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HISTOCHEMICAL STUDIES ON HYDROLYTIC ENZYMES IN ARTIFICIAL CARIES OF THE DENTINE

by
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INTRODUCTION

Strong arylaminopeptidase activity has been recently demonstrated histochemically in human carious dentine (*Larmas, Mäkinen & Scheinin*, 1968). In these studies no reaction was seen in sound dentine but the areas in the vicinity of histologically normal dentine displayed considerable enzyme activity. Even acid phosphatase and arylsulphatase activity was observed in carious dentine (*Larmas*, 1968). Activity of phosphatases, proteinases, and disulfide reductases in normal and carious human dentine has also been recently demonstrated by biochemical means (*Mäkinen, Larmas & Scheinin*, 1969; *Mäkinen*, 1970). These observations supported the hypothesis that the same enzymes could be active in the hydrolysis of dentine as well as in the resorption of bone.

Experiments to produce artificial dental caries *in vitro* have been numerous since the original experiments of *Miller* (1890), where ordinary foodstuffs and mixed mouth organisms were used as the media. Subsequent experiments have been made with pure cultures of microorganisms. Acids, buffers, and other chemicals have also been used as attacking agents. An artificial mouth has also been developed (*Pigman*, 1952). These *in vitro* experiments have been recently reviewed by *Darling* (1970). Caries-like lesions, produced

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in vitro under different conditions, have been compared with carious lesions, for example, by means of microradiographic and polarized-light microscopic examination.

In the present study, the enzyme activities studied were considered as indicators of the propagation of dentinal caries. An attempt was made to determine the conditions favouring the *in vitro* formation of hydrolytic enzyme activities in sound dentine.

The synthetization of the enzymes in microbial cells during bacterial growth in various media infected by microorganisms from carious dentine was also studied, as well as the liberation of the hydrolytic enzymes in their growth media.

MATERIAL AND METHODS

1. *Dentine.* Pieces of sound coronal dentine were prepared from several caries-free teeth extracted mainly for orthodontic reasons. The teeth were immediately placed in cold (+4°C) and stored for 3—6 hours. Pieces of sound dentine were thereafter prepared by a diamond bur with a water spray and pieces of about $3 \times 3 \times 3$ mm were cut. These sound dentine pieces were stored in cold (+4°C). When a sufficient amount of dentine had been obtained (during a period of two weeks) the samples were placed into a culture medium and sterilized by different methods, described later in detail.

2. *Microbiological media.* The TSHGA-medium, rich in nutrients, was composed of the following ingredients per 1000 ml distilled water: 5 g sucrose, 5 g Bacto-tryptone (Difco Laboratories, Detroit, Michigan, USA), 10 g glucose, 5 g lactose, 5 g yeast extract (Difco), 2.5 g gelatine, and 0.7 g ascorbic acid. The pH of the medium was adjusted to 6.7—6.8.

The TSHGA-lactic-acid medium was prepared as above, but the pH of the medium was adjusted to 4.0 (about 4.6 ml/lactic-acid per 1000 ml TSHGA-medium).

The GSHT-medium was composed of the following ingredients per 1000 ml distilled water: glucose 10 g, sodium citrate 10 g, yeast-extract 5 g (Difco), and Bacto-tryptone 5 g (Difco). The pH of the medium was adjusted to 6.8. When GSHT-lactic-acid-medium was used, the pH was adjusted to 4.0 with lactic-acid (21.4 per 1000 ml GSHT).

The mono- and disaccharide free, amino acid and vitamin rich liver-meat extract broth was prepared as follows: 1 kg fresh homogenous beef-liver was boiled in 6 l meat extract broth for 30 min and filtered. The 1000 ml meat extract broth was prepared from the following ingredients: 10 g peptone, 5 g sodium chloride, 5 g yeast-extract (Difco) and 2 g phosphorus. The pH

was adjusted to 7.2. When the liver meat extract lactic-acid medium was used, the pH was adjusted to 4.0 with lactic-acid (6.5 ml lactic acid/1000 ml liver-meat extract).

The following solid culture media were used for identification of microorganisms: ten per cent blood agar, chocolate agar (McLeod agar), and Rogosa agar.

3. *Microorganisms.* The microorganisms used in this investigation were obtained from active carious lesions. Sterile microbiological media were prewarmed to +37°C and inoculated with microorganisms from carious lesions. Microorganisms were taken with a sterile excavator from human carious teeth *in situ* after first having rinsed the teeth with sterile water. The identification of microorganisms in the cultivation media was made as follows: after 24 hours growth at 37°C the aqueous media were diluted to 1:100, 1:10.000 and 1:100.000 with the same sterile media and a volume of 100μl of the diluted media was subcultivated either on blood-agar, McLeod agar, or Rogosa agar media. The subcultivations were carried out at 37°C aerobically or microaerobically in vacuo. The identification of microorganisms from the mixed cultures was performed by routine methods only, based on the morphology, staining reactions and cultural characteristics of the individual organisms, the appearance of the colonies to the naked eye being the primary criteria.

