Acta odont. scand. 1972 *30:* 555—573

Keywords:
Dental caries
Enzymes
Hydrolases
Oxidoreductases
Transferases

From:
The Institute of Dentistry,
University of Turku,
Finland

ENZYMES IN CARIOUS HUMAN DENTINE
A HISTOCHEMICAL AND BIOCHEMICAL STUDY

MARKKU LARMAS

Human carious dentine was excavated *in situ* or obtained from extracted teeth and sound dentine samples were prepared from extracted teeth. The dentine samples were cut in a cryostat for histochemical studies or crushed in a mortar for biochemical analyzes. Hydrolysis of numerous naphtol derivates was histochemically detected in dental plaque and in the tubules of carious dentine, indicating the occurrence of the following hydrolases in the structures: arylaminopeptidases (EC 3.4.1.1.—3.4.1.3.), nonspecific esterases (EC 3.1.1.), alkaline phosphatase (EC 3.1.3.1.), acid phosphatase (3.1.3.2.), ATPase (3.6.1.3.), phosphoamidase (EC 3.9.1.1.), α-glucosidase (EC 3.2.1.20.), β-glucosidase (EC 3.2.1.21.), βgalactosidase (EC 3.2.1.23.), β-glucuronidase (EC 3.2.1.31.), and arylsulphatase (EC 3.1.6.1.). Aryliminopeptidase (EC 3.4.1.4.) was observed only in dental plaque. Chitobiase activity (EC 3.2.1.29.) was not demonstrable in any structures. None of these enzyme activities was histochemically demonstrable in sound human dentine, whereas acid and alkaline phosphatase and α-glucosidase activities were additionally observed in the predentine. Histochemical studies further revealed the occurrence of the following oxidoreductases in dental plaque and in the tubules of carious dentine: lactate dehydrogenase (EC 1.1.1.27.), glucose 6-phosphate dehydrogenase (EC 1.1.1.49.), succinate dehydrogenase (EC 1.3.99.1.), glutamate dehydrogenase (EC 1.4.1.2.), glutathione reductase (EC 1.6.4.2.), and lipoamide reductase (EC 1.6.4.3.). Cystine reductase (EC 1.6.4.1.), alanine (EC 2.6.1.2.) and aspartate aminotransferases (EC 2.6.1.1.) were biochemically demonstrated in carious human dentine. These observations were thought to map a part of the metabolism catalyzed by enzymes in dentine during the propagation of caries.

The morphological features of dentine have been extensively studied both in normal and pathological conditions. These studies are important for understanding the structural changes occurring in the tissue during caries. However, little information is available on the normal metabolism of dentine or catabolism leading to destruction of the tissue, i.e. the initiation and propagation of, for example, dentine caries.

The importance of enzymes as catalysts of molecular interactions in cells

Received for publication, August 30, 1972.

and tissues in general is readily recognized, but until recently no information was available on enzymes occurring in carious dentine.

The present study was planned to map a part of the enzyme spectrum of normal and carious dentine. Certain enzyme activities* were considered as markers of various metabolic pathways in order to shed light on the anabolism and catabolism of the tissue. Tissue destruction is a primary characteristic of dentine caries and thus enzymes demonstrable only in carious dentine were thought to indicate mainly the primary or secondary catabolic metabolism, whereas enzymes demonstrable only in mature sound dentine and predentine were thought to be mainly anabolic in nature. Enzyme-chemical studies (connected with the more frequent analyses of the changes in the substrate) were thought to serve as a basis on which to build a better understanding of the processes involved in health and disease.

The present study covers a series of investigations on the occurrence and behaviour of certain enzymes in normal and carious human dentine, and was carried out in order to:

1) find out if certain enzyme activities are demonstrable in normal or carious dentine by present enzyme assay methods;
2) determine if the hydrolysis of peptide bonds of the organic stroma of the dentine can occur enzymatically, and if so, the localization and characterization of the enzymes involved;
3) demonstrate the eventual occurrence of esterases, phosphatases, phosphoamidases, pyrophosphatases, sulphatases, and glycosidases in carious dentine;
4) determine the eventual enzymic aminotransferase reactions in sound and carious dentine;
5) ascertain if hydrogen transfer reactions occur in carious dentine, and especially to determine if LDH occurs in the lesion;
6) determine if it is possible to distinguish between normal turnover metabolism and catabolism of the dentine during caries by estimating certain enzyme activities.

The final aim of the present study was to map the metabolism of carious dentine. It was hoped that this might reveal the key processes leading to the

---

* The classification and nomenclature of enzymes recommended by the international Commission on Enzymes *(EC., Florkin & Stotz,* 1965) is followed in this treatise, although it is realized that by the methods used the enzymes were not thoroughly characterized and thus one particular enzyme may in fact be a group of enzymes or even a quite different enzyme with a wide substrate specificity. For the simplification of the presentation in this treatise, for example, »alkaline phosphatase-like enzymes» or more correctly »the enzymatic hydrolysis of a phosphate monoester at an alkaline pH value» will be merely termed »alkaline phosphatase».

destruction of dentine so that in the future these may be controlled clinically by chemical means.

MATERIAL AND METHODS

MATERIAL

For the biochemical studies carious dentine was excavated either (1) from freshly extracted permanent and deciduous teeth or (2) from living persons. Care was taken to avoid pulp perforation during this procedure. The dentine fragments were ground at +4°C in a porcelain mortar and all the samples were weighed (fresh) and the enzymes extracted as described in detail elsewhere (V). For the histochemical studies the material was either (1) excavated *in situ* or (2) carious and sound dentine samples were cut with a highspeed drill under a continuous jet of cold water. In both cases the samples were cut in a cryostat as described in detail elsewhere (V).

The possible liberation of the enzymes during the drilling of the samples under the continuous jet of cold water was additionally controlled as follows:

This study is based on the following papers which are referred to in the text as I, II, III etc.

