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STUDIES ON DOG SALIVA

III. OBSERVATIONS ON ENZYMES ACTING ON ESTER BONDS AND GLYCOSYL COMPOUNDS

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Hydrolases acting on ester bonds and glycosyl compounds were studied in the supernatant and sediment fraction of centrifuged dog saliva. When comparing the values obtained to those of man the most interesting deviations were: (1) the lack of α-amylase in dog saliva. (2) the low activity in dog saliva towards 6-bromo-2-naphthyl-α-D-glucoside and glucuronide, (3) the low activity towards 1-naphthyl phosphate at the acidic pH in dog saliva, and (4) the lack of enzymes hydrolyzing 6-bromo-2-naphthyl sulphate in the dog saliva supernatant fraction.

The enzymes of centrifuged dog saliva and its sediment were fractionated with Sephadex G-200 and DEAE-cellulose columns. DEAE-cellulose chromatography brought out three enzyme peaks with phosphomonoester hydrolase activity, one of which was active at a wide pH range, one at the acidic side and one at the alkaline side. Two enzyme peaks hydrolyzing 6-bromo-2-naphthyl sulphate were obtained with DEAE-cellulose chromatography from the sediment fraction of dog saliva. The hydrolysis of carboxylic acid esters was observed in several enzyme peaks both from the supernatant and sediment fractions. The chromatographic results resembled those obtained with man.

One of the most interesting differences in the salivary enzyme spectrum between man and dog is that α-amylase (α-1.4-Glucan 4-glucano-hydrolase, EC 3.2.1.1.), the enzyme catalyzing the hydrolysis of α-4-glucosidic linkages of polysaccharides, is not at all demonstrable in dog saliva although it is the most active in human saliva (*Scheunert & Trautman*, 1921; *Schwarz & Rasp*, 1928; *Rosebom & Patton*, 1929; *Schneyer*, 1956; *Schneyer & Schneyer*, 1960, *Chayncey, Henriques & Tanzer*, 1963). Information on other enzymes acting on glycosyl compounds is more limited. *Chayncey et al.* (1963) reported that dog saliva had a marked β-D-galactosidase activity (β-D-galactoside galactohydrolase, EC 3.2.1.23.) in the submandibulary secretions and moderate activity in the parotid fluid. On the other hand this group

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reported that human saliva appeared to be devoid of β-D-galactosidase activity. *Mäkinen* (1968), however, observed β-galactosidase activity in human whole saliva. He also demonstrated high enzyme activity against 6-bromo-2-naphthyl-α-D-glucoside at pH 7.0 in enzyme preparations derived from human whole saliva, indicating α-D-glucosidase activity (α-D-glucoside glucohydrolase, EC 3.2.1.20) (*Mäkinen*, 1966c).

The occurrence of phosphoric monoester hydrolases both in human and dog saliva has been reported (*Chayncey et al.* 1963). Thus acid phosphatase (ortophosphoric monoester phosphohydrolase, EC 3.1.3.2) was found in both human and dog saliva, the activity seemed to be similar in both species. On the other hand, alkaline phosphatase activity (ortophosphoric monoester phosphohydrolase, EC 3.1.3.1) was not apparent in the saliva of either species. *Mäkinen* (1966 d) observed, however, that sodium l-naphthyl-phosphate was cleft at nearly the same rate at pH values of 4.5, 7.0, and 9.0, which would indicate the occurrence of alkaline phosphatase-type activity in human mixed saliva. Information on the occurrence of other enzymes acting on ester bonds in dog saliva was not found in the literature.

In a previous paper (*Larmas & Scheinin*, 1971 b) the existence of aryl-aminopeptidases in dog saliva was demonstrated and the results were compared to those obtained from man. In the present paper some hydrolases acting on ester bonds (EC 3.1.) and on glycosyl compounds (EC 3.2.) of dog saliva have been studied in principle by the same methods as used earlier in this laboratory on human saliva (*Mäkinen*, 1966 d). The results were compared to those obtained from human material.

The aim of the present investigation was to map the enzyme spectrum of dog saliva and compare it to that of man, in order to have reference material in studies on oral enzymes.

MATERIAL AND METHODS

Test animals. In this study 3 male and 4 female mongrel dogs were used and their diets were the same as in the earlier studies (*Larmas & Scheinin*, 1971 a, b).