4. *Sterilization of dentine.* The pieces of sound dentine were sterilized by boiling for 30 min at 100°C in an open vessel, or by autoclaving either for 20 min at 121°C or by intermittent exposure for 1 hour at 60°C on each of three successive days. The sterilizations were performed in the culture media. Because no difference could be observed between the results obtained with the different sterilization methods, autoclaving at 121°C was most frequently used. The TSHGA, GSHT and meat extract media were sterilized by autoclaving for 20 min at 121°C, the liver-meat extract medium was sterilized by boiling for 30 min at 100°C.

5. *Cultivation of microorganisms.* 30 ml sterilized media with pieces of sound dentine were inoculated with microorganisms from carious dentine as described above. After 24 hours growth the infected dentine pieces were transferred aseptically into flasks containing 30 ml of the same sterile medium. This transfer was repeated daily during a period of 3 to 12 weeks. The pH of the culture medium was measured after the transfer of the dentine into the fresh medium. All measurements of the pH of the media were made with a glass-calomel electrode at room temperature (about 22°C). The semiquantitative count of microorganisms was performed after 24 hours growth in the aqueous media, then subcultivated for 24 and 48 hours on blood

agar, McLeod agar, and Rogosa agar. This procedure was thereafter repeated weekly during the experiment, as stated earlier.

Because the mutual ratio of the main micro-organisms cultivated *in vitro* conditions seemed to change, the sterile media without dentine pieces were reinoculated once a week by new microorganisms from carious dentine from the same person. The microorganisms were cultivated for 12 hours and thereafter the dentine pieces, after being washed with sterile saline, were transferred to the infected medium and allowed to grow for another 12 hours. The transfer to fresh medium was then performed as earlier. This procedure was repeated once a week during the whole experiment.

As a control, sterile pieces of dentine were aseptically transferred to the same media without microorganisms and the subsequent incubations were performed as in the case of the infected media.

When studying the synthetization of the enzymes in microbial cells and the liberation of these enzymes in growth media, microorganisms from carious lesions were disseminated into 30 ml prewarmed TSHGA, GSHT, and liver-meat extract, and allowed to grow for 12 hours. Fifteen ml was then transferred aseptically to 3000 ml of the same medium, and the remaining 15 ml to another 3000 ml of the same medium. The first medium was aerated by agitating with a mixer (Heidolp Laborrührer, Typ RZR, Heidolp Elektro KG, Kelheim/Donau, Germany) using a steel propeller at 150 r.p.m.: the other medium was not aerated. Growth was followed turbidimetrically with a Klett-Summerson colorimeter, employing filter No. 62. When the biosynthesis of aryl-aminopeptidases, arylsulphatases and phosphatases in the cells of the microorganisms of the mixed cultures from the carious lesions was studied, 30 ml samples were taken from the 3000 ml TSHGA, GSHT, and liver-meat extract medium. The cells from these samples were harvested by centrifugation at 19.500 r.p.m. (45.900 g) for 10 min at 4°C in rotor SS-34 of the Sorvall Superspeed RC-2B centrifuge.

The cells were then washed twice with 5 ml of cold (about 4°C)0.9 % NaCl solution in the centrifuge tubes. After pouring off the last washing solution, 2 ml of cold sterile water was added to the pellet, and the cells were suspended in it. The resultant suspension was then treated 10 min with the MSE ultrasonic disintegrator (Measuring & Scientific Equipment Ltd, London, England), 100 Watt Model, using a titanium probe, end diameter 19 mm. During the treatment the sample was kept in ice to prevent overheating. The resultant solution was centrifuged at 19.500 r.p.m. (45.900 g) for 15 min at 4°C, and the supernatant fluid was designated the enzyme preparation and stored at 4°C between experiments. The clear growth media were saved for investigation and the enzyme activities were determined from them.

6. *Histochemical demonstration of arylaminopeptidase, phosphatase, and arylsulphatase activity in cryostat cut sections.* After the incubation at 37°C for 20—90 days in the growth media, the samples of dentine were cut in a cryostat (International Equipment Company, Model CTI, Needham Heights, Mass., USA) into sections of 10 μ without any preceeding decalcification as described earlier (*Larmas et al.*, 1968; *Larmas*, 1968).