I *Larmas, M., K. K. Mäkinen* and *A. Scheinin,* 1968: Histochemical studies on the arylaminopeptidase activity in human carious dentine. Acta odont. scand. *26:* 127—126.

II *Larmas, M.* and *K. K. Mäkinen,* 1972: Histochemical demonstration of enzymes hydrolyzing N-L-arginyl- and N-L-prolyl-2-naphthylamine in human carious dentine. Caries Res. *6:* 60—65.

III *Mäkinen, K. K., M. A. Larmas* and *A. Scheinin,* 1969: Activity of arylaminopeptidases, phosphatases, and L-cystine cleaving enzymes in normal and carious human dentine. Caries Res. *3:* 134—148.

IV *Larmas, M.* and *K. K. Mäkinen,* 1971: The ability of various microorganisms to produce histochemically determinable enzyme activity in human dentine. Acta odont. scand. *29:* 471—486.

V *Larmas, M.,* 1972: A chromatographic and histochemical study of nonspecific esterases in human carious dentine. Arch. oral Biol. *17:* 1121—1132.

VI *Larmas, M.,* 1968: Histochemical studies on phosphatase and arylsulphatase activity in human carious dentine. Acta odont. scand. *26:* 357—367.

VII *Larmas, M.,* 1972: Histochemical studies on enzymes acting on glycosyl compounds, phosphoryl-containing anhydrides, and phosphoamides in human carious dentine. Acta odont. scand. *30:* 201—218.

VIII *Larmas, M.,* 1972: Alanine and aspartate aminotransferases in sound and carious human dentine. Arch. oral Biol. *17:* 1121—1132.

IX *Larmas, M.,* 1972: Histochemical demonstration of various dehydrogenases in human carious dentine. Arch. oral Biol. *17:* 1143—1153.

the jet of cold water was directed onto the carious lesion without drilling for 1 min., and the water collected (18 ml.). The hydrolysis of *N*-L leucyl-2-naphthylamine was biochemically estimated both from the water and from the carious dentine after homogenization and it was observed that less than 5 % of the enzymes hydrolyzing this substrate was extracted from the samples during drilling of the tissue for histochemical purposes.

For histochemical studies 254 carious teeth were used, for experimental procedures sound coronal dentine was obtained from 48 intact teeth and for the biochemical analyses 29.56 g (fresh weight) of carious dentine and 61.43 g (fresh weight) of sound coronal dentine were used. The complete material consisted of carious dentine from several thousand permanent teeth and sound dentine from several hundred teeth, extracted mainly for orthodontic reasons.

METHODS

Morphological methods

The frozen unfixed samples were cut in a cryostat and the localization of enzymes was detected using various histochemical assay methods. The sections were also stained with haematoxylin and eosin for reference and by the Gram method to detect and localize the microorganisms. The sections were also studied under a polarizing microscope to detect the demineralized structures.

The carious dentine was classified as active or arrested according to *Miller* and *Massler* (1962). The classification of various zones in carious dentine was as described elsewhere (VII).

For the histochemical estimation of hydrolytic enzymes the azo-coupling principle (*Menten, Junge & Green*, 1944) was selected as the main method, because: (1) it is simple to use, (2) it is highly sensitive, (3) there was no need for electron density because only light microscopic studies were involved, and (4) the tissue itself did not contain naphthol derivates and thus no intrinsic factor interfered markedly with the coupling reagent. The metal salt procedures (*Gomori*, 1939; *Takamatsu*, 1939), for example, seemed to be less suitable because the tissue itself evidently contained free phosphate groups (possibly of the polyphosphate type, *Ebel*, 1952) which also reacted nonenzymatically with the trapping agent (VII). The dehydrogenases were assayed according to the tetrazolium procedure (*Kuhn & Jerchel*, 1941) in which the reduction of the tetrazolium yields a coloured formazan precipitate. The particular modification of the methods described above used in assaying individual enzymes is given in detail in papers I to IX.

Many factors must be considered when analyzing the results of reactions on tissue sections (*Pearse*, 1960; *Barka & Anderson*, 1963; *Lillie*, 1965; *Chayen et al.*, 1969). In order to test some of the variables which may cause false results the following parameters were controlled: (1) the substrate, coenzyme, diazonium and tetrazolium salt concentrations, (2) incubation time, (3) pH of the medium, (4) the effect of the diazonium and/or tetrazolium salts without the substrate, and (5) the avidity of the end products to certain sites of the tissue, as described in detail elsewhere (II, IX). Additionally, tests to determine the diffusion of enzymes hydrolyzing *N*-L alanyl-2-naphthylamine and *N*-L leucyl-2-naphthylamine were made. Inactivated sections (heating 100°C for 30 min) were incubated at +4°C in buffer solution in contact with active sections, and thereafter both sections were incubated separately as in the normal arylaminopeptidase assay (I, II). It was observed that no diffusion of enzymes was histochemically demonstrable, whereas the biochemical enzyme assay (III) from the buffer solution revealed that about 20 % of the enzymes were soluble. The diffusion of the end product of the arylaminopeptidase assay was controlled as follows: inactivated sections were incubated in contact with active sections as in the normal arylaminopeptidase assay (I, II) and both sections were examined separately. It was observed that a weak colour reaction occurred in inactivated sections in this case, this activity localizing in the tubules of carious but not of normal dentine, indicating that the end product diffundated slightly from the site of formation.

Biochemical methods

Enzyme assay methods

The azo-coupling technique (*Goldberg & Rutenburg*, 1958) was selected when possible to estimate the activity of hydrolytic enzymes because of the possibility of referring histochemical results to biochemical. The aminotransferase activity was assayed using 2,4-dinitro phenylhydrazine (*Reitman & Frankel*, 1957) or an azo dye (6-denzamido-4-methoxy-m-toluidine diazonium chloride, *Babson et al.*, 1962) as the colour developer.

