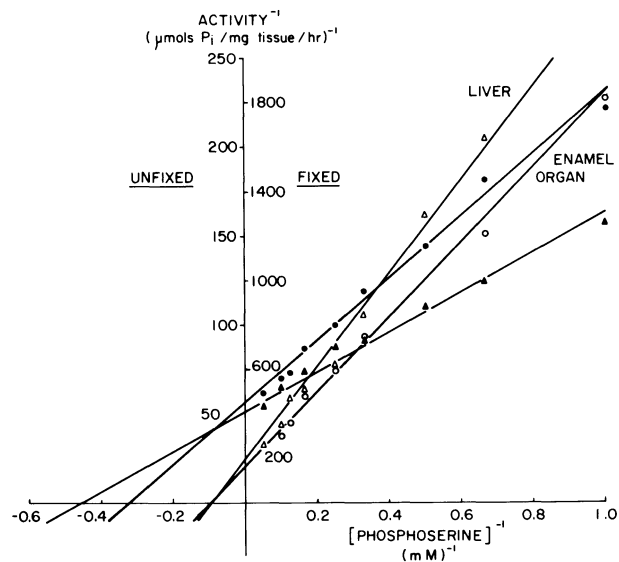
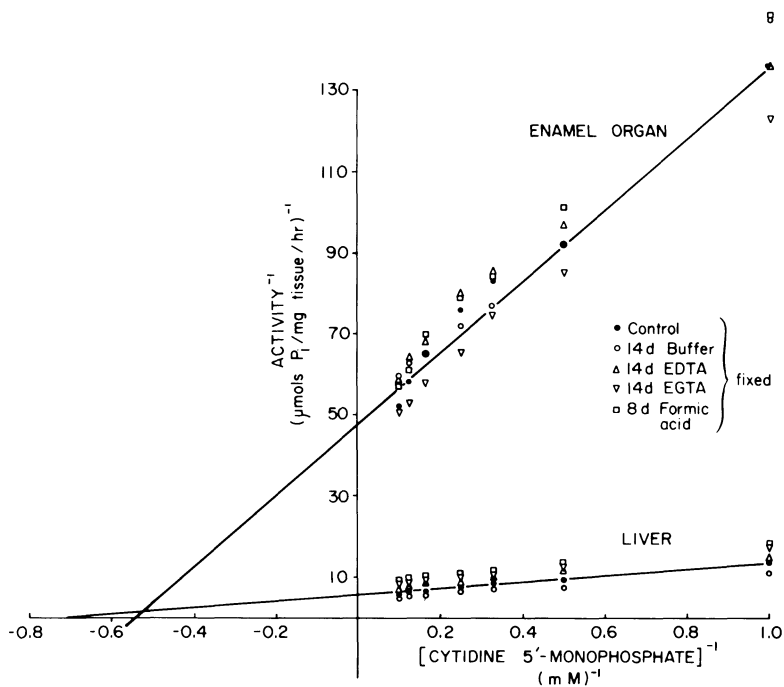


Figure 9. Comparison by double reciprocal plots of enzyme activity toward 5'-CMP in enamel organ and liver following treatment with washing buffer, EDTA, EGTA, or FA for the number of days indicated. Control refers to tissue fixed with glutaraldehyde and rinsed for 24 hr in buffer prior to enzyme assay.



were made for enzymes in the decalcifying solutions (Figures 9, 10), for example, by the addition of Mg or Mn ions to the chelating agents as suggested by Kirkeby and Vilmann (34), since it was the intent of this study to evaluate APH activity under the least ideal conditions. The data in Figures 9 and 10 clearly suggest that brief exposure to decalcifying agents does not alter directly the kinetics of substrate hydrolysis in fixed tissue despite the chelation of cations by compounds such as EDTA and EGTA (6, 11, 34, 49).