In the demonstration of arylaminopeptidase, acid and alkaline phosphatase and arylsulphatase activity, the same methods were used as earlier (*Larmas et al.*, 1968; *Larmas*, 1968). The substrates used were leucyl-2-naphthylamine and 6-bromo-2-naphthylsulphate (Mann Research Laboratories Inc., New York, N.Y., USA) naphthol AS-MX and AS-GR phosphate (Sigma Chemical Company, St. Louis, No., USA) and naphthol AS-TR phosphate (Nutritional Biochemical Corporation, Cleveland, Ohio, USA). In coupling of the liberated 2-naphthylamine or naphthol, Fast Blue B (tetrazotized odianiside, L. Light Co. Ltd., Colnbrook, England), Red Violet LB salt (diazotized 5-benzamido-4-chloro-2-toluidine, Sigma Chemical Company), and Blue BBN salt diazotized 4-amino-2.5-diethoxybenzanilide, Verona Dyestuffs, Inc., Springfield, USA) were used. The histochemical demonstration of leucine aminopeptidase activity was carried out according to *Nachlas et al.* (1957), acid and alkaline phosphatase activity according to *Burstone* (1960), and arylsulphatase activity according to *Rutenberg et al.* (1952). The effect of the azo dyes alone on the tissue sections was studied in a similar reaction mixture as above without the substrates.

The semiquantitative estimation of the enzyme activity was based on the activity density on the tissue sections, not on the intensity of the activity, because microscopic examination revealed only coarse differences of intensity.

7. *Determination of enzyme activity in the microbial cells and growth media.* The estimation of arylaminopeptidase activity was performed in a reaction mixture containing the following ingredients: 0.3 ml of buffer, (0.01 M tris-HCl pH 7.0) 0.1 ml of 10^{-3} M aqueous substrate solution, 0.1 ml of water and 0.1 ml of enzyme preparation. The water soluble substrates were the N-L-2-naphthylamines of L-arginine, L-leucine, L-lysine, and L-proline (Mann Research Laboratories). N-L-alanyl-2-naphthylamine (Mann Research Laboratories) was dissolved well in 5 ml ethanol and was not precipitated when made up to 100 ml with water. The mixtures were incubated for various periods of time at 37°C and the enzyme reactions were stopped by adding 0.2 ml of 0.1 % Fast Garnet GBC Salt (diazotized 4-amino-3:1'-dimethylazobenzene) in 1 M acetic buffer, pH 4.2, which contained 10 % Tween 20 (v/v). The colour intensity was read on a Hitachi

Perkin Elmer 139 Spectrophotometer at 525 mμ. A standard curve was prepared with free 2-naphthylamine (Sigma Chemical Company).

When naphthylesters 6-bromo-2-naphthyl sulphate and 1-naphthyl phosphate were used as substrates, the enzyme activities were estimated in an incubation mileu described above. The method was slightly changed in that the hydrolysis of naphthylesters was stopped by adding, first 0.05 ml of 0.1 % Fast Garnet GBC Salt, and then, after 10 minutes standing, 0.15 ml of 1 M acetic buffer pH 4.2, which contained 10 % Tween 20 (v/v). The colour intensity was read as described above. A standard curve was made in this case with free 1- or 2-naphthol (Sigma Chemical Company). In the controls the enzymes were replaced by water. The buffers, when the hydrolysis of 1-naphthyl phosphate was studied, were 0.02 M borate buffer, pH 9.0, 0.01 M tris-HCl pH 7.0, and 0.05 M β,β-dimethylglutaric acid buffer pH 4.6.

The rate of hydrolysis of the substrates used were in most experiments expressed as extinction. Unless otherwise stated, all chemicals used in this study were purchased from E. Merck AG, Darmstadt, Germany.

RESULTS

1. *Microbiological observations.* The microorganisms cultivated in these experiments are listed in Tables I and II. The results show that on the first experimental day streptococci were the main microorganisms in all of the media used. In these *in vitro* conditions the mutual ratio of various microorganisms was changed in a few days and after one week candida became dominant in some culture media, as can be seen in Table I. At this stage the sterile media were inoculated by new microorganisms and the original ratio was approximately obtained. On the other hand, these procedures did not maintain the original ratio during the whole experiment. Table II thus shows the main microorganisms after the last inoculation and at the end of the experiment. In the conditions employed only a few microorganisms tended to dominate, although the reinoculation with new microorganisms was made weekly.

When the microbial growth was studied in greater detail in various growth media, it was observed that different microorganisms were dominant at different times in the total growth of the mixed culture. As a rule, neisseria and streptococci began their growth first and reached their exponential growth phase in 15 hours (the first peak in the total growth curve, as seen in Fig. 1). After 25—35 hours candida and lactobacilli reached the exponential

Table I.

Microorganisms isolated from the mixed cultures. Approximate percentage of various microorganisms counted from the isolated total flora on blood-agar and McLeod agar cultures during the first experimental week