Determination of protein, amino acid, sodium, and keto acid concentrations

The Folin-Ciocalteu method was selected for the determination of protein concentration (*Layne*, 1963) because of its high sensitivity. Bovine serum albumine (Sigma Chemical Company, St. Louis) was used as a standard. The amino acids were analyzed with an amino acid analyzer (III), sodium by

atomic adsorption spectrophotometry (III), and keto acids colorimetrically (*Friedemann & Haugen*, 1943).

Chromatographic methods

Molecular exclusion chromatography was conducted on Sephadex columns, and ion-exchange chromatography on DEAE-cellulose columns. Gas-liquid cromatographic analyses were conducted on a gas chromatograph (V).

Experimental methods

In order to determine the possible extrinsic sources of certain hydrolytic enzymes an experimental study was undertaken. Pieces of coronal dentine from extracted sound human teeth were sterilized and the possible enzyme activities in the tissue thus destroyed. These pieces of sound dentine were incubated for 28—68 days at +37°C in various culture media which were infected by one species or strain of microorganism at a time. The infected dentine pieces were transferred daily to fresh media. After the pieces were sufficiently softened, cryostat sections were made and enzyme activities demonstrated histochemically in the dentine (IV).

RESULTS AND DISCUSSION

HYDROLASES IN CARIOUS DENTINE

α-Amino-acyl-peptide hydrolases (EC 3.4.1.)

Ultrastructural studies indicate that the changes in dentine which is affected by caries seem to be a demineralization process which is followed by dissolution of organic material (*Johansen*, 1963, 1965; *Johnson, Taylor & Bergman*, 1969). The mechanism of protein hydrolysis in dentine is not known. Because no information on proteolytic enzymes in carious dentine was available, a group of peptide hydrolases (arylamino and arylaminopeptidases) was selected as a marker of possible enzymatic protein hydrolysis.

Results

Distribution of arylaminopeptidases in dentine (I, II). Histochemically it was possible to demonstrate the hydrolysis of *N*-L-leucyl, *N*-L-methionyl, *N*-L-alanyl and *N*-L-arginyl-2-naphthylamine in human carious dentine but not in normal dentine. No hydrolysis of *N*-L-aspartic acid and *N*-L-lysine-2-

napthylamine could be observed. Hydrolysis of all the substrates was observed in the dental plaque layer and in the superficial infected parts of the dentine. The enzyme activity occurred as narrow stripes along the dentinal tubules, and in some cases a more noticeable hydrolysis could be observed in deeper parts of carious dentine than in superficial layers. Even on the borderline between the histologically normal and carious dentine the hydrolysis of *N*-L-leucyl, *N*-L-alanyl and *N*-L-arginyl-2-naphthylamine could be observed, but in normal mature dentine and predentine no hydrolysis was ever observed by histochemical methods.

Biochemical properties of arylaminopeptidases in dentine (III). The most rapidly hydrolyzed aminoacyl 2-naphthylamines were those of L-arginine, L-lysine, L-alanine, L-ornithine, and L-leucine by crude enzyme preparations both from carious and sound dentine. Hydrolysis rates calculated per mg protein in the samples were at least ten times higher in carious than in sound dentine. The pH optimum of the hydrolysis was 7.0 and the enzymes were inactive close to about pH 5.0. Various divalent metal cations such as Zn^{2+} and Fe^{2+} ions inhibited totally, and Mn^{2+}, Ca^{2+} and Co^{2+} ions inhibited strongly the hydrolysis of *N*-L-alanyl- and N-L-arginyl-2-naphthylamines. Other experiments did not reveal the importance of imidazole as a nucleophile in the action of any of the enzymes, nor did they reveal the participation of an active serine residue.

Discussion

Peptidyl hydrolases have not been fully characterized owing to their overlapping specificities and thus in this treatise a less specific trivial nomenclature *arylamino-* or *arylaminopeptidases* was selected as suggested by *Mäkinen* (1969). Histochemically arylaminopeptidases have been observed in odontoblasts in experimental animals during odontogenesis (*Burstone*, 1960; *Korhonen & Näätänen*, 1963) and by biochemical methods it was possible to demonstrate weak arylaminopeptidase activity also in sound dentine (III). These findings do not exclude the possibility that enzymes in carious dentine in mature teeth might be of endogenous origin. However, a primary exogenous origin seemed to be more probable because: (1) a negative arylaminopeptidase reaction in the odontoblasts of experimental animals has also been reported (*Quintarelli*, 1961; *Yoshiki*, 1962; *Hammarstrom, Toverud & Hanker*, 1971), (2) enzyme activity calculated per mg protein was at least ten times higher in carious than in sound dentine, (3) no histochemically demonstrable enzyme activity was observed in sound dentine, (4) caries-like lesions produced *in vitro* revealed that oral candida and lactobacilli were the

most potent producers of arylaminopeptidases, the distribution of enzyme activity being similar to that in natural carious lesions (IV).

These enzymes were thought to play a role in the enzymatic hydrolysis of peptide linkages of dentine matrix proteins and thus, for example, they may be related to the preinflammatory peptide formation as suggested by *Hopsu et al.* (1966), and *Raekallio* and *Mäkinen* (1967).

Carboxylic ester hydrolases (EC 3.1.1.)

Two degenerative processes have been reported to occur in dentinal tubules preceding the decalcification of the caries process: a calcification and a fatty degeneration resulting in the formation of sclerotic dentine (*Berggren,* 1965). *Willner* (1936) reported a special layer of fatty degeneration under the formation of the zone of dentinal sclerosis, but *Wilson* (1940) and *Miller* (1969) did not find fatty degeneration at all. Because of the possible enzymatic fatty degeneration occurring under and in the carious lesion carboxylic acid esterases were studied.