Chemicals. Unless otherwise stated all chemicals and their sources were the same as in earlier publications (*Larmas & Scheinin*, 1971 a, b; *Mäkinen*, 1966 a-e).

Collecting and pretreating of saliva. The saliva was collected from unanesthetized animals and the pretreating performed as described in detail in the earlier publications (*Larmas & Scheinin*, 1971 a, b).

Determination of enzyme activity. The hydrolysis of 1- and 2-naphthol esters was estimated in principle according to the method of *Goldberg & Rutenburg* (1958) in reaction mixtures consisting of the following ingredients: 0.3 ml of buffer, 0.1 ml of substrate solution, 0.1 ml of water (which could be replaced by solutions of various enzyme inhibitors or other compounds) and 0.1 ml of enzyme solution. Unless otherwise stated the substrate concentration was 0.167×10^{-3} M in the reaction mixture and the experiments were carried out in various buffers of various pH values at +37°C, as given in more detail in »Results»-section. The enzyme reactions were stopped in an ice bath by adding Fast Garnet GBC Salt (diazotized 4-amino-3:1'-dimethylazobenzene; G.T. Gurr, London, (0.05 ml as 0.5 % solution in water). After 10 min, 0.15 ml of 1 M acetate buffer of pH 4.2 (containing with 10 % Tween-20 v/v) was added. The colour intensity was read on a Hitachi Perkin Elmer 139 Spectrophotometer at 525 nm. A standard curve was prepared with 1- or 2-naphthol (Sigma Chem. Co.).

RESULTS

1. *Hydrolysis of various naphthyl esters and glycosyl compounds by crude enzyme preparations*

Fig. 1 shows the results obtained when testing the ability of enzymes in centrifuged dog saliva and its sediment to catalyze the hydrolysis of various glycosidic linkages and ester bonds. These reactions were carried out at pH 4.6, 7.0, and 9.0 for various periods of time, but the results are normally given only for pH 7.0, where the highest rate of hydrolysis was observed with most substrates. In the case when phosphoric monoester hydrolase activity was tested, the rate of hydrolysis is presented at all the pH values tested, because enzyme activity could be observed at a wide pH range. The results are calculated in each case as liberated μmoles of liberated 1- or 2-naphthol. Most of the naphthol esters tested were hydrolyzed to some extent. The substrate most rapidly cleft was 6-bromo-2-naphthyl sulphate by the crude enzyme preparation from the saliva sediment fraction, indicating the occurrence of arylsulphatase (arylsulphate sulphohydrolase, EC 3.1.6.1) activity in dog saliva. On the other hand, no clear hydrolysis of this substrate was observed by enzyme preparation from the supernatant fraction of dog mixed saliva. Remarkable hydrolysis of 1- and 2-naphthyl phosphates was also observed at all pH values tested. The most rapidly cleft carboxylic ester substrates were 1- and 2-naphthyl acetate, 1-naphthyl propionate, and 2-naphthyl valerate at pH 7.0. In repeated experiments quantitative variations