Medium	Microorganism	Aerobic		Microaerophilic	
		1. day	*6. day*	*1. day*	*6. day*
TSHGA	Strept. α-haemol.	67—88	67—68	25—37	20—40
	Strept. non-haemol.	11—32	2—6	63—75	62—75
	Candida	<1—1	26—29	<1	7—14
	Lactobacillus	<1	3—6	—	—
	Neisseria	3—6	—	—	—
	Other	<1	<1	<1	2—7
TSHGA+ LACTIC-ACID	Candida	42—70	72—80	1—4,5	10—17
	Strept. α-haemol.	27—20	10—14	14,5—63	20—32
	Lactobac.	4—15	5—10	7—16	14—17
	Strept. non-haemol.	12—16	3—10	35—81	42—60
GSHT	Strept. α-haemol.	2—7	3—7	12—41	14—24
	Strept. non-haemol.	91—98	61—70	80—94	60—90
	Candida	<1	32—47	<1	12—30
	Staph. albus	<1	—	—	—
	Other	1—2	3—6	—	—
GSHT+ LACTIC-ACID	Candida	36—70	—	Candida	47—60
	Strept. non-haemol.	42—60	—	Strept. hac-mol.	21—72
LIVER-MEAT EXTRACT	Strept. α-haemol.	6—54	6—37	12—32	10—14
	Strept. non-haemol.	45—89	38—70	49—80	41—90
	Candida	<1	25—43	<1	14—20
	Neisseria	<1	—	—	—
	Lactobacil.	2—4	—	—	—
	Enterobact.	<1	<1	—	—
	Other	<1	<1	—	—
LIVER-MEAT EXTRACT+ LACTIC-ACID	Candida	4—6	17—36	2—4	12—21
	Strept. α-haemol.	12—53	29—47	8—43	17—30
	Lactobac.	1—3	—	—	—
	Strept. non-haemol.	40—80	50—75	60—70	56—80
CONTROLS	—	—	—	—	—

Table II.

Micro-organisms isolated from the mixed cultures. Approximate percentage of various micro-organism counted from the isolated total flora on blood-agar and McLeod agar cultures during the last experimental week

Medium	Microorganisms	Aerobic The last experimental week		Microaerophilic The last experimental week	
		In the beginning	At the end	In the beginning	At the end
TSHGA	Strept. non-haemol.	14—86	20—60	47—70	36—81
	Strept. α-haemol.	8—14	8—12	11—17	14—30
	Candida	31—42	37—66	21—30	29—35
	Neisseria	2—5	3—7	—	—
	Other	<1	<1	—	—
TSHGA+ LACTIC-ACID	Candida	60—90	85—94	40—70	30—80
	Strept. non-haemol.	8—24	4—17	6—48	11—39
	Strept. α-haemol.	3—6	1—2	3—4	2—17
GSHT	Strept. α-haemol.	12—30	17—21	—	—
	Strept. non-haemol.	46—81	12—27	78—91	64—70
	Candida	16—37	39—82	12—18	25—40
	Other	<1	<1	—	—
GSHT+ LACTIC-ACID	Candida	45—76	69—84	43—55	50—60
	Strept. non-haemol.	55—70	36—69	71—90	60—70
LIVER-MEAT EXTRACT	Strept. non-haemol.	39—48	41—68	47—72	36—74
	Strept. α-haemol.	11—32	8—17	10—	6—21
	Candida	47—68	51—70	36—49	21—49
LIVER-MEAT EXTRACT+ LACTIC-ACID	Candida	58—81	72—90	36—68	42—77
	Strept. non-haemol.	35—47	12—31	29—41	36—62
CONTROLS	—	—	—	—	—

growth phase, and when aerated, candida dominated and only a few other microorganism species could be isolated from the mass of candida (the last peak in the total growth curve, especially in the aerated culture as seen in Fig. 1). The various micro-organisms isolated from the TSHGA medium by subcultivation on blood agar, McLeod agar, and Rogosa agar at 10 and 40 hours after the inoculum are seen in Table III. Similar results were obtained by the other media used.

