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HISTOCHEMICAL STUDIES ON ENZYMES ACTING ON GLYCOSYL COMPOUNDS, PHOSPHORYL-CONTAINING ANHYDRIDES AND PHOSPHOAMIDES IN HUMAN CARIOUS DENTINE

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Fresh, undecalcified sections from (1) *in situ* excavated carious dentine or from (2) specimens with carious and sound dentine from extracted human teeth were cut in a cryostat and the localization of various hydrolytic enzymes demonstrated histochemically. It was observed that the hydrolysis of β-glucuronidase, α-glucosidase, β-glucosidase, β-galactosidase, ATPase, and phosphoamidase substrates could be demonstrated (a) in dental plaque, (b) in the superficial necrotic layer and in the infected zone of carious dentine, and (c) also in the decalcified carious dentine, where Gram staining revealed no microorganisms. Additionally, the hydrolysis of β-glucuronidase and α-glucosidase substrates could be observed (d) in the predentine layer. No hydrolysis of chitobiase substrate could be observed in any zone of carious dentine, nor any of the substrates in normal sound dentine. These findings were thought to further map the catabolic events during the propagation of dentine caries, especially in the enzymic degradation of mucopolysaccharides of the organic stroma of the dentine.

Previous studies on the carious metabolism in dentine revealed the occurrence of arylaminopeptidases (*Larmas, Mäkinen & Scheinin,* 1968, *Mäkinen, Larmas & Scheinin,* 1969, *Larmas & Mäkinen,* 1972) and endopeptidases, such as collagenase (*Mäkinen,* 1970a, *Larmas,* unpublished) in human carious dentine. Enzymatic hydrolysis of ester bonds (phosphate, sulphate, and carboxylic acid esters) takes place in carious dentine (*Larmas,* 1968, *Mäkinen et al.,* 1969, *Mäkinen* 1970b, *Larmas,* 1972b). The occurrence of enzymes forming keto acids mainly from L-cysteine and related compounds (*Mäkinen,* 1968, *Mäkinen* et al., 1969) and from alanine and aspartic acid (*Larmas,* 1972c) has been demonstrated in carious dentine. Further, the occurrence of several dehydrogenases in carious dentine provides possibilities for both aerobic and anaerobic glycolysis and dehydrogenation of glutamate, linked protein and carbohydrate metabolism etc. (*Larmas,* 1972a). All these studies

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would indicate a versatile metabolism during the propagation of dentine caries. However, no information is available on the enzymatic hydrolysis of glycosyl compounds. Further, it is apparent that knowledge on the enzymatic cleavage of some phosphate groups (other than phosphate esters) by carious dentine enzymes is still limited, although *Mäkinen* (1970b) reported some preliminary observations.

This paper provides information on various glycosidases and phosphohydrolases in human carious dentine. β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31) was studied because it is believed to catalyze the hydrolysis of acid mucopolysaccharides. It also catalyzes glucuronotransferase reactions (*Florkin & Stotz*, 1965). α- and β-Glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20 and β-D-glucoside glucohydrolase EC 3.2.1.21), β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), and chitobiase (chitobiose, acetamidodeoxyglucohydrolase, EC 3.2.1.29) were selected because they are thought to release the corresponding sugars from the glycoprotein and thus may have a function in the catabolic hydrolysis of glycoproteins. Further, adenosine triphosphatase (ATP phosphohydrolase, EC 3.6.1.3) was studied because of its fundamental importance in the cellular metabolism. The enzymes acting on phosphoamide bonds in creatine and arginine phosphates are classified as phosphoamidases (phosphoamide hydrolase, EC 3.9.1.1). According to the Enzyme Commission (*Florkin & Stotz*, 1965) this enzyme may be identical with phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16), which has been claimed to liberate phosphate from enamel (*Kreitzman et al.*, 1969).

In view of the factors mentioned above this study was undertaken. The aim of the present investigation was (1) to shed some light on certain hydrolytic reactions in carious dentine, (2) to find the localization of these enzymes, and (3) to clarify some properties of the enzymes in order to further map the catabolic reactions during the propagation of dentine caries.

MATERIAL AND METHODS

Dentine samples

Two different methods were used in the collecting of carious dentine as described earlier in detail (*Larmas*, 1972c). First, carious dentine was excavated *in situ* from human molars (15 altogether) with advanced caries lesions. Second, carious and normal dentine samples were cut from freshly extracted carious teeth with a high speed drill under a continuous jet of

cold water. Samples from altogether 40 teeth were used. In each case sections of 10 μm were cut in a cryostat (International Equipment Company, Model CTI, Needham Heights, Mass. USA).

The carious dentine as occurring in the tissue sections was classified by using the following nomenclature (Fig. 1): (1) the dental plaque layer existing in close proximity to carious dentine, (2) the carious dentine layer, (3) the sclerotic and sound dentine (termed normal dentine) layer existing below and around the carious dentine, and (4) the predentine layer (seen only in some sections). The criteria for the layering of the sections were as described earlier (*Larmas, Mäkinen & Scheinin,* 1968, *Larmas,* 1972c, *Larmas & Mäkinen,* 1971).