Results (V)

The distribution of the hydrolysis of l-naphthyl acetate was the same as described for *N*-L-leucyl-2-napthylamine, and thus no hydrolysis could be observed histochemically in normal dentine (including sclerotic dentine). By biochemical methods, practically no enzyme activity was obtained from sound dentine. The water extractable fraction of carious dentine revealed the highest enzyme activity towards 1- and 2-naphthyl acetate, 1-naphthyl valerate, and 1-naphthyl propionate. Fractionation by DEAE-cellulose revealed two enzymes with esterolytic activity. The pH optimum of both enzymes was 7.4. Hg^{2+}, L-cysteine, and dithiothreitol (9.34×10^{-4}M) inhibited totally the hydrolysis of l-naphthyl acetate.

Discussion

Knowledge on esterases in biological material is not so complete and the nomenclature varies from one author to another so much that in this treatise all the enzymes with carboxylic esterase activity found in dentine are termed nonspecific esterases. The origin of nonspecific esterases in carious dentine might be endogenous because esterase activity has been described in odontoblasts at least during odontogenesis (*Yoshioka et al.*, 1960a, *Quintarelli*, 1961, *Yoshiki*, 1962). On the other hand, *Burstone* (1957, 1960) and *Burstone* and *Keyes* (1957) observed no activity in the odontoblasts of experimental animals and the present findings that practically no activity was found in

sound dentine would indicate that these enzymes in carious dentine are of exogenous origin.

The natural substrates in dental tissues for these types of enzymes were thought to be the membrane lipids and glycolipids of the odontoblast processes.

Phosphoric monoester hydrolases (EC 3.1.3.)

Many kinds of biologically important phosphate esters occur in mineralized dentine structures. In order to obtain information on the possible enzymatic phosphate ester hydrolysis, acid and alkaline phosphatase-like enzyme activities were selected as marker enzymes of the metabolism involved.

Results (III, VI)

The hydrolysis of l-naphthyl phosphate at acidic pH values was observed to occur in the same areas as *N*-L-leucyl-2-naphthylamine. Additionally, hydrolysis was observed in the predentine. No hydrolysis of l-naphthyl phosphate was observed at alkaline pH values in decalcified zones of carious dentine, whereas the predentine layer revealed marked hydrolysis. In the case, however, when the carious process had reached the predentine, marked alkaline phosphatase activity was found to be spreading from the exposure area into the layer of decalcification in dentinal tubules. The rate of hydrolysis of l-naphthyl phosphate by crude enzyme preparation from carious dentine was higher at pH 9.2 than at pH 7.0. Mg^{2+} ions did not cause a higher rate of the hydrolysis of l-naphthylphosphate.

Discussion

Though some overlap of alkaline and acid phosphatase can occur, the presence and characteristics of each can be distinguished. However, in this treatise these enzymes are dealt with together because they catalyze the hydrolysis of the same bond. An activation by Mg^{2+} ions is a general characteristic of alkaline phosphatases, and thus the enzyme(s) hydrolyzing l-naphthylphosphate on the alkaline side were not true »alkaline phosphatases». On the other hand, more recent studies (*Mäkinen*, 1970) revealed that 1 mM $MgCl_2$ slightly activated most of the catalysis of various phosphates by crude enzyme preparations from carious dentine, indicating the possible presence of an alkaline phosphatase in the tissue. Thus the phosphatase spectrum of dentine seems to be wide and specific nomenclature especially concerning phosphatases in carious dentine cannot be obtained.

It seems probable that phosphoric monoester hydrolases in carious dentine originate both from the bacterial plaque and from the odontoblasts. The intrinsic origin is supported by the facts that (1) alkaline phosphatase activity was demonstrable in odontoblasts during odontogenesis (*Nuki & Bonting*, 1961; *Burstone*, 1960, *Zerosi & Baratieri*, 1954; *Symons*, 1962; *Yoshiki & Kurahashi*, 1971), (2) acid phosphatase activity has also been reported in odontoblasts (*Quintarelli*, 1961; *Yoshiki*, 1962) and in predentine (*Burstone*, 1960; *Yoshiki*, 1962), (3) hydrolysis of l-naphthyl phosphate was observed in the predentine of mature human tooth and in the case when carious process reached the pulp, intense hydrolysis was observed to be spreading from the site of exposure (VI). On the other hand, an exogenous origin also seems to be possible, although the microbiological experimental study did not reveal histochemically demonstrable enzyme activity in artificial caries of dentine (IV). It may be due to technical difficulties as, for example, the enzymes might be so soluble that they are histochemically undemonstrable. Although negative phosphatase reactions have been reported in odontoblasts (*Sasso & Castro*, 1957; *Ten Cate*, 1962) this may be due to different assay methods (metal salt procedures contra azo dye technique, *Ten Cate*, 1962) and inter species variations, and thus these enzymes seem to be both intrinsic and extrinsic.

The significance of phosphoric monoester hydrolases in dentine was thought to be connected with (1) catalysis of the destruction processes of phosphate esters in dentine during caries and (2) catalysis of the response mechanisms of the tissue against caries (mineralization and sealing off the affected dentinal tubules, synthetic phenomena in the formation of secondary dentine proteins etc.).

Phosphoamidases (EC 3.9.1.1.) and pyrophosphatases (EC 3.6.1.)

The phosphoamide bond in creatine or arginine may store energy, as does the pyrophosphate bond in adenosine triphosphate. In order to obtain information on enzymes hydrolyzing these bonds, which hydrolysis is important in energy utilizing processes in carious dentine, phosphoamidase and ATPase activity was studied.

Results (VII)

Hydrolysis of naphthyl AS-BI phosphodiamide was distributed in the carious lesion as described earlier for arylaminopeptidases, i.e. occurring as distinct stripes along the dentinal tubules in infected and sterile zones of decalcification in carious dentine. No enzyme activity could be observed in sound dentine

nor in predentine. When p-chloroanilido phosphoric acid was used as substrate no differences between actual and control sections could be observed. By the lead method possible enzymatic hydrolysis of ATP could be observed in infected zones of carious lesion, whereas sterile layers, sound dentine and predentine revealed a very marked non-enzymatic precipitation of lead, and thus no differences between actual and control sections could be observed.