The experiments involving lead (Figure 12) raise questions concerning the reliability of the assay for free P_i released by enzymes in media which also contain lead ions. That is, one would expect that lead phosphate was formed and precipitated in the reaction mixture during incubation (12, 29), and it could be argued that the data in Figure 12 do not show lead-induced inhibition of enzyme activity, but simply indicate the amount of free P_i that was removed from the supernate of the reaction mixture by precipitation. It should be noted that according to the assay procedure followed in this study an equal volume of 10% TCA was added to each incubation tube before the concentration of P_i in the reaction mixture was estimated. Independent experiments established that precipitates of lead phosphate formed in acetate buffer at pH 5.0 dissolve completely into solution when mixed with an equal volume of 10% TCA (determined by measuring turbidity and P_i concentration in tubes containing various mixtures of lead acetate and sodium phosphate; unpublished). This finding suggests that all P_i released by enzymatic hydrolysis of a substrate was capable of being detected, and hence, the results in Figure 12 probably represent a true inhibitory effect of lead, and/or accumulated deposits of lead phosphate, on enzyme activity (8, 62).

The original purpose in using four structurally different substrates (Figure 1) was to investigate whether glutaraldehyde depressed equally within a tissue APH activity toward different classes of phosphorylated molecules, that is, molecules related broadly to nucleotides (5'-CMP), sugars (βGP, 5'-CMP), lipids (βGP, PC, PS), or amino acids (PS). The data in Figure 4 support this contention, although it is evident that APH activity toward PS was more severely depressed than toward the other substrates in liver. The data in Table 1 further show that relative differences between K_m or V_{max}, which characterize hydrolysis of each substrate in a tissue, are altered on a substrate-to-substrate basis following fixation. It is not clear if the enamel organ retained less APH activity than liver after fixation (Figure 4, Table 1) because this tissue was sensitive to aldehydes (21) or because the enamel organ perfused more completely than the liver via the ascending aorta (see ref. 4).

It is difficult on the basis of the techniques used in this study (unfractionated homogenates) to determine whether each substrate (Figure 1) was hydrolyzed by similar or distinct enzymes. On the one hand, the findings of high APH activity toward 5'-CMP and βGP, but low APH activity toward PC and PS, in whole homogenates (Figure 2) suggest less "non-specific" hydrolysis of the latter substrates than the former substrates (14, 26, 41, 47). On the other hand, the findings of substantial fluoride inhibition of APH activity toward 5'-CMP, βGP, and PS, but not toward PC (Figure 11), and chelator-enhanced lead inhibition of APH activity toward PC, but not toward 5'-CMP, βGP, and PS (Figure 12), suggest that 5'-CMP, βGP, and PS are hydrolyzed by enzymes with similar properties, while PC is hydrolyzed by a different enzyme(s)

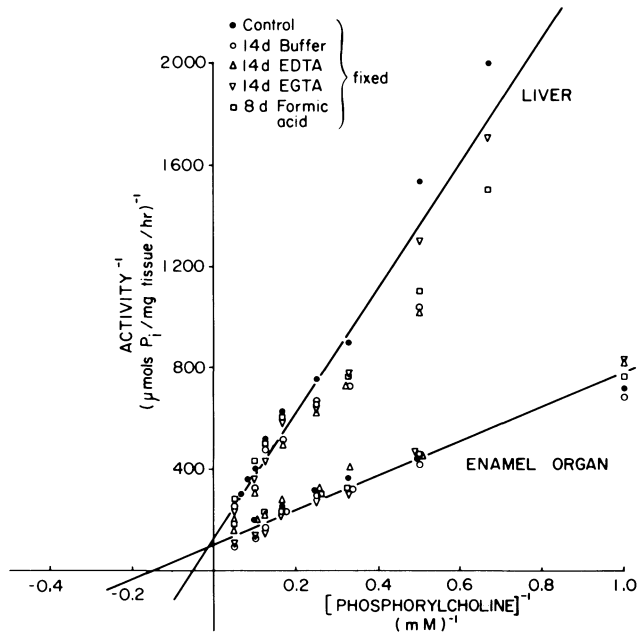


Figure 10. Comparison by double reciprocal plots of enzyme activity toward PC in enamel organ and liver following treatment with washing buffer, EDTA, EGTA, or FA for the number of days indicated. Control refers to tissue fixed with glutaraldehyde and rinsed for 24 hr in buffer prior to enzyme assay.