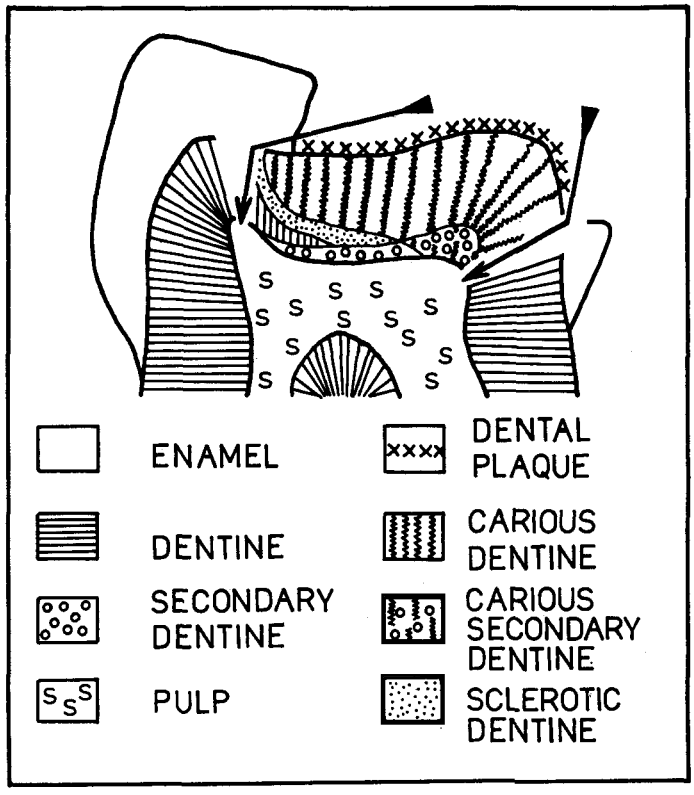

Fig. 1. Diagrammatic representation of dissection procedure used to obtain samples from caries dentine from a human molar with an advanced carious lesion. The zones in the lesion are presented schematically. The enamel was first removed and the samples dissected as shown by the arrows. Haematoxylin and eosin, and Gram stainings were used as reference material from every third section (transverse or longitudinal to tubule direction).

Enzyme assay methods

All the enzyme activity determinations were performed using unfixed cryostat sections from human dentine, in spite of the recommendations of the assay methods used, presented below. The β-glucuronidase activity was assayed according to the postcoupling method of *Fishman* and *Goldman* (1965). The substrate used was naphthol AS-BI β-D-glucuronic acid (2×10^{-4} or 0.5×10^{-4} moles/litre in the reaction mixture). The pH of the reaction medium was adjusted from 3.5 to 6.5 with 0.1 M acetate buffer. The diazonium salts used were Fast Garnet GBC (4-amino-3:1'-dimethyl azobenzene, G.T. Gurr, Ltd., London, England), Fast Dark Blue R (diazotized product of 2:6-Dichloro-4-nitroaniline and 2:5-dimethoxyaniline, Sigma Chem. Company, St. Louis, Mo. USA), or Fast Red Violet LB salt (5-chloro-0-toluidine-4-benzanilide, Sigma), of which a saturated solution in 0.01 M phosphate buffer (pH 7.4) was made. The enzyme activity was also assayed according to the post-coupling method of *Seligman et al.* (1954). The substrate used in this case was 6-bromo-2-naphthyl-β-D-glucuronide, 1.5×10^{-3}, 1.3×10^{-3}, and 0.7×10^{-3} moles/litre in the reaction mixture. The pH was adjusted from 4.0 to 6.5 with 0.1 M citric acid. The diazonium salt was Fast Blue B (tetrazotized-0-dianisidine, Sigma), 1 mg per ml of 0.02 M phosphate buffer, pH 7.5.

The post-coupling method of *Rutenburg et al.* (1960) was followed in the determination of α- and β-glucosidase activity. The substrates used were 6-bromo-2-naphthyl-α-D-glucoside (Sigma) or 6-bromo-2-naphthyl-β-D-glucoside (Sigma), both at concentrations ranging from 10^{-4} to 10^{-3} moles/litre in the reaction mixture (pH 6.5). The diazo salt used was Fast Blue B (1 mg/ml). This method was also modified to a simultaneous coupling procedure by including the diazonium salt in the incubation medium.

The β-galactosidase activity was assayed according to the method of *Rutenburg et al.* (1958). The substrate used was 6-bromo-2-naphthyl-β-D-galactopyranoside (Mann Research Laboratories, New York, N.Y., USA), 2×10^{-3}, 10^{-3}, and 5×10^{-4} moles/litre in the reaction mixture. The pH was adjusted to 5.0. The diazonium salt was Fast Blue B 1 mg/ml.

The chitobiase activity was estimated according to the method of *Pugh* and *Walker* (1958). The substrate used was N-acetyl-β-D-glucosamine naphthol AS-LC (Sigma), 5×10^{-3}, 3×10^{-3}, and 10^{-3} moles/litre in the reaction mixture, pH 4.6. Fast Garnet GBC was used in the coupling of the liberated naphthol.

The ATPase activity was assayed by the lead method of *Wachstein* and *Meisel* (1956), the substrate was adenosine-5'-triphosphate, disodium salt (Fluka AG, Buchs, Switzerland), 10^{-3}, 0.8×10^{-3}, and 0.5×10^{-3} moles/litre in the reaction mixture, pH 7.2. The liberated lead phosphate was converted

into lead sulphide by treatment with ammonium sulphide (0.5 % v/v). Additionally, the calcium method was used (*Padykula & Herman*, 1955a, b): the substrate used was adenosine-5'-triphosphate, disodium salt (3×10^{-3}, 10^{-3}, and 8×10^{-4} moles/litre in the reaction mixture, and the liberated phosphate was trapped with calcium ions with three washes in 1 % (w/v) calcium chloride solution. The calcium phosphate was converted to cobalt phosphate with 1 % (w/v) cobalt nitrate solution and this was transformed into cobalt sulphide with 0.5 % (v/v) ammonium sulphide.