Discussion

By the histochemical methods some evidence of the hydrolysis of ATP and phosphodiamide in carious dentine could be demonstrated and thus the histochemical findings were in agreement with the biochemical (*Mäkinen*, 1970; *Larmas*, 1972). However, the presence of true ATPase and phosphoamidase in the dentine has not yet been established. The intrinsic source of these enzyme in carious dentine is supported by the observation that at least during the dentinogenesis ATPase activity has been reported in odontoblasts and in the subodontoblast layer (*Pourtois*, 1962, a, b; *Heyden & From*, 1969a). Recent quantitative biochemical observations, however, revealed considerably higher enzyme activity in carious than in sound dentine (*Mäkinen*, 1970; *Larmas*, 1972) and thus these enzymes were thought to be produced by microorganisms. *Paunio, Mäkinen* and *Scheinin* (1968) and *Mäkinen* and *Paunio* (1969) reported that certain commercial preparations of proteolytic enzymes were able to liberate phosphate from enamel powder. *Kreitzman et al.* (1969) also reported that enzymes probably with phosphoprotein phosphatase activity catalyze the rapid demineralization of tooth enamel. This liberation of phosphorus was at least partly due to the presence of ammonium sulphate in the commercial enzyme preparation (*Mäkinen & Paunio*, 1970; *Paunio & Mäkinen*, 1971). Because the phosphoamidase may be identical to phosphoprotein phosphatase (*Florkin & Stotz*, 1965) and certain proteolytic enzymes can also cleave phosphoamide bonds (*Holter & Li*, 1950), the possibility that the hydrolysis of phosphoamide bonds may play a role in the degradation of dentine structures cannot be excluded. The natural substrate might be the phosphoprotein of dentine matrix (*Veis & Perry*, 1967).

Glycosidases (EC 3.2.1.) and arylsulphatase (EC 3.1.6.1.)

Complex polysaccharides, glycoproteins, and glycolipids comprise an important part of cellular membranes. The enzymatic hydrolysis of these glycosaminoglycans seems to be of central importance in cell injury, and thus hydrolytic enzymes acting on these compounds may also play an important role in dentine catabolism.

Results (VII)

By histochemical methods it was possible to demonstrate the hydrolysis of α- and β-glucosidase, β-glucuronidase, β-galactosidase and arylsulphatase substrates in the same areas as arylaminopeptidases. Additionally, the hydrolysis of β-glucuronidase and α-glucosidase substrates could be observed 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. The hydrolysis of 6-bromo-2-naphthyl-β-D-glucuronide and 6-bromo-2-napthyl-α-D-glucoside by enzyme preparations obtained by DEAE-cellulose chromatography from human carious dentine was observed to occur. (V).

Discussion

On the basis of their distribution, glucosidases and arylsulphatases in carious dentine were thought to originate from microorganisms in the tissue. An experimental microbiological study also revealed that many oral microorganisms were able to produce arylsulphatase activity. Oral candida, lactobacilli and streptococci produced abundantly histochemically demonstrable activity in dentine (IV). However, possible intrinsic sources of glycosidases cannot be excluded because enzyme activity has been reported in odontoblasts at least during odontogenesis (*Yoshioka et al.*, 1960b; *Quintarelli*, 1961; *Yoshiki*, 1962) and also in mature predentine (VII).

The possible functions of these enzymes were thought to be connected with the catabolism of acid mucopolysaccharides in dentine. Histochemically the loss of acid mucopolysaccharides in human carious dentine has been demonstrated (*Toto*, 1966; *Takuma et al.*, 1967), and thus the enzymatic hydrolysis of these compounds can partly cause the propagation of dentine caries.

TRANSFERASES IN CARIOUS DENTINE

Aminotransferases (EC 2.6.1.)

The pyridoxal dependent transamination reaction is intimately associated with amino acid metabolism both in its anabolic and catabolic phases. In some lactic acid bacteria such reactions provide the only available route for synthesis of many of the amino acids (*Broquist & Snell*, 1949; *Holden et al.*, 1951). The participation of transamination in the reversible formation of alanine, aspartic and glutamic acids from the corresponding keto acids provides an important link between carbohydrate and protein metabolism. The aminotransferase reactions were studied in dentine in order to obtain further information on the metabolism of the tissue.

Results (VIII)

It was observed that aspartate aminotransferase activity was higher than alanine aminotransferase activity both in sound and carious dentine. Both enzyme activities calculated per weight of the sample were higher in carious than in sound dentine. When the enzyme activities were calculated per mg protein in the sample, those activities in sound dentine were higher than, or equal to, those observed with carious dentine, indicating that only nonstructural proteins, such as many enzymes, were water-extractable from sound dentine. By DEAE-cellulose chromatography one main enzyme peak representing aspartate aminotransferase activity was obtained from carious dentine. The pH optimum was 7.4. This enzyme was stable for one hour at temperatures from +35° to +50°C. The optimum temperature was +37°C. Hg^{2+} ions (2.0 mM), L(+) cysteine (2.0 mM), and dithiothreitol (0.2 mM) inhibited totally the enzymatic transamination of aspartate.

Discussion

The origin of these enzymes was thought to be both extrinsic and intrinsic. *Armstrong* and *Atkinson* (1951) reported the occurrence of alanine and aspartate aminotransferases in sound human dentine and thus the present findings were in agreement with their observation. Possible microbial origin, however, cannot be excluded.

These enzyme activities in carious dentine were connected: (1) with transdeamination processes leading to the degradation of excess structural amino acids of dentine, resulting in the formation of corresponding keto acids and ammonia, (2) with transreamination, which was thought to provide the only available route for the synthesis of many amino acids in some lactic acid bacteria in the infected carious dentine.