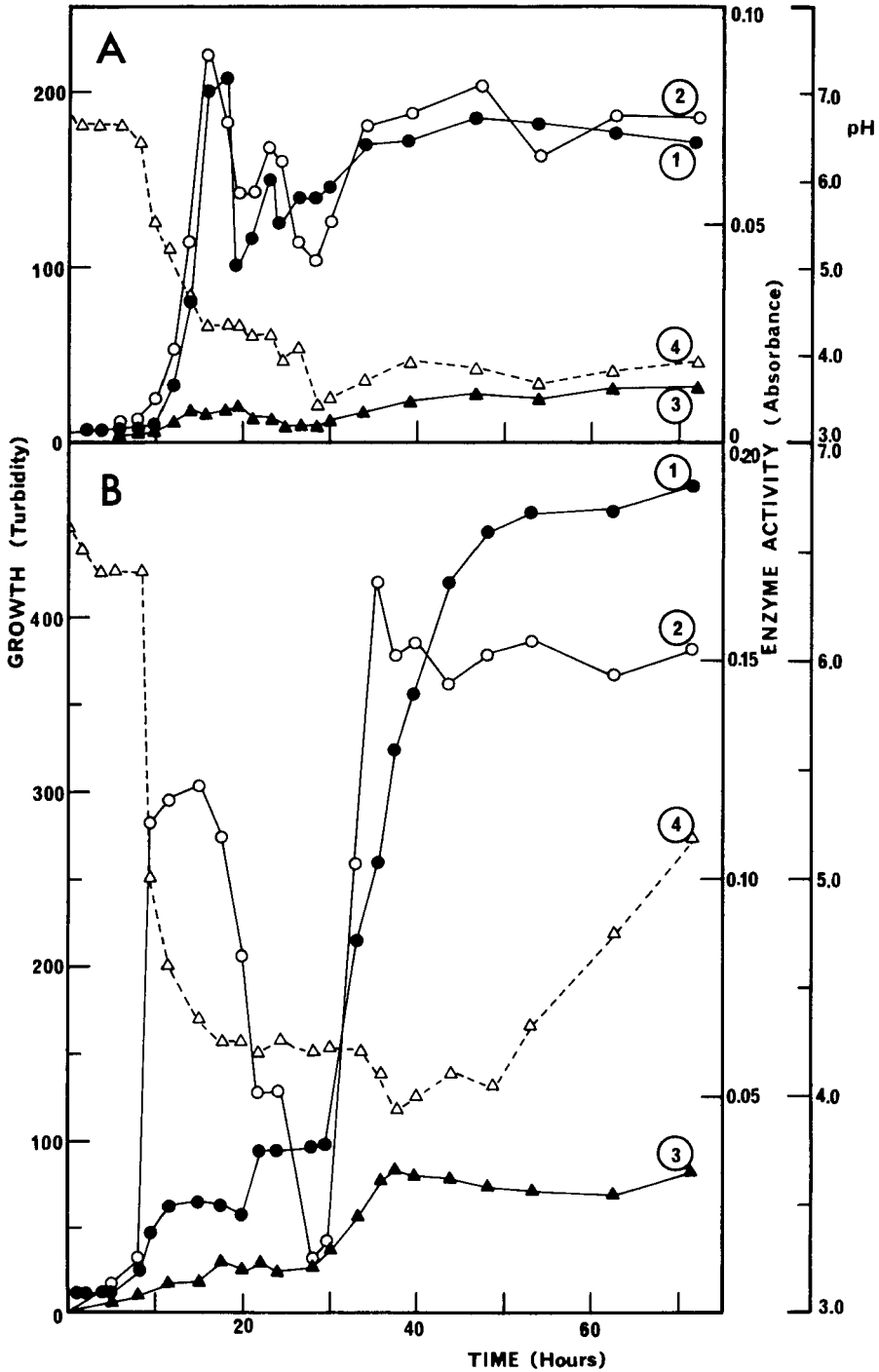

Fig. 1. Formation of enzymes hydrolyzing N-L-leucyl-2-naphthylamine by microbial cells during the growth of a mixed culture from caries cavity in the TSHGA medium. A: not aerated, B: aerated culture. 1: growth curve (lefthand scale); 2: enzyme activity in the cells; 3: enzyme activity in the growth medium; 4: pH of the medium.

Table III.

The micro-organisms isolated from the mixed culture in the TSHGA medium by aerobic subcultivation on solid media, after various dilutions at 10 hours and 40 hours after the inoculation. Appr. percentage of various micro-organisms counted from the isolated total flora on blood agar and McLeod agar at different dilutions (1:100, 1:10.000, 1:100.000)

	Aerated TSHGA		Nonaerated TSHGA	
	10 h.	40 h.	10 h.	40 h.
Str. viridans	45—60	3—5	45—70	50—70
Neisseria	20—40	0	20—30	<1
Enterobacteria	<1	<1	3—5	<1
Candida	<1	85—95	<1	4—9
Lactobacilli	<1	2—4	<1	15—30
Staph. albus	3—7	0	1—3	0
Other	2—4	<1	2—3	1—2

The pH values of the media after the consecutive 24 hours incubation periods can be seen in Fig. 2. The pH fell daily after the microbial growth from about neutral to pH 5—4. The final pH value attained seemed to depend on the medium used and the microorganisms isolated from that medium.

When studying the production of hydrolytic enzymes of the microorganisms in the mixed cultures, the enzyme activity was calculated per ml of growth medium and also the relative enzyme content of the cells was estimated. Fig. 1 shows the results from the experiments in which the biosynthesis of aryl-amino-peptidase in the mixed cultures was studied. It shows that the formation of enzymes started simultaneously with the cell division, and the highest enzyme activity in the cells was found during the exponential growth phase of the different microorganisms in the mixed culture, mostly when the cell division of candida started. The leucine aminopeptidase activity in the medium was not found in any restricted growth phase but rather during the whole life cycle. Similar observations were made of the production of arylsulphates and the results were similar, regardless of the medium used. When aerated, the growth of candida was stimulated and the pH of the medium rose, as also seen in Fig. 1.