The phosphoamidase activity was assayed by the lead method of *Gomori* (1952) as modified by *Chayen et al.* (1969). The substrate used was *p*-chloroanilido phosphonic acid (Sigma), 1.5×10^{-2} moles/litre in the reaction mixture, pH 5.4. The lead phosphate formed was converted into lead sulphide by 0.5 % (v/v) ammonium sulphide. Additionally, a simultaneous coupling azo dye technique was used in principle as suggested by *Burstone* (1958). The substrate used was naphthol AS-BI-phosphodiamide (Sigma). The reaction mixture was made by dissolving 4 mg of the substrate in 0.1 ml dimethylformamide and diluting this solution to 25 ml with 0.2 M acetate buffer, pH 5.4. Finally, 35 mg of diazonium salt (Fast Garnet GBC, Fast Blue B, Fast Dark Blue R, or Fast Red Violet LB) was added. The mixture was filtered before use. Incubation was carried out at +37°C and the sections were mounted after washing with water in Farrant's medium.

Control tests for all the assay methods were as follows: (1) the effect of the diazo salts or trapping agents alone on the tissue sections was studied in all cases in a similar reaction mixture as that used in the usual tests, but omitting the substrates, (2) heating the sections for 30 min at 100°C in 0.9 % (w/v) (or 0.154 M) sodium chloride solution before the normal incubation. The azo-dye technique was additionally controlled by (3) an incubation with the end product of the reaction: Free 1- or 2-naphthol (at 1.0, 0.5, 0.1, 0.05, and 0.01 mM) replaced the substrate in the normal incubation with the tissue sections.

Other methods

The following chemical compounds were used to characterize the enzyme activity: p-chloromercuribenzoate (Calbiochem, Luzern, Switzerland) $HgCl_2$, $AgNO_3$, $CaCl_2$, $MgCl_2$, $ZnCl_2$, NaF, dithiothreitol (Calbiochem, Los Angeles, Calif., USA), L-cysteine hydrochloride (Nutritional Biochemicals Corp., Cleveland, Ohio, USA), all at concentrations of 5×10^{-3} and 10^{-3} moles/litre in the reaction mixture.

Sections stained with haematoxylin and eosin, or which were Gram stained, served as reference material. The lead method (*Ebel*, 1952) was used in the histochemical determination of polyphosphates. Unless otherwise stated, all chemicals were purchased from E. Merck AG, Darmstadt, Germany.

RESULTS

The first experiments with the enzymes studied were performed to determine the proper substrate concentrations for the tests. This was done to ensure that the substrate concentrations were high enough to saturate or nearly saturate the enzymes in all experiments. Tests to determine the proper incubation time and diazo salt concentration were also performed. Table I shows the concentrations used in the actual tests. The results obtained when studying the distribution of the enzyme activity in the sections are summarized in Table I.

Fig. 2 shows the localization of the enzymatic hydrolysis of 6-bromo-2-naphthyl-α-D-glucoside in human carious dentine. The enzyme activity occurred in the dental plaque layer and as distinct stripes along the dentinal tubules of carious dentine, but never in the normal dentine layer (see also Fig. 3). Additionally, enzyme activity was also seen in the predentine layer below the carious lesion (Fig. 3), Hg^{2+}, Ag^{+}, Zn^{2+} ions (5×10^{-3} M) inhibited the enzyme activity. Similar distribution of the enzymic hydrolysis of 6-bromo-2-naphthyl-β-D-glucoside and 6-bromo-2-naphthyl-β-D-galacto-pyranoside was obtained for β-galactosidase activity as seen in Fig. 4. On the other hand, no hydrolysis of naphthol AS-LC *N*-acetyl-β-D-glucosamine could be observed in any sites of the sections. Hg^{2+} and Ag^{+} ions (5×10^{-3} M) totally inhibited the enzyme activity.

The distribution pattern of the hydrolysis of 6-bromo-2-naphthyl-β-D-glucuronide was similar to that described earlier for glucosidases and galactosidases. Similar results were obtained when naphthol-AS-BI-β-D-glucuronic acid was used as the substrate (Fig. 5). In this case, however, the enzyme activity density was more noticeable than in the case described earlier for glucosidases and galactosidases. In Fig. 6 the localization of the hydrolysis is presented in a higher magnification. The enzymic hydrolysis occurred (1) as long stripes along the dentine tubules, and (2) as distinct small spots in the dentine tubules. The localization of the spots was observed to occur in regions where Gram staining revealed gram positive micro-organisms. Enzyme activity was also observed in the predentine layer. Hg^{2+}, Ag^{+}, and p-chloromercuribenzoate ions (5×10^{-3} M) inhibited the enzyme activity.

Table I.

Distribution of the enzyme activity on tissue sections

Enzyme	Substrate	Localization	Enzyme activity
β-Glucuronidase	Naphthol AS-BI β-D-glucuronic acid	Dental plaque	+++
		Carious dentine	+++
		Normal dentine	0
		Predentine	+
β-Glucuronidase	6-bromo-2-naphthyl-β-D-glucuronide	Dental plaque	++
		Carious dentine	++
		Normal dentine	0
		Predentine	0
α-Glucosidase	6-bromo-2-naphthyl-α-D-glucoside	Dental plaque	++
		Carious dentine	++
		Normal dentine	0
		Predentine	++
β-Glucosidase	6-bromo-2-naphthyl-β-D-glucoside	Dental plaque	+
		Carious dentine	+
		Normal dentine	0
		Predentine	0
β-Galactosidase	6-bromo-2-naphthyl-β-D-galactopyranoside	Dental plaque	+
		Carious dentine	+
		Normal dentine	0
		Predentine	0
Chitobiase	N-acetyl-β-D-glucosamine naphthol AS-LC	Dental plaque	0
		Carious dentine	0
		Normal dentine	0
		Predentine	0
ATPase (Lead method)	Adenosine 5'-triphosphate disodium salt	Dental plaque	++
		Carious dentine	++
		Normal dentine	—
		Predentine	—
Phosphamidase (diazo method)	Naphthol AS-BI-phosphodiamide	Dental plaque	+++
		Carious dentine	+++
		Normal dentine	0
		Predentine	0

Explanations:

+++ Abundant enzyme activity density in all sections

++ Abundant enzyme activity density in some sections

+ Slight enzyme activity

0 No enzyme activity in any of the sections

— No differences between actual and control sections and thus the occurrence of enzyme activity remained obscure.