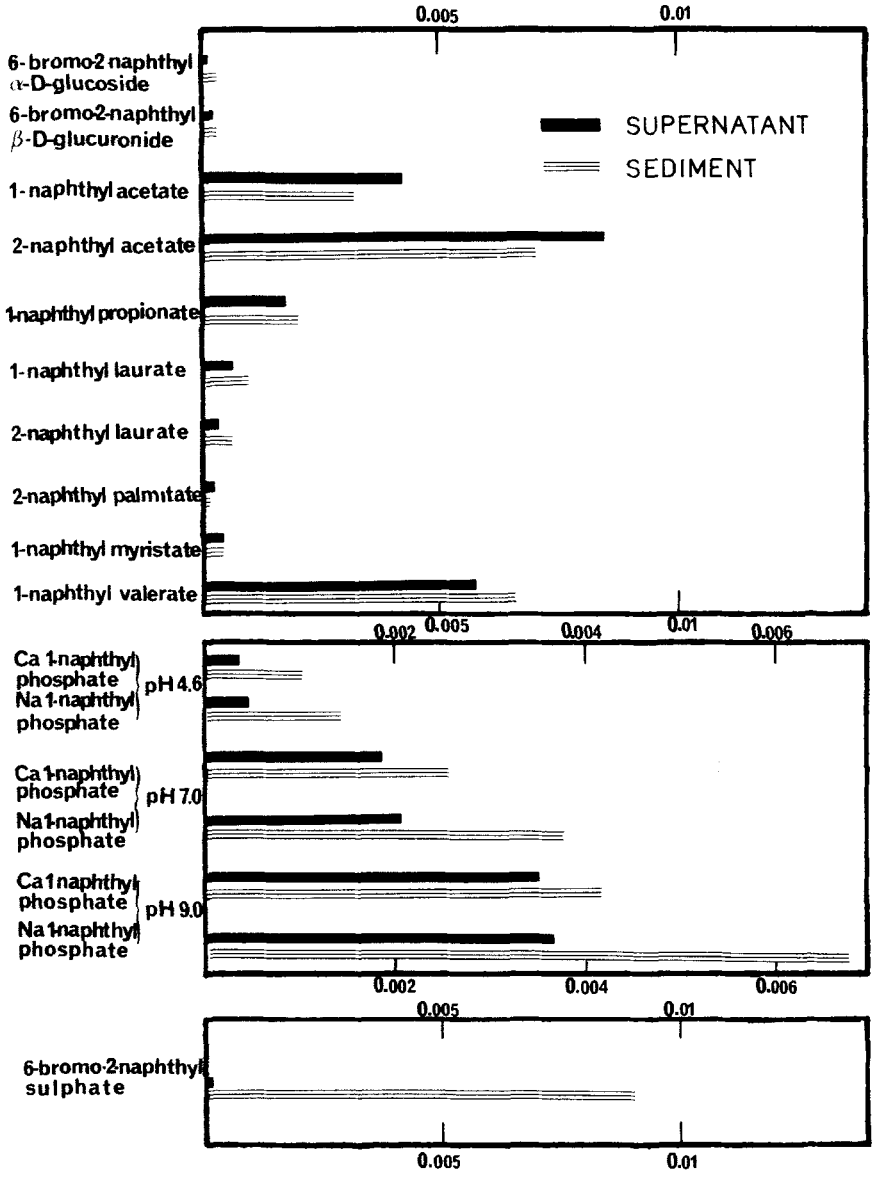

Fig. 1. Hydrolysis of various glycosidase, as well as carboxylic acid, phosphate, and sulphate ester substrates by centrifuged dog saliva and its sediment in 0.01 M tris-HCl pH 7.0 or 9.0, or 0.05 M $\beta.\beta$-dimethylglutarate buffer, pH 4.6. The activity is expressed as liberated μmoles of 1- or 2-naphtol per mg protein per min. Substrate concentration 0.167×10^{-3} M in the reaction mixture. Black columns, supernatant; hatched columns, sediment.