OXIDOREDUCTASES IN CARIOUS DENTINE

Lactate (EC 1.1.1.27.), glucose 6-phosphate (EC 1.1.1.49.) and succinate dehydrogenases (EC 1.3.99.1.)

These enzymes were selected as marker enzymes of possible lactic acid formation, pentose shunt pathway, and citric acid cycle in order to obtain information on the carbohydrate metabolism in dentine during caries.

Results (IX)

By histochemical methods lactate, glucose 6-phosphate, and succinate dehydrogenase activity was observed in the dental plaque layer and in the

carious dentine, SDH additionally in the predentine. Normal dentine revealed no enzyme activity. $HgCl_2$ (10^{-3} M) inhibited totally all the enzymes mentioned, p-chloromercuri benzoate (5×10^{-3}M) and $AgNO_3$ (10^{-3} M) additionally the activity of LDH and G 6-PD and NaF (5×10^{-3} M) the activity of SDH. No activity of LDH could be observed below pH 5.5.

Discussion

Because no enzyme activity was normally demonstrable in sound dentine, the microbial origin of these enzymes in carious dentine seemed to be most probable. However, it also seems to be highly probable that these enzymes at least partly originate from the cell organelles in dentine because: (1) they are demonstrable in odontoblasts during odontogenesis (*Nuki* & *Bonting*, 1961; *Balogh*, 1962, 1963; *Fullmer*, 1963; *Fullmer* & *Martin*, 1964; *Heyden* & *From*, 1969b; *Schwarz* & *Yaeger*, 1961; *Pourtois*, 1962b), (2) the predentine below a carious lesion also revealed enzyme activity (IX), (3) LDH was observed by histochemical methods (*Godt* & *Lehmann*, 1971a, b.) and by biochemical assay (*Bosia et al.*, 1970) also in sound bovine dentine.

These observations were thought to indicate that aerobic and anaerobic glycolysis and pentose shunt pathway would be possible in carious dentine metabolism. Recent gas-liquid chromatographic analyses, however, revealed that lactate was the only metabolite which gave a peak of those citric acid cycle metabolites studied (*Rosenqvist* & *Larmas*, 1973). This observation was thought to indicate, for example, that demonstration of an enzyme in a metabolic pathway does not necessarily mean that the pathway is active unless changes in the substrate are also detected. Many microorganisms in carious dentine may behave both aerobically and anaerobically, but in the carious tissue they seem to behave mainly anaerobically.

Glutamate dehydrogenase (*EC 1.4.1.2.*)

This enzyme has a key role in cellular metabolism because it also links the amino acid metabolism to the citric acid cycle, and this enzyme activity is closely linked with transaminase activity in damaged cells (*Bitensky*, 1967). This dehydrogenase activity has been found to be a very sensitive indicator of cellular damage (*Kirkby*, 1965) The enzyme activity was studied in order to obtain information on amino acid metabolism in carious dentine.

Results (*IX*)

The distribution of this enzyme was similar to that described for SDH. A very marked activity was observed in the liquefaction foci of the carious

dentine. The enzyme activity was totally inhibited by Ag^{+}, Hg^{2+}, and p-chloromercuri benzoate ions (all 10^{-3} M).

Discussion

The enzyme activity was thought to be linked with the transaminases to the amino acid metabolism of carious dentine and this would then partly explain the noncorrelated results between arylaminopeptidase activity and amino acid analyses in carious dentine (III). The activity in the predentine was thought to be linked with the damage of the odontoblasts during the carious process.

Cystine (EC 1.6.4.1.), glutathione (EC 1.6.4.2.), and lipoamide reductase (EC 1.6.4.3.)

Enzymes acting on reduced forms of the coenzymes act in the cells as a part of the hydrogen transport chain linking the reduced coenzyme with O_2. They are comparatively unspecific in regard to the other reactant. Because this type of tissue enzyme system is also essential for histochemical demonstration of dehydrogenase activity, these enzymes were studied.

Results (III, IX)

By histochemical methods it was observed that reduced forms of NAD and NADP were enzymatically oxidized (1) in the dental plaque layer, (2) in carious dentine and (3) in the predentine layer, but not in the sound dentine. By biochemical methods it was also observed that pyridoxal 5-phosphate dependent keto acid formation occurred.

Discussion

Based on the results mentioned above it seems probable that human carious dentine contains enzymes capable of oxidating the reduced NAD or NADP, making possible the histochemical tetrazolium procedure for dehydrogenases. Biochemical studies also supported the observation of *Mäkinen* (1968) that human carious dentine contains an enzyme, disulphide reductase, known to convert L-cystine to L-cysteine. Further, these studies gave evidence of the existence of desulfhydrase enzymes in carious dentine, which convert L-cysteine to pyruvic acid, ammonia and hydrogen sulphide.

Acknowledgements. Financial support provided by the *Finnish Dental Society*, the *Yrjö Jahnsson Foundation* and the *National Council for Research in Medical Sciences*, Finland, is gratefully acknowledged.

REFERENCES

Armstrong, W. G. & H. F. Atkinson, 1951: Transamination in the human teeth. Nature (Lond.) *98:* 695—696.

Babson, A. L., P. O. Shapiro, P. A. R. Williams & C. E. Phillips, 1962: The use of a diazonium salt for the determination of glutamic-oxalcetic transaminase in serum. Clin-chim. Acta *7:* 199—205.

Balogh, K., 1962: Decalcification with versene for histochemical study of oxidation enzyme systems. J. Histochem. Cytochem. *10:* 232—233.

Balogh, K., 1963: Histochemical study of oxidative enzyme systems in teeth and periodontal tissues. J. dent Res. *42:* 1457—1466.

Barka, T. & P. J. Anderson, 1963: Histochemistry. Theory, practice and bibliography. Hoeber Medical Division, New York, Evanston, and London.

Berggren, H., 1965: The reaction of the translucent zone of dentine to dyes and radio-isotopes. Acta odont. scand. *23:* 197—213.