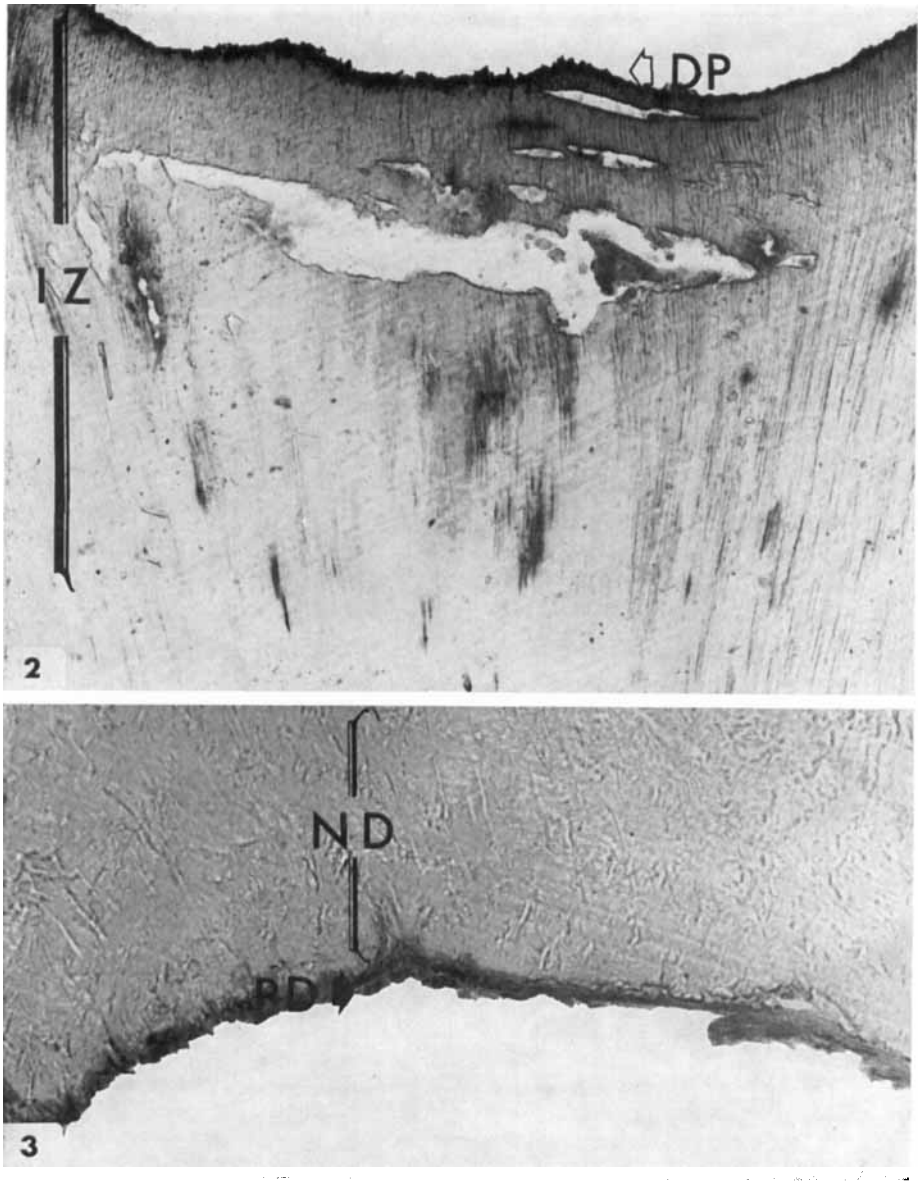

Fig. 2. α-Glucosidase. A longitudinal section from a human molar tooth with a clinically active type of occlusal caries lesion. The enzyme activity occurs as distinct dark lines along the dentinal tubules from the superficial infected parts to the deeper zones of the lesion, where Gram staining revealed no microorganisms. Substrate: 6-bromo-2-naphthyl-α-D-glucoside, 10^{-3} moles/litre in the reaction mixture. Fast Blue B. DP = dental plaque, IZ = infected zone of carious dentine (Gram staining revealed microorganisms). (48×)

Fig. 3. α-Glucosidase. A higher magnification from the tooth seen in Fig. 2. The borderline between predentine (PD) and normal (ND) dentine below the lesion is seen. (260×)