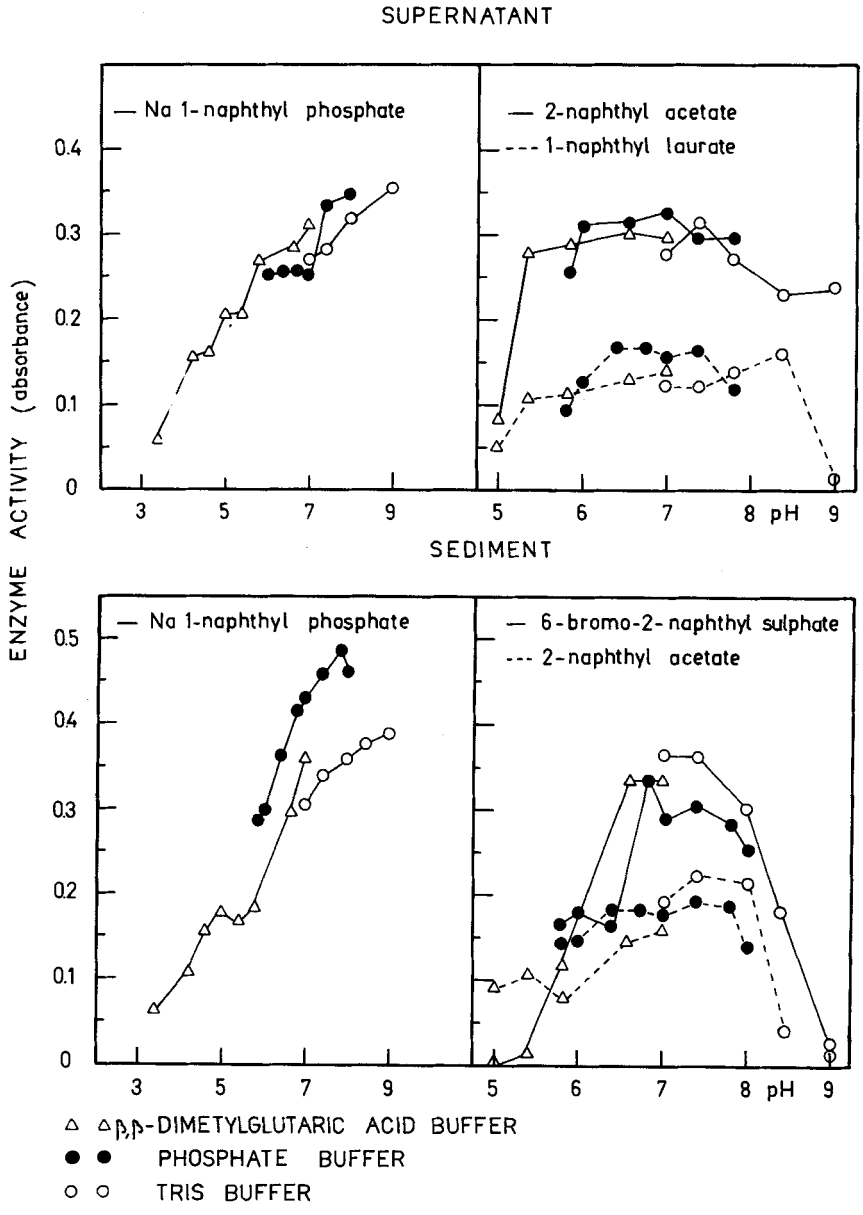

Fig. 2. Effect of pH on the hydrolysis of phosphate, sulphate, and carboxylic acid ester substrates by enzyme preparation from dog saliva supernatant (to the left) and its sediment (to the right). The test buffers: 0.05 M β,β-dimethylglutaric acid buffer from pH 3.4—7.0, 0.05 M phosphate buffer from 5.8—7.8 and 0.1 M tris-HCl buffer from 7.0—9.0. Substrate concentration: 0.167×10^{-3} M in the reaction mixture.

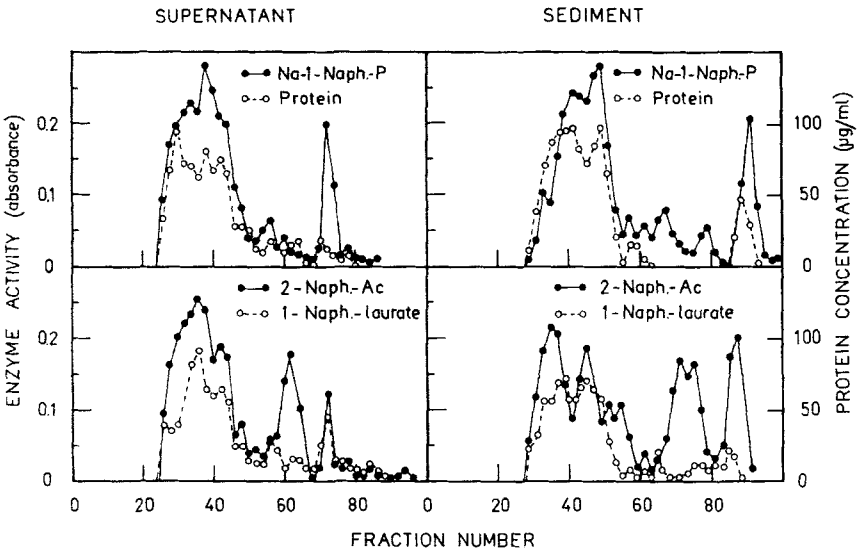

Fig. 3. Separation of phosphomonoester hydrolases and carboxylic acid esterases of dog saliva supernatant (to the left) and sediment (to the right) with a Sephadex G-200 column (90×2.5 cm). Elution: 0.1 M tris-HC; buffer, pH 7.0; flow rate: 0.18 ml/min, hydrostatic pressure: 15 cm H O; temperature 0—3 C; fraction volume 3.0 ml. Blue Dextran was fractionated into tubes 25—30. The protein concentration and enzyme activity were estimated every second fraction. Abbreviations: 1-naph. -P = 1-naphthyl phosphate, 2-Naph-Ac = 2-naphthyl acetate.