Bitensky, L., 1967: Histochemistry on experimental immunology. In: Handbook of Experimental Immunology. Ed by *Weir,* D.M., Blackwell, Oxford.

Bosia, A., P. Arese & M. Pezzoli, 1970: Glycolytic enzyme activities in bovine hard dental tissue at different ages. Calcif. Tiss. Res. *6:* 93—102.

Broquist, H. P. & E. E. Snell, 1949: Studies of the mechanisms of histidine synthesis in lactic acid bacteria. J. biol. Chem. *180:* 59—71.

Burstone, M. S., 1957: Esterase activity on developing bones and teeth. A.M.A. Arch. Path. *63:* 164.

Burstone, M. S., 1960: Hydrolytic enzymes in dentinogenesis and osteogenesis. In: Calcification in Biological Systems. Ed. by *Sognaes,* R.F.) pp. 117—243. Am. Ass. for Advancement of Sci.

Burstone, M. S. & P. H. Keyes, 1957: Studies on calcification. I. The effect of inhibition of enzyme activity on developing bone and dentin. Amer. Path. *33:* 1229.

Chayen, J., L. Bitensky, R. G. Butcher & L. W. Poulter, 1969: A Guide to Practical Histochemistry. Oliver & Boyd, Edinburgh.

Ebel, J. P., 1952: Recherches sur les polyphosphates contenus dans diverses cellules vivantes. IV. Localisation cytologique et rôle physiologique des polyphosphates dans la cellule vivante. Bull. Soc. Chim. biol. (Paris) *34:* 498—505.

Friedemann, T. E. & G. E. Haugen, 1943: Pyruvic acid. II. The determination of keto acids in blood and urine. J. biol. Chem *147:* 415—442.

Fullmer, H. M., 1963: Dehydrogenases in developing teeth of rats. J. Histochen. Cytochem. *11:* 641—644.

Fullmer, H. M. & G. R. Martin, 1964: On the activity of D(—)-p-hydroxybutyric dehydrogenase in scruvy. Nature (Lond.) *202:* 302.

Florkin, M. & E. H. Stotz, 1965: Enzyme Nomenclature. Comprehensive Biochemistry, Vol. 13. Elsevier, Amsterdam.

Godt, H. & J. Lehmann, 1971a: Zur Lokalization der Lactat-Dehydrogenase im Dentin mitteils histochemischen Nachweises. DDZ *25:* 37—39.

Godt, H. & J. Lehmann, 1971b: Histochemische Enzym-Lokalisation am Dentinschliff. Dtsch. zahnärztl. Z. *26:* 987—991.

Goldberg, J. A. & A. M. Rutenburg, 1958: The colorimetric determination of leucine aminopeptidase in urine and serum of normal subjects and patients with cancer and other disease. Cancer *11:* 283—291.

Gomori, G., 1939: Microtechnical demonstration of phosphatase in tissue sections. Proc. Soc. exp. Biol. N.Y. *42:* 23.

Hammarstrom, L. E., S. U. Toverud & J. S. Hanker, 1971: Naphthylamidase in ameloblasts during enamel maturation. Calcif. Tiss. Res. *7:* 267—276.

Heyden, G. & S. H. From, 1969a: A histochemical demonstration of ATPase activity during tooth ontogeny in the mouse. Archs oral Biol. *14:* 1225—1232.

Heyden, G. & S. H. From, 1969b: A histochemical study of some oxidative enzymes in mouse molar ontogeny. Arch. oral Biol. *14:* 1233—1241.

Holden, J. T., R. B. Wildman & E. F. Snell, 1951: Growth promotion of hydroxyacids and its relation to vitamin B_6. J. biol. Chem. *191:* 559—576.

Holter, H. & S-O. Li, 1950: Phosphoamidase of some proteolytic enzymes and rennin. Acta chem. scand. *4:* 1321—1322.

Hopsu, V. K., K. K. Mäkinen & G. G. Glenner, 1966: Formation of bradykinin from kallidin-10 by aminopeptidase B. Nature (Lond.) *212:* 1271—1272.

Johansen, E., 1963: Ultrastructure and chemical observations on dental caries. In: Mechanisms of Hard Tissue Destruction, pp. 187—211, Amer.Ass. Advance. Sci., Washington.

Johansen, E., 1965: Electron microscopic and chemical studies of carious lesions with reference to the organic phase of affected tissue. Ann. N.Y. Acad. Sci. *131:* 776—785.

Johnson, N. W., B. R. Taylor & D. S. Berman, 1969: The response of deciduous dentine to caries studied by correlated light and electron microscopy. Caries Res. *3:* 348—368.

Kirkby, W. W., 1965: The use of histochemical examination of maintenance-cultured tissue in assessing potential cytotoxic substances. Biochem. J. *94:* 24—25.

Korhonen, L. K., & E. Näätänen, 1963: Leucine aminopeptidase in the developing teeth of the mouse. Acta anat. (Basel) *52:* 130—138.

Kreizman, S. N., S. Irving, J. M. Navia & R. S. Harris, 1969: Enzymatic release of phosphate from rat molar enamel by phosphoprotein phosphatase. Nature (Lond.) *223:* 520—521.

Kuhn, R. & D. Jerchel, 1941: Über Invertseifen. VIII. Reduction von Tetrazoliumsalzen durch Bakterien, gärende Hefe und keimende Samen. Ber. dtsch chem. Ges. *74B:* 949—952.

Larmas, M., 1972: Phospho-amidases in sound and carious human dentine. Caries Res. *6:* 274—275.

Layne, E., 1963: Spectrophotometric and turbidimetric methods for measuring proteins. In: Methods in Enzymology (Ed. by *Colowick, S. P. & N. O. Kaplan*) Vol. III. pp. 448—450. Academic Press, New York and London.

Lillie, R. D., 1965: Histopathologic Technic and Practical Histochemistry, 3rd ed., McGraw-Hill, New York.