2. *Histochemical demonstration of enzymes.* Fig. 3 shows abundant leucine aminopeptidase activity in a transversal section obtained from dentine kept in GSHT medium, this activity being similar to that observed *in vivo* (*Larmas et al.*, 1968). The enzyme activity occurred in and along

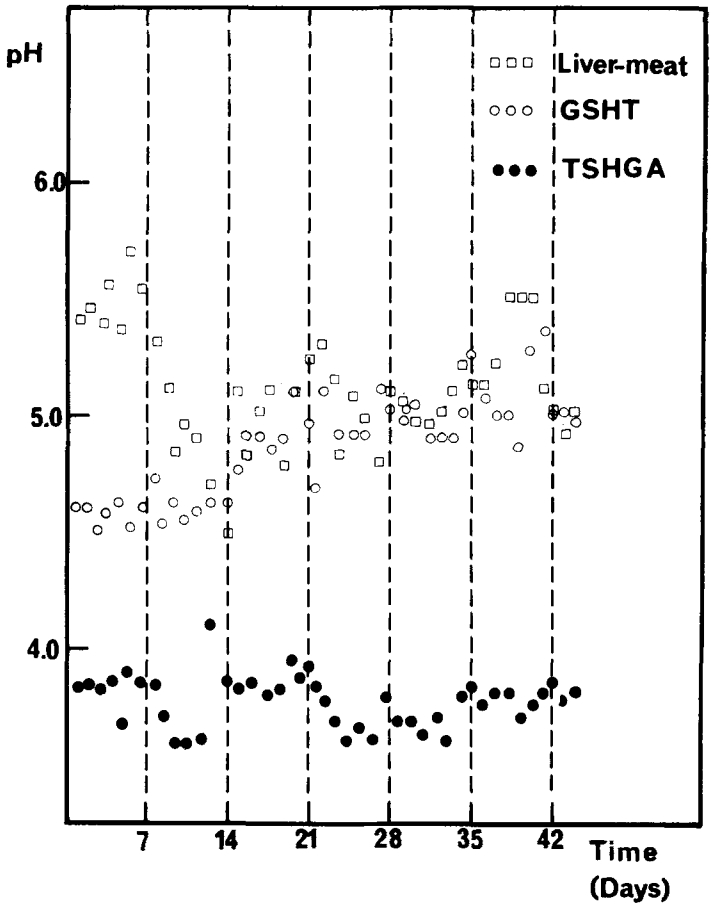

Fig. 2. The pH values of the media after the 24 hours incubation. The reinoculations are shown as hatched lines.

the dentinal tubules. The activity also occurred in uninfected parts of the dentinal tubules. Fig. 4 shows considerable arylsulphatase activity in a section cultivated in the meat-liver extract medium. The localization of the activity differed from that of arylaminopeptidases, as in this case the activity occurred in the superficial layer of the dentine. Arylaminopeptidase activity occurred mostly in the central part of the dentine sections (Fig. 3). In some sections obtained with TSHGA-medium a distinct zone of marked arylsulphatase activity could be observed on the borderline of histologically normal middle parts of the dentine pieces. No enzyme activity could be

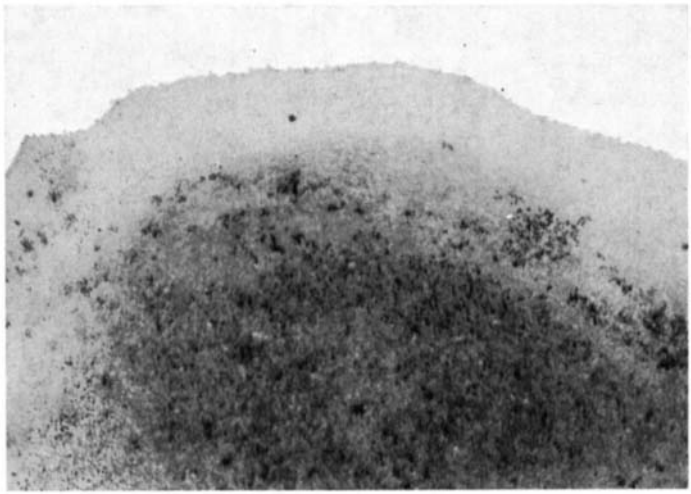

Fig. 3. Abundant leucine aminopeptidase activity in a transversal section from dentine kept in GSHT medium. Activity is seen as dark areas in the dentinal tubules all over the section. Note that the strongest activity density was in the inner part of the section. Incubation time 30 min. (×20).

Fig. 4. Strong arylsulphatase activity in dentine kept in meat-liner extract broth. The activity occurs in the superficial layer of the dentine as dark stripes along the dentinal tubules. Incubation time 3 hours. (×20).

Table IV.