occurred in the rate of the hydrolysis of each individual substrate, depending on the experimental dog and the time of day when the sample was taken. However, the relative ratio of the hydrolysis rates between the substrates seemed to be constant. In Fig. 1. the mean values of the rate of hydrolysis from the seven dogs are represented.

Practically no hydrolysis of starch could be observed (not seen in Fig. 1) indicating that dog saliva did not possess any amylase (EC 3.2.1.1 and 3.2.1.2) or glucoamylase (EC 3.2.1.3) activity.

The hydrolysis of 6-bromo-2-naphthyl sulphate at the concentration used was observed to be linear with time up to 30 min, with calcium 1-naphthyl phosphate up to 5 hours, and with the esterase substrates to twenty hours.

The rate of the hydrolysis of phosphatase substrates, catalyzed by enzyme preparations both from supernatant and sediment fraction of dog saliva, was highest at alkaline pH values. (Fig. 2). The hydrolysis of 2-naphthyl acetate seemed to be rapid over a wide pH range, and thus no exact pH optimum could be observed in the conditions employed. The optimum pH for the hydrolysis of 6-bromo-2-naphthyl sulphate was between 7.0—7.4.

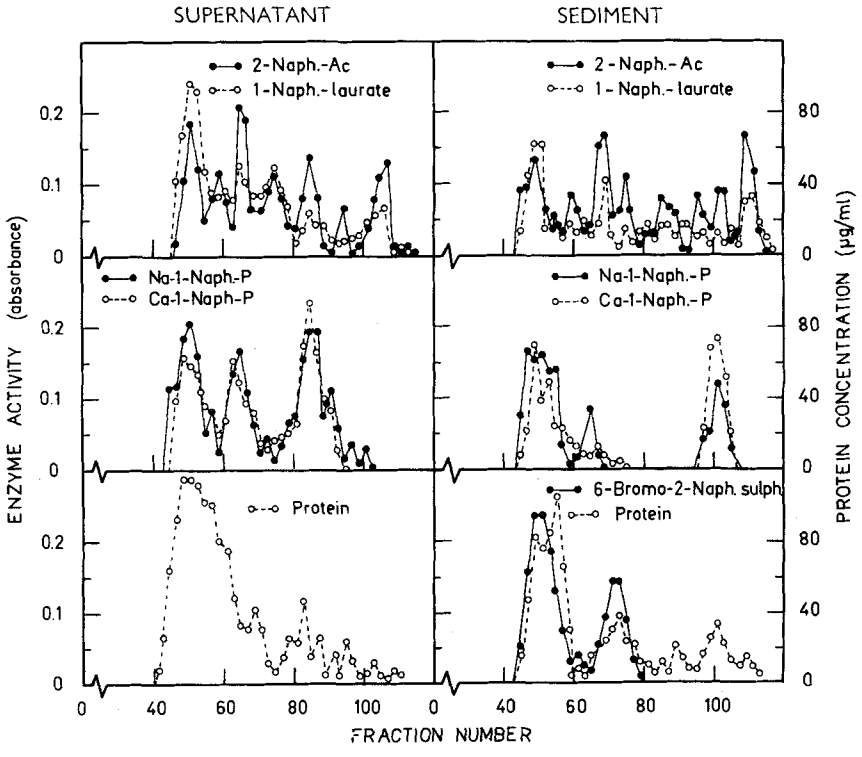

Fig. 4. DEAE-cellulose fractination of hydrolases from dog saliva supernatant (to the left) and its sediment (to the right). Sample 15.6 ml protein solution (the initial 5.0 ml enzyme preparation was first transferred to 0.005 M tris-HCl buffer, pH 7.0 by passing it through a Sephadex G-25 column (16×1.5 cm) with the buffer mentioned, and was marked with Blue Dextran, when the volume collected was 15.6 ml). DEAE-cellulose column: 30.0× 1.5 cm; elution buffer: 0.005 M tris-HCl, pH 7.0, containing a NaCl gradient from 0.005 to 0.5 M, which was thereafter raised directly to 1.0 M; flow rate: appr. 0.05 ml/min; hydrostatic pressure: 100 cm H_2O; temperature 0—3°C; fraction volume 1.5 ml. In each fraction the protein concentration and enzyme activity were estimated from every second fraction. Abbreviations as in Fig. 3.