Menten, M. L., J. Junge & M. H. Green, 1944: A coupling histochemical azo dye test for alkaline phosphatase in the kidney. J. biol. Chem. *153:* 471—477.

Miller, W. A., 1969: Fat staining in carious dentin. J. dent. Res. *48:* 109—113.

Miller, W. A. & M. Massler, 1962: Permeability and staining of active and arrested lesions in dentine. Brit. dent. J. *112:* 187—197.

Mäkinen, K. K., 1968: Studies on oral enzymes. VII. Pyridoxal 5-phosphate dependent cleavage of L-cystine and related compounds by enzyme preparations from human oral cavity and certain microorganisms. Acta odont. scand. *26:* 445—471.

Mäkinen, K. K., 1969: The proline iminopeptidases of the human oral cavity. Partial purification and characterization. Acta chem. scand. *23:* 1409—1438.

Mäkinen, K. K., 1970: Hydrolysis of phosphates by enzyme preparations derived from carious dentine, bacterial plaque and saliva. Caries Res. *4:* 14–22.

Mäkinen, K. K. & I. K. Paunio, 1969: Enzymatic release of phosphorus from human dental enamel by enzyme preparations of *E. coli*. A preliminary report. Acta odont. scand. *27:* 477–480.

Mäkinen, K. K. & I. K. Paunio, 1970: The pH-dependent liberation of phosphate from human dental enamel and dentine by ammonium sulphate. Acta chem. scand. *24:* 1541–1550.

Nuki, K. & S. L. Bonting, 1961: Quantitative histochemistry of the developing hamster tooth: alkaline phosphatase and lactic dehydrogenase. J. Histochem. Cytochem. *9:* 117–125.

Paunio, I. K. & K. K. Mäkinen, 1971: Effect of sulphate ion on the solubility of human enamel and dentin. J. dent. Res. *50:* 862–866.

Paunio, I. K., K. K. Mäkinen, & A. Scheinin, 1968: Liberation of phosphate from human dental enamel by enzymes. Caries Res. *2:* 217–332.

Pearse, A. G. E., 1960: Histochemistry. Theoretical and Applied. J. A. Churchill, Ltd. London.

Pourtois, M., 1962a: Contribution a l'étude des bourgeons dentaires chez la Souris. II. Phases de cytodifférenciation, d'élaboration organique et de minéralisation. Arch. Biol. (Liège) *73:* 225–309.

Pourtois, M., 1962b: Le minéralisation de la dentine et de l'émail dans ses rapports avec le métabolisme des tissues générateurs. Archs. Biol. (Liege) *73:* 491–520.

Quintarelli, G. C., 1961: Enzyme histochemistry of tooth germs. Arch ital. Biol. orale. *2:* 1–125.

Raekallio, J. & P.-L. Mäkinen, 1967: Aminopeptidases in serum and skin of rats during early normal healing. Ann. Med. exp. Fenn. *45:* 224–229.

Reitman, S. & S. Frankel, 1957: A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Amer. J. clin. Path. *28:* 56–63.

Rosenqvist, H. & M. Larmas, 1973: Gas-liquid chromatographic analyses of lactate and citric acid cycle metabolites in human dentine. Proc. ninth European Symp. Calcif. Tis., Wiener Med. Akad. Vienna (in press).

Sasso, W. S. & N. M. Castro, 1957: Histochemical study of amelogenesis and dentinogenesis. Oral Surg. *10:* 1323–1329.

Schwartz, E. M. & J. A. Yager, 1961: Succinic dehydrogenase activity in odontoblasts. J. dent. Res. *40:* 1291.

Symons, N. B. B., 1962: A histochemical study of the odontoblast process. Arch. oral Biol. *7:* 455–462.

Takamatsu, H., 1939: Histochemische Untersuchungen der Phosphatase und deren Verteilung in verschiedenen Organen und Gewebe. Trans. Soc. path. jap. *29:* 492.

Takuma, S., U. Sunohara, K. Sekiguchi & I. Egawa, 1967: Electron microscopy of carious lesion in human dentine. Bull. Tokyo dent. Coll. *8:* 143–165.

Ten Cate, A. R., 1962: The distribution of alkaline phosphatase in the human tooth germ. Archs. oral Biol. *7:* 195–205.

Toto, P. D., 1966: Softening of dentin in caries. J. dent. Res. *45:* 1562.

Veis, A. & A. Perry, 1967: The phosphoprotein of the dentin matrix. Biochemistry *6:* 2409–2416.

Willner, H., 1936: Untersuchungen über des Vorkommen von Fett in der Zahn Pulpa. Z. Stomat. *24:* 1084–1092.

Wilson, H., 1940: Fatty changes in tooth pulps and dentin. J. dent. Res. *19:* 325.

Zerosi, C. & A. Baratieri, 1954: Recherches histochimiques sur les localisations de la phosphatase alcaline dans la pulpe dentaire, dans ses rapports avec la formation de la dentine. Minerva stomat. *3:* 226—235.

Yoshiki, S., 1962: Histochemistry of various enzymes in developing bone, cartilage and tooth of rat. Bull. Tokyo dent. Coll. *3:* 14—28.

Yoshiki, S. & Y. Kurahashi, 1971: A light and electron microscopic study of alkaline phosphatase activity in the early stage of dentinogenesis in the young rat. Arch. oral. Biol. *16:* 1143—1154.

Yoshioka, W., M. Mori, T. Mizushima & T. Amatsu, 1960a: Histochemical studies of esterase activity in the developing teeth. Arch. histol. jap. *20:* 523—527.

Yoshioka, W., M. Mori, T. Mitzushima & S. Deguchi, 1960b: Histochemical studies of glycosidase (β-glucuronidase, β-galactosidase and β-glucosidase) activity in the developing teeth in rat. Arch. histol. jap. *20:* 529—533.

Address:
Institute of Dentistry,
University of Turku,
Lemminkäisenkatu 2,
SF-20520 Turku 52, Finland