The histochemical demonstration of enzymes in dentine sections after microbiological growth in various media

MEDIUM	Arylamino-peptidase	Arylsul-fatase	Acid phos-phatase	Alkaline phosphatase
TSHGA	++++	+++++	—	—
TSHGA+				
LACTIC-ACID	+	+	—	—
GSHT	+++++	++	—	—
GSHT+				
LACTIC-ACID	—	+	—	—
LIVER-MEAT				
EXTRACT	+	+++	—	—
LIVER-MEAT				
EXTRACT+				
LACTIC-ACID	++	++++	—	—
CONTROLS:				
TSHGA*	—	—	—	—
GSHT*	—	—	—	—
LIVER MEAT				
EXTRACT*	—	—	—	—
TSHGA+				
LACTIC-ACID	—	—	—	—
GSHT+	—	—	—	—
LIVER-MEAT				
EXTRACT+				
LACTIC-ACID	—	—	—	—

+++++ Very rich activity in all sections from different layers of the dentine
++++ Rich activity in all sections
+++ Rich activity in some sections, moderate activity in the others
++ Moderate activity in some sections
+ Poor activity in some sections
— No activity in any sections

observed in dentine pieces incubated without microorganisms in the same microbiological media, or when incubated without the substrates. No phosphatase activity could be observed histochemically in any section. The results are summarized in Table IV. Different enzyme activity could be observed in various media, the strongest arylaminopeptidase activity being found in sections obtained with GSHT-medium, whereas arylsulphatase activity was prominent in those obtained with TSHGA-medium, the latter medium providing in general the most noticeable histochemical enzyme activity of all sections.

DISCUSSION

For obvious reasons these experiments were made with extracted human teeth; thus the environment differed from that in the mouth and the metabolism of the teeth was drastically disturbed. Therefore, these experiments are open to criticism. The results show, however, that the lesions produced were macroscopically, histologically, and histochemically indistinguishable from advanced natural caries. The discussion can be limited to comparisons, first of the substrate (i.e. the pieces of dentine) with natural dentine, and second of the *in vitro* environment (i.e. the microorganisms and their culture media) with that of the oral cavity.

It is to be noticed that although freshly extracted teeth were used, certain post mortem changes may have occurred in the dentine. As for the preparation and sterilization of the pieces of dentine, certain types of physical irritation occurred, for example heating, dehydrating etc. However, the structural characteristics of the mineralized tissue did not change, and thus the results can be considered as indicative of, or at least comparative with earlier experiments.

In this experimental system many microorganisms from the oral normal flora did not grow, (for example the spirochetes), and the information available was thus limited. On the other hand, the microorganisms grown in this system were the same as normally observed in carious dentine and dental plaque, and thus this experimental system did not show any drastic differences when compared to those *in vivo*.

The experimental environment seemed to have some similarities to those *in vivo*. It was first to be observed that the pH of the media changed daily from about neutral to pH 5—4, depending on the medium used. Thus the hydrogen ion concentration corresponded to the physiological limits in the oral cavity. The media were selected so that they contained various amounts of refined carbohydrates and other nutritients, as is the situation in the oral cavity. Accumulation of the microorganisms on and in the dentine also occurred, and the enzyme spectrum in the environment was very similar to that of saliva and dental plaque. The concentration of calcium, phosphorus and some trace elements did not, on the other hand, correspond to those *in vivo*, as was the situation with the buffering capacity etc. Some similarities as well as some differences thus occurred between this experimental system and conditions *in vivo*.

The distribution of the hydrolytic enzyme in the caries-like lesions was similar to that in natural carious lesions. It was of interest that the enzyme activity occurred as distinct stripes along the dentinal tubules, not as dis-

tinct spots in the infected regions. This would suggest that the enzymes involved may even be extracellular, or at least that cellular damage of microorganisms occurs in the infected dentine and the intracellular microbial enzymes are thus liberated into the dentinal tubules. Hydrolytic processes may take place in the dentine, preceding the invasion of microorganisms, as was the situation in the present series of experiments.

The *in vitro* system presented seems to be sufficient, however, for studying the interrelationship between specific strains of microorganisms and specific enzyme activities in caries-like lesions in human dentine. Thus it will be possible to determine from which species or strain the enzymes involved originate in human dentine.

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SUMMARY

The present study was carried out in order to elucidate the circumstances under which the arylaminopeptidases, phosphatases, and arylsulphatases appeared histochemically demonstrable in the dentine. Pieces of sound coronal dentine were incubated at 37°C in various culture media (TSHGA, GSHT, and liver-meat extract broth). The media with the sterile piece of normal dentine were infected with a mixed microorganism culture from a carious human tooth and allowed to grow for 24 hours. After that period the infected dentine pieces were transferred to corresponding fresh media and the microorganisms allowed to grow for another 24 hours. This procedure was repeated daily for a month or longer. During this period the pieces of dentine were softened and sections could be cut in a cryostat.

Leucine aminopeptidase and arylsulphatase activity could be demonstrated in the dentine, the distribution of the activity being similar to that in the case of carious dentine. No acid or alkaline phosphatase activity could be observed by the methods used. No enzyme activity was seen in the control sections, which were cut from dentine incubated without microorganisms. The *in vitro* system presented seemed to be sufficient when comparing artificial and natural caries lesions, at least when the hydrolytic enzyme activities were considered as indicators of the propagation of the process.