2. *Fractionation of the enzymes*

The results from molecular exclusion chromatography on Sephadex® G-200 gel with supernatant and sediment fractions of dog mixed saliva are shown in Fig. 3. The following results require consideration: a) In general, two main enzyme peaks with phosphomonoesterase activity were encountered in the chromatograms; b) The first main fraction was eluted from the column in the same volume as Blue Dextran, used in testing the void volume of the gel columns. These enzymes may be proteins possessing a molecular weight of about 100.000 or more (based on the known exclusion limit of Sephadex

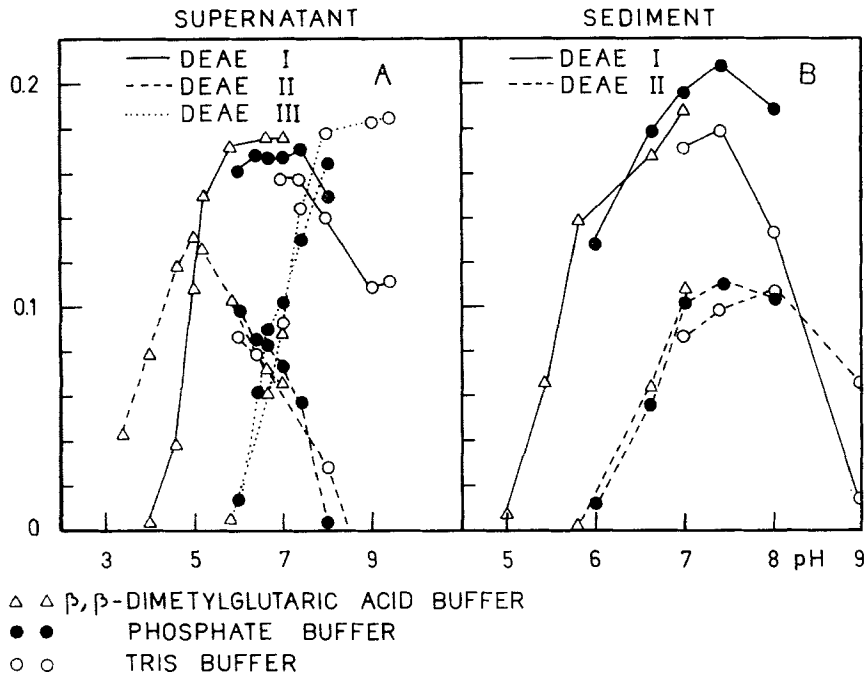

Fig. 5. Effect of pH on the hydrolysis of 1-naphtyl phosphate (to the left) from enzyme preparations obtained from the supernatant fluid of dog saliva by DEAE-cellulose chromatography, pooled 45—55 (DEAE I). 60—70 (DEAE II), and 80—90 (DEAE III) and the effect of pH on the hydrolysis of 6-bromo-2-naphthyl sulphate (to the right) form enzyme preparations from the sediment of dog saliva by DEAE cellulose chromatography, pooled from 45—58 (DEAE I) and 80—90 (DEAE II). The test buffers: 0.05 M β,β-dimethylglutaric acid buffer from pH 3.4—7.0, phosphate buffer from 5.8—7.8, and 0.1 M tris-HCl buffer from 7.0—9.0. Substrate concentration: 0.166×10^{-3} M.

G-100 gel, not seen in the figure). DEAE cellulose chromatography (Fig. 4) brought out three enzyme peaks with phosphomonoester hydrolase activity both from the supernatant and sediment fraction of dog saliva. Two enzyme peaks hydrolyzing 6-bromo-2-naphthyl sulphate were obtained from the sediment fraction. The hydrolysis of carboxylic ester substrates was observed by several enzyme peaks both from the supernatant and sediment fraction of dog saliva. Similar fractionation patterns were obtained by molecular exclusion chromatography.