(14, 26, 41, 44, 47, 50). Although controversial, it is generally implied that β GP is hydrolyzed by acid phosphatase (E.C. 3.1.3.2), but 5'-CMP is hydrolyzed by acid nucleotidase (E.C. 3.1.3.31) (14, 16, 26, 41, 56). Numerous biochemical studies have shown that while the "acid phosphatase" and "acid nucleotidase" activities in a tissue can be resolved into separate fractions with distinct molecular weights, substrate specificity and/or inhibitor sensitivities (reviewed in refs. 24 and 56) (3, 5, 55), both activities contain fractions capable of hydrolyzing substantial amounts of either substrate (5, 24, 56). Hence, from a practical standpoint the findings from cytochemical localizations involving these two molecules are generally comparable (14). Little information is available about the hydrolysis of PS at acid pH. Richardson et al. (44) have shown, however, that acid phosphatase can hydrolyze P_i from the phosphoserine residues of intact phosphoprotein. It is noteworthy that the enamel organ showed 3-fold higher activity toward PS than liver (Table 1; Figure 2), because this molecule constitutes the only substrate of the ones used in this study with obvious physiological significance to the enamel organ. That is, ameloblasts of the enamel organ produce an extracellular protein rich in phosphoserine residues (15), and it has long been suspected that acid phosphatase may play some role in the turnover of these proteins during formation and/or maturation of the enamel (reviewed in ref. 52). Lastly, despite a controversy over terminology and subcellular localization, there appears to be general agreement from the studies by Serrano et al. (50, 51) and Schofield et al. (47, 48) that PC is hydrolyzed by an enzyme(s) that differs from "acid phos-

phatase" (which hydrolyzes β GP) and some cells appear to contain more of this "acid phosphorylcholine phosphatase" activity than others. While the significance of the 10-fold higher activity toward PC in enamel organ compared to liver is not clear, it is of interest that the kinetics for hydrolysis of this molecule appeared substantially different between the two tissues (Table 1). Furthermore, Schofield et al. (47) have noted an apparent association of acid phosphorylcholine phosphatase with cells which release acid phosphatase extracellularly (sebaceous and prostate epithelium and osteoclasts).

Taken together, the results of this study provide additional evidence that the spectrum of acid-dependent phosphatase activities, as well as their preferred substrates, appears slightly different in the enamel organ than in other tissues (3, 18, 19, 22, 55). They also draw attention to the difficulties that can be encountered in attempting subcellular localizations of these enzymes in the enamel organ using the lead capture technique. That is, neither the relative strength of the fixative procedure or the concentration of lead used in this study was excessive, yet both caused substantial inhibition of enzyme activity in the enamel organ compared to liver taken from the same animals. Since some glutaraldehyde is generally required in the fixative to achieve adequate ultrastructural preservation of cells in the enamel organ and between 2 to 4 mM lead appears necessary to trap P_i and prevent diffusion artifacts (11, 12, 22, 41), the alterations in standard procedures that could provide some improvement in the intensity of weak cytochemical localizations in decalcified specimens include 1) using organic acids instead of chelating agents for decalcification (especially when

Figure 11. Effect of 10 mM sodium fluoride on enzyme activity in enamel organ and liver. The height of each column represents the mean \pm SD for five or more separate experiments. The results were identical for unfixed tissue, fixed tissue, and fixed tissue treated with any of the decalcifying agents. Substrates: 5'-CMP and PS (4 mM); β GP and PC (15 mM).