When the synthetization of enzymes in microbial cells during bacterial growth was studied, it was observed that the formation of enzymes started simultaneously with the cell division, and enzymes were demonstrable during the whole life cycle of the microorganisms in the mixed culture.

RÉSUMÉ

ÉTUDES HISTOCHIMIQUES SUR LES ENZYMES HYDROLYTIQUES DANS LA CARIE ARTIFICIELLE DE LA DENTINE HUMAINE

La présente étude a été effectuée dans le but de mettre en lumière les circonstances dans lesquelles la mise en évidence histochimique des arylaminopeptidases, des phosphatases et des arylsulfatases pouvait être faite dans la dentine. Des fragments de dentine coronaire saine ont été incubés à 37°C dans différents milieux de culture (TSHGA, GSHT, et bouillon d'extrait de foie-viande). Les milieux contenant les fragments stériles de dentine normale ont été infectés par une culture mixte de micro-organismes provenant d'une dent humaine cariée et se sont developpés pendant 24 heures. Après cette période, les fragments de dentine infectée ont été transplantés dans un milieu correspondant fraîchement préparé, et les micro-organismes ont pu se développer pendant encore 24 heures. Ce procédé a été répeté tous les jours pendant un mois ou plus. Pendant cette période, les fragments de dentine se sont ramollis, et des coupes ont pu être faites dans un cryostat.

L'activité leucine-aminopeptidase et leucine-arylsulfatase a pu être mise en évidence dans la dentine, la répartition de cette activité étant analogue à celle qu'on trouve dans le cas de la dentine cariée. Les méthodes utilisées n'ont pas permis de constater d'activité phosphatase alcaline ni acide. Les coupes témoins, faites à partir de dentine incubée sans micro-organismes ne présentaient pas d'activité enzymatique. Le système *in vitro* présenté ici semblait être suffisant pour la comparaison des caries artificielles et naturelles, tout au moins quand les activités enzymatiques hydrolytiques étaient considérées comme des indicateurs de la propagation du phénomène.

Lorsqu'on étudiait la synthèse des enzymes dans les cellules microbiennes pendant la croissance bactérienne, on observait que la formation des enzymes commençait en même temps que la division cellulaire, et que des enzymes pouvaient être mis en évidence pendant tout le cycle vital des micro-organismes dans la culture mixte.

ZUSAMMENFASSUNG

HISTOCHEMISCHE UNTERSUCHUNGEN ÜBER HYDROLYTISCHE ENZYME BEI ARTIFIZIELLEM DENTINKARIES

In vorliegender Untersuchung wurde ein Klarlegen der Verhältnisse angestrebt, unter denen das Erscheinen von Arylaminopeptidase, Phosphatasen und Arylsulphutasen mit histochemischen Methoden nachgewiesen werden konnten.

Zu diesem Zweck wurden gesunde, sterile Kronendentinstücke bei 37°C in Nährböden verschiedener Art inkubiert (TSHGA, GSHT, Leber-Fleischbrühe). Als Nächstes wurde der Nährboden mitsamt dem darin steckenden Kronendentinstücke mit einer aus kariösem humanen Zahngewebe gezüchteten Mischkultur infiziert. Nach dem Verlauf von 24 Stunden verpflanzte man diese ursprünglich gesunden, mit der Mikrobenkultur infizierten Dentinstücke für weitere 24 Stunden auf frische Nährböden entsprechender Art. Im Verlauf von 1 Monat bzw. sogar einer längeren Zeit wurde dieses täglich wiederholt, bis die Dentinstücke soweit erweicht waren, dass die Herstellung von Kryostatpräparaten möglich war. Durch histochemische Enzymbestimmungsmethoden gelang es, in den Dentinstücken das Vorhandensein von sowohl Leucin-Aminopeptidase als Arylsulphatase aufzuzeigen, mit einer Distribution der Aktivität gleich derjenigen des kariösen Dentins in situ.

Mit den von uns angewendeten histochemischen Methoden gelang es nicht, das Vorhandensein alkalischer bzw. saurer Phosphatasen nachzuweisen. In den Kontrollpräparaten, hergestellt aus Dentinstücken, die für die Dauer entsprechender Zeitspannen in sterilen Nährböden inkubiert wurden, konnten keinerlei Enzymaktivitäten beobachtet werden.

Die von uns angewendetet in vitro-Methode erwies sich — insoweit die Aktivität einiger histochemisch nachweisbarer Enzyme als Kariesindikator gültig ist — für das Anstellen von Vergleichen zwischen Karies in vivo und in vitro als befriedigend.

Die Untersuchungen über die Synthetisierung der Enzyme in den Mikrobenzellen während des Bakterienwachstums führten zu der Erkenntnis, dass Enzymbildung und Zellteilung gleichzeitig stattfanden, und dass Enzyme in der Mischkultur nährend des gesamten darauffolgenden Wachstumszyklus nachgewiesen werden konnten.

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