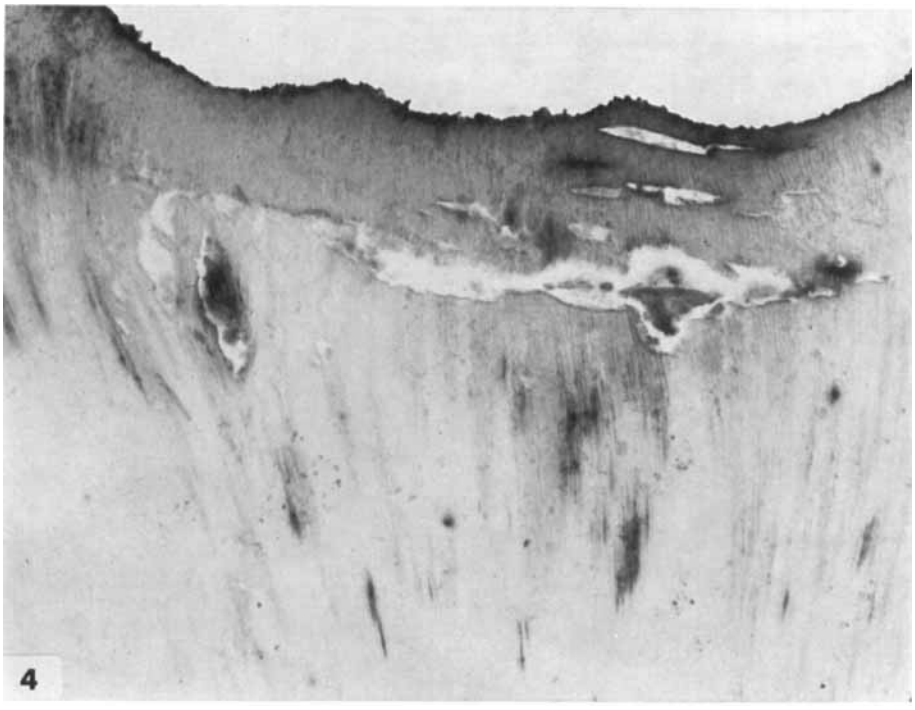

Fig. 4. β-Galactosidase. A longitudinal section from the same tooth as in Fig. 2. The enzyme activity occurs as distinct dark lines along the dentinal tubules. Substrate: 6-bromo-2-naphthyl-p-D-galactopyranoside (10^{-3} moles/litre in the reaction mixture), Fast Blue B. 2. (48×)

Fig. 7 shows the localization of the hydrolysis of ATP assayed by the lead method (*Wachstein & Meisel*, 1956). Lead sulphide precipitation occurred as distinct stripes along the tubules in the carious dentine layer. However, a very marked precipitation of lead sulphide could be observed on the borderline between carious and normal dentine. This precipitation was observed to occur nonenzymatically and it could also be seen in the control sections incubated without the substrate (Fig. 8). The difference between the actual and control sections was not great but a higher magnification revealed enzymatic hydrolysis of ATP in the dentinal tubules of carious dentine layer (Fig. 9). F-, Hg-, Ag- and β-chloromercuribenzoate ions (5×10^{-3} M) inhibited the enzyme. The difference between the test and control sections was undetectable when the modified calcium method was used (*Padykula & Herman*, 1955a, b), and thus the results from that series of tests could not be taken into account.

Fig. 10 shows the localization of the hydrolysis of naphthyl AS-BI phospho-

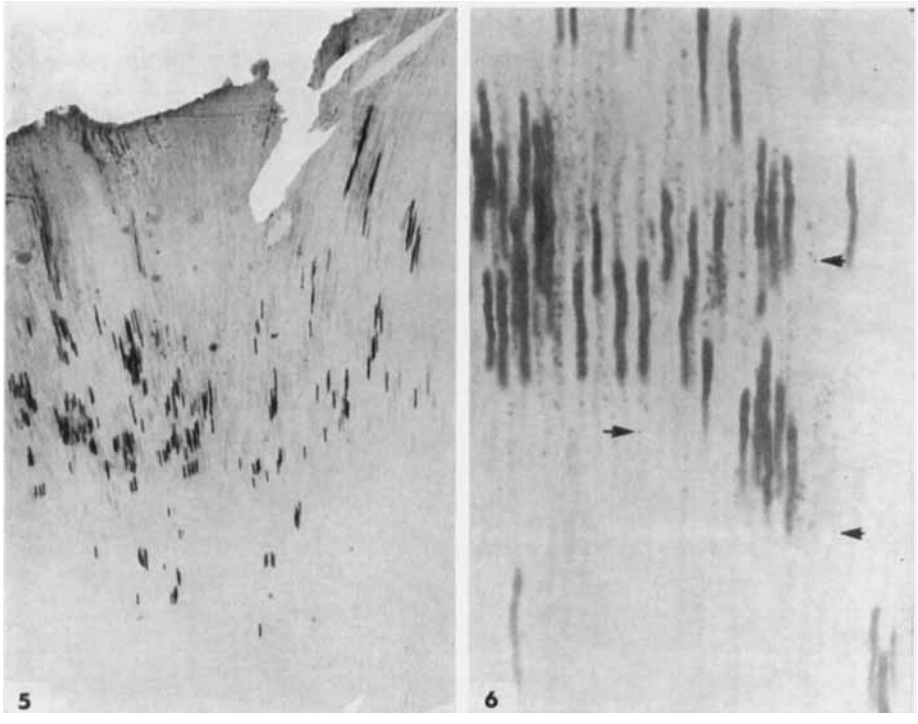

Fig. 5. β-Glucuronidase. A longitudinal section from a human premolar with advanced occlusal caries lesion of clinically active type. Reaction is seen in the carious lesion as dark stripes and spots in the dentinal tubules (some of which are marked with arrows). Substrate: naphthol AS-BI-β-D-glucuronic acid (10^{-4} moles/litre in the reaction mixture), Fast Red Violet LB salt. (40×)

Fig. 6. β-Glucuronidase. A higher magnification from Fig. 5. Note the spots (Gram + microorganisms, some of which are marked with arrows), which could be detected in the reference sections. (395×)

diamide in human carious dentine, the distribution of the catalyzing enzyme being similar to that of glycosidases, described earlier. No enzyme activity could in this case be observed in the predentine layer. The difference between the test and control sections was undetectable when β-chloroanilido phosphonic acid was used as substrate and thus the results of that series of tests could not be considered either.