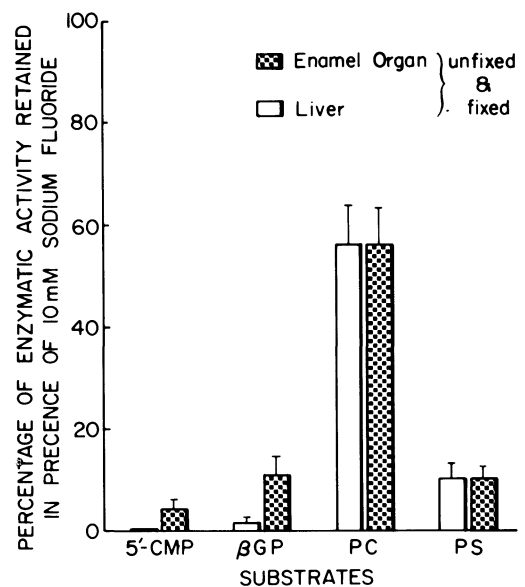
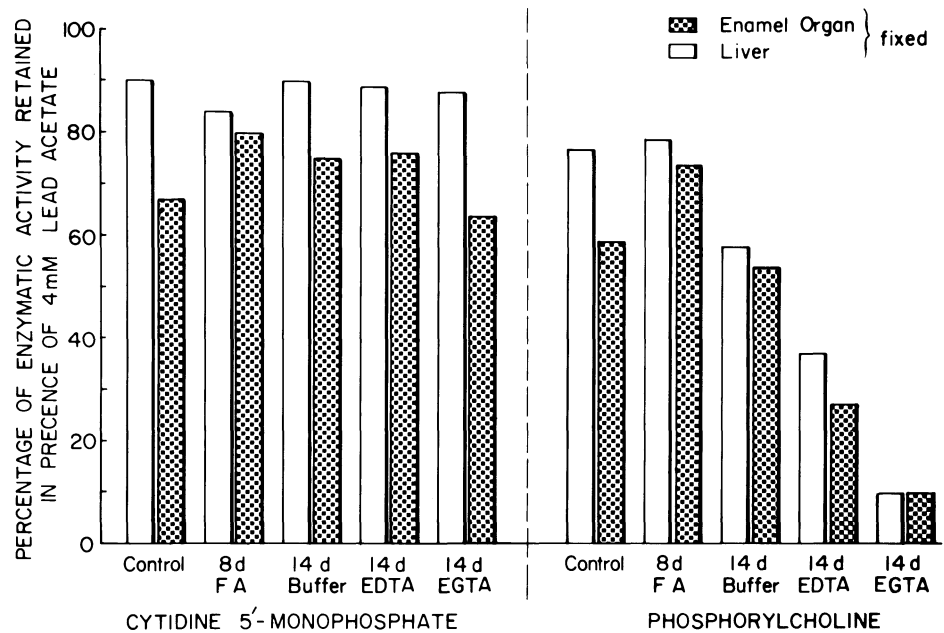


Figure 12. Effect of 4 mM lead acetate on enzyme activity in enamel organ and liver before (control) and after treatment with either washing buffer, FA, EDTA, or EGTA for the number of days indicated. The height of each column represents the mean of two separate experiments. Control refers to tissue fixed with glutaraldehyde and rinsed for 24 hr in buffer prior to enzyme assay. Substrates: 5'-CMP (4 mM); PC (15 mM).



PC is used as substrate) and 2) increasing the concentration of substrate in the incubation medium. According to Table 1, conventional cytochemical media in their present composition achieve only half-saturation of enzymes in fixed enamel organ (7, 16, 41, 47, 50).

Acknowledgment

The author gratefully acknowledges the helpful advice given by Dr. J.J.M. Bergeron concerning the biochemical assay of phosphatases. The author also thanks Dr. P.S.W. Bai and Dr. M.C. Greenblatt, former dental students, for their technical assistance in perfusing animals and in obtaining the data shown in Figures 2, 4, and 11.

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