When the Ebel's test for polyphosphate was carried out, it was observed that the black lead sulphide precipitation gradually increased in the deeper parts of the dentine, until, in the sclerotic and normal dentine layers, it suddenly decreased (Fig. 11), and the demarcation line was sharply distinguishable.

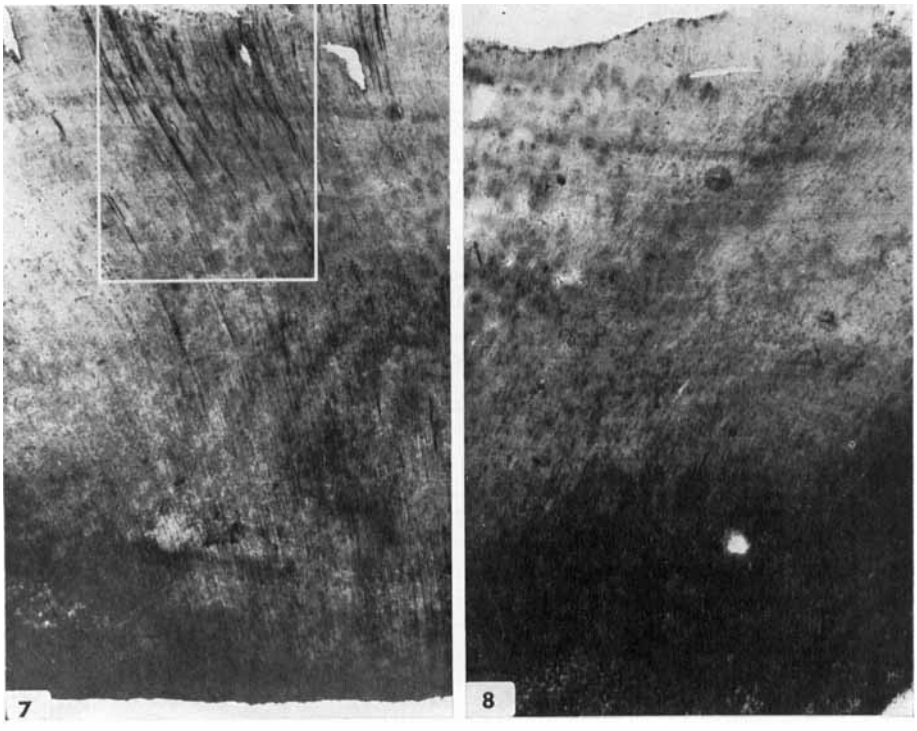

Fig. 7. Adenosine triphosphatase. A longitudinal section from a human molar tooth with a clinically active type of caries. The enzyme activity occurs as black precipitation of lead sulphide in the upper part of the tissue. The noticeable precipitation of lead sulphide was observed to occur nonenzymatically in the deeper parts of the lesion, as seen in Fig. 8. Substrate: Adenosine-5'-triphosphate, disodium salt (3×10^{-3} moles/litre in the reaction mixture). (40×)
Fig. 8. Control section from the tooth seen in Fig. 7, incubated without the substrate. Note the nonenzymic precipitation of lead sulphide in the lower part of the picture and the lack of dark stripes in the upper part of the picture. (40×)

No hydrolysis of any of the substrates mentioned above could be observed after heating the sections at +100°C for 15 min. None of the diazo methods described earlier revealed any colour formation when incubated without the substrates. The lead and calcium methods which were used in the determination of ATPase activity revealed nonenzymatic colour precipitation after heating and when incubated omitting the substrates. Incubation with free 1- or 2-naphthol (representing end products of the hydrolysis of some of the substrates) in the reaction mixtures instead of the substrate revealed that the colour formed had no avidity to certain sites on the tissue. Thus the azo dye formed was considered to occur at the site where the catalyzing enzymes occurred. The Gram staining revealed that the hydrolysis of all the

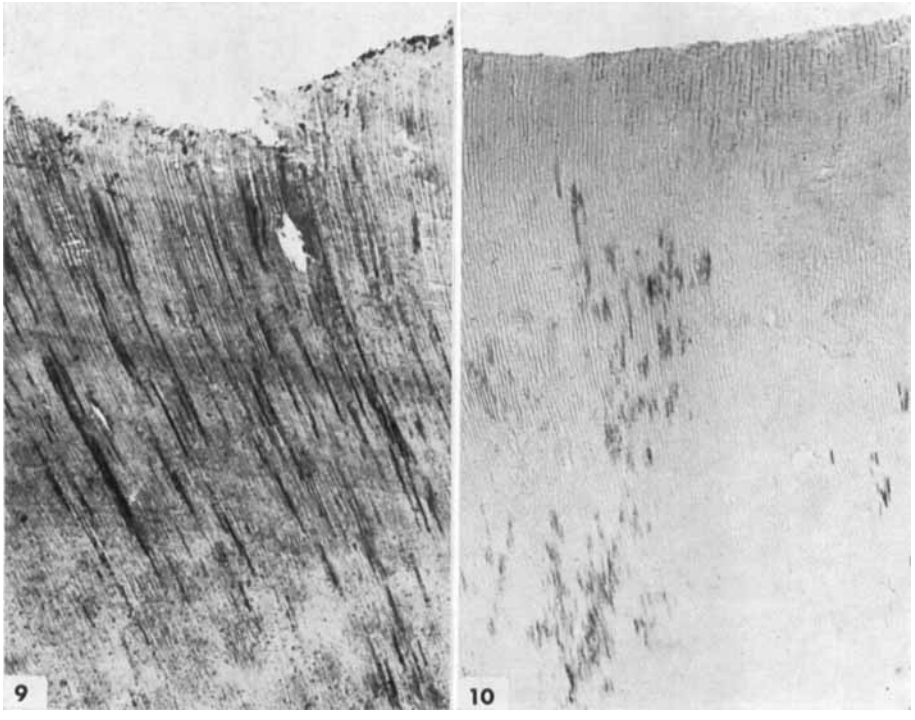

Fig. 9. Adenosine triphosphatase. A higher magnification from the area seen in Fig. 7. revealed the precipitation of lead sulphide in the superficial parts of the carious dentine to be enzymic. (100×)

Fig. 10. Phosphoamidase. A longitudinal section from a human molar tooth with occlusal caries. The enzyme reaction is seen as dark stripes along the dentinal tubules all over the section. Substrate: naphthol AS-BI-phosphodiamide 4 mg/25 ml of reaction mixture. Fast Blue B. (100×)

substrates (except ATP, which could not be observed because of the non-enzymic precipitation) also occurred in regions where Gram staining revealed no microorganisms.

DISCUSSION

The characterization of an enzyme by histochemical methods so exact that it would satisfy the criteria for the nomination of an enzyme according to the Enzyme Commission's Nomenclature, is impossible. In this study, however, the enzyme(s) acting on, for example, 6-bromo-2-naphthyl-β-D-glucuronide and Naphthol AS-BI-β-D-glucuronic acid were classified as β-

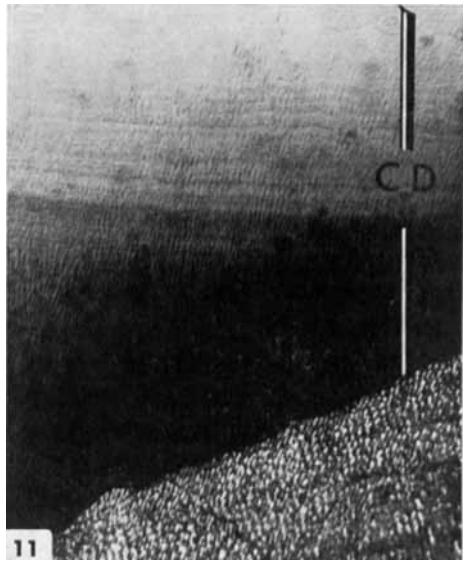

Fig. 11. Polyphosphate staining. The borderline between the carious (CD) and normal dentine can be seen as a sharply defined demarcation line. Ebel's polyphosphate staining, acetate buffer, pH 4.5. (120×)

glucuronidase (EC 3.2.1.31), but this does not mean that the enzyme really was the enzyme named. Rather, it may represent a nonspecific enzyme with glucuronidase activity. Further, demonstration of an enzyme does not mean it is active *in vivo*, as, for example, demonstrated earlier for the succinate dehydrogenase in carious dentine (*Larmas*, 1972c, *Larmas & Rosenquist*, unpublished). Thus no exact enzymechemical results can be expressed and only some cariological aspects of this general subject are discussed here.

Tappel (1969) presented the hydrolytic pathways of glycoproteins catalyzed by lysosomal enzymes, as seen in Fig. 12. It is thought that the sequential action of lysosymal sialidase, β-galactosidase, chitobiase, and α-mannosidase releases the corresponding sugars from the glycoproteins, but the pathways are not known in detail. When considering the possible catabolic pathways in dentine during the propagation of dentine caries, the present study revealed the occurrence of many of the glycosidases in carious but not in normal dentine, presented above in the lysosomal pathway. The hydrolysis of carious dentine polysaccharides (including acid polysaccharides) has been observed earlier by histochemical methods (*Toto*, 1966). It can be catalyzed by the glycosidases presented in this paper and by the arylsulphatase-like enzymes in carious dentine observed earlier (*Larmas*, 1968). Further, it is thought

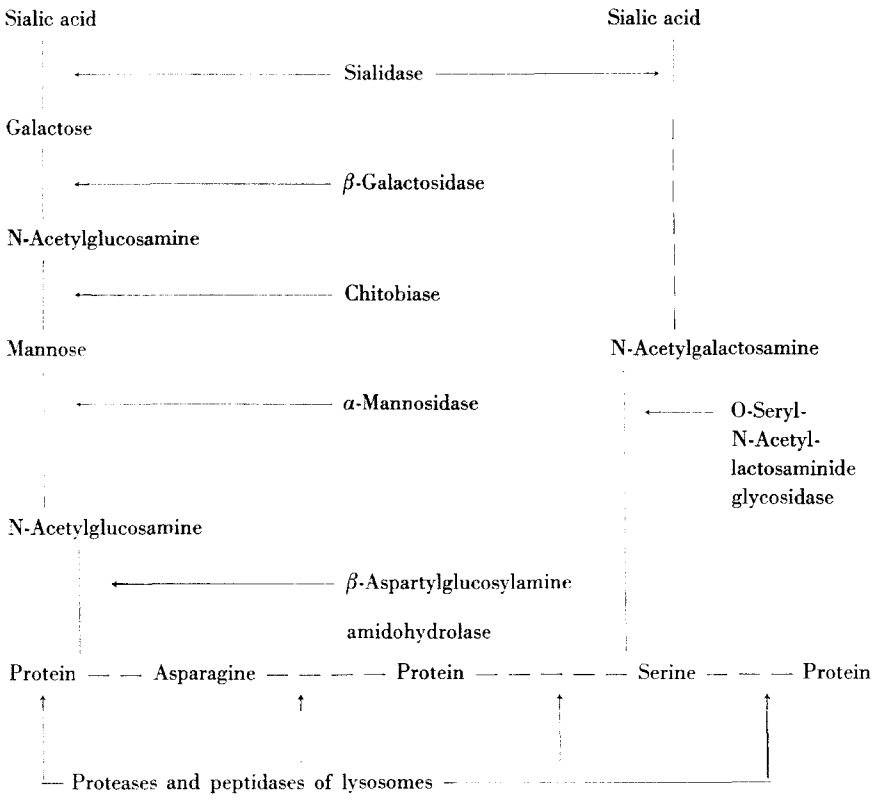

Fig. 12. Degradation of glycoproteins by lysosomes (*a.m.* Tappel, 1969).

that a number of glycosidases and proteolytic enzymes are involved in the degradation of mucopolysaccharideprotein complexes, as are esterolytic and proteolytic enzymes in the degradation of glucolipids and simple lipids by lysosymal hydrolytic enzymes (*Tappel*, 1969). The occurrence of peptide hydrolases (*Larmas, Mäkinen & Scheinin*, 1968, *Mäkinen, Larmas & Scheinin*, 1969, *Larmas & Mäkinen*, 1972), nonspecific esterases (*Larmas*, 1972a), and the occurrence of glycosidase like activity would indicate that the lysosymal hydrolytic pathways presented above may fulfil the logical requirements even for the carious dentine catabolism during the propagation of dentine caries.

The origin of these hydrolytic enzymes in carious dentine is probably microbial, because normal dentine revealed no enzyme activity. On the other

hand, the observation that enzyme activity was demonstrable also in infected but sterile layers of carious dentine would indicate that the enzymes diffuse ahead of the organisms which produce them, as discussed earlier (*Larmas, Mäkinen & Scheinin,* 1968; *Larmas,* 1968; *Larmas & Mäkinen,* 1972; *Larmas,* 1972a, b). Experimental studies also revealed that the hydrolytic enzymes probably are microbial (*Larmas,* 1971; *Larmas & Mäkinen,* 1971). However, the possibility that the enzymes in carious dentine (which process can be considered as sublethal or lethal cell injury) are lysosomal cannot be excluded, although no experimental works give support to that view.

The central importance of the phosphorus liberation in the carious process by acid dissolution has been known for a long time. More recent studies have shown an additional possibility for phosphorus liberation catalyzed by enzymes. It has been observed that certain commercial preparations of proteolytic enzymes were able to liberate phosphate from enamel and dentine powder (*Paunio, Mäkinen & Scheinin,* 1968; *Mäkinen & Paunio,* 1969). This liberation of phosphorus was at least partly, or perhaps totally due to the presence of ammonium sulphate in the commercial enzyme preparations (*Mäkinen & Paunio,* 1970; *Paunio & Mäkinen,* 1971). *Kreitzman et al.* (1969) have claimed that phosphate was enzymatically released from rat molar enamel by a phosphatase. The above findings of *Mäkinen & Paunio,* (1970, 1971), however, contradict this claim at least in part. Recently, *Kreitzman, Fritz* and *Saffir* (1970) reported that a phosphoprotein phosphatase liberated phosphorus from bone, too. *Holter* and *Li* (1950) reported that crystalline pepsin, trypsin, chymotrypsin and rennin are able to cleave phosphoamide bonds. Such enzyme activity does not seem to fit into the Enzyme Commission numbering, but is interesting because of the observations quoted above on enzymic release of phosphate from dental tissues. It is to be noticed that the Enzyme Commission classifies enzymes acting on phosphoamide bonds in creatine or arginine phosphates as phosphoamidases (phosphoamide hydrolase, EC 3.9.1.1) but suggests a possibility that the substrate specificity may be identical to that of phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16). Present findings revealed the occurrence of enzyme(s) acting on naphthol AS-BI-phosphodiamide, which would indicate the occurrence of phosphoamidase activity in human carious dentine. This phosphohydrolase and those reported earlier (*Larmas,* 1968; *Mäkinen, Larmas & Scheinin,* 1969; *Mäkinen,* 1970b) might hydrolyze the organic and inorganic components of dentine. One natural substrate to enzymes of this type might be the phosphoprotein of the dentine matrix (*Veis & Perry,* 1967).

The presence of ATP in cells is associated with a reciprocal loss of oxidative

phosphorylation, and thus in the anaerobic conditions ATPase has a central role in the energy utilizing processes (*Kielley*, 1961). Recent studies revealed the occurrence of succinate dehydrogenase (an enzyme of the Kreb's cycle) in carious dentine (*Larmas*, 1972b), but gasliquid chromatographic analyses revealed that no citric acid cycle acids were demonstrable in carious dentine. Lactate, on the other hand, was present (*Larmas & Rosenquist*, unpublished). These results were thought to indicate that the carious dentine metabolism was anaerobic, and thus the ATPaselike enzyme activity observed may be of central importance in the propagation of dentine caries in the energy utilizing processes. The presence of real ATPase in carious dentine was not established, because of the considerable non-enzymatic precipitation of lead sulphide on tissue sections. The polyphosphate staining (*Ebel*, 1952) was thought to indicate that in the deeper parts of carious dentine there are free acidic phosphate groups of polyphosphate type. In the decalcification process of dentine caries calcium is first removed, leaving free active phosphate groups in the inorganic and organic components of the dentine. The phosphate present at that stage of carious attack is still reactive and can be trapped.

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