The DEAE-fractions from 45 to 55, from 60 to 70 and from 80 to 90 were pooled from the dog saliva supernatant for further studies and designated as DEAE I, II, and III respectively. From the sediment fractionation, fractions from 45 to 58 and 65 to 77 were pooled and designated as DEAE I and II, respectively.

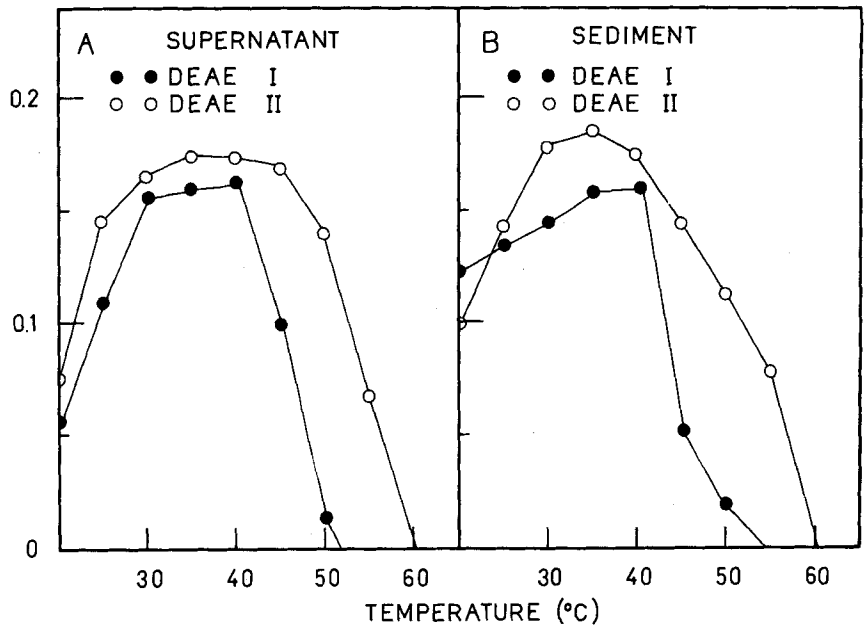

Fig. 6. Effect of temperature on the hydrolysis of 1-naphthyl phosphate by enzyme preparations from dog saliva supernatant (to the left) and of 6-bromo-2-naphthyl sulphate by enzyme preparations from sediment fraction (to the right). The preparations were pooled as seen in Fig. 5. Buffer: 0.1 M tris-HCl buffer, pH 7.0, substrate concentration 0.166×10^{-3} M in the reaction mixture.

The curves in Figs. 3—4 were selected from several others as illustrations of the varying enzyme spectrum of dog saliva. It was not possible to obtain identical fractionation of samples collected from different test animals, not even from one and the same animal at different collecting times.

3. *Characterization of phosphate and sulphate esters*

The three phosphomonoesterase and two sulphuric ester hydrolase peaks obtained by DEAE cellulose chromatography were studied for some enzymic properties: the effect of temperature, pH and some affectors. All experiments were carried out with a substrate concentration of 0.166 mM.

The pH dependence curves for the enzymes from dog saliva supernatant hydrolyzing 1-naphthyl phosphate are shown in Fig. 5 a. The enzyme preparation DEAE I was active at a wide pH range. DEAE II had its pH optimum at an acidic value, close to pH 6, and DEAE III at an alkaline value, pH 9.

Table I

The effect of various chemical compounds on the rate of hydrolysis of sodium l-naphthyl phosphate (0.166 mM), catalyzed by enzyme preparations obtained with DEAE-cellulose chromatography from dog saliva supernatant fluid. The effects are given in percentage inhibition or activation (+) as compared to the effect observed without added affector (water instead)

Compound	Concentration (mM)	DEAE I	DEAE II	DEAE III
$CaCl_2$	1.0	0	+ 10	+ 70
	0.5	0	0	+ 30
$CuCl_2$	1.0	0	0	0
	0.5	0	0	0
$HgCl_2$	1.0	100	80	80
	0.5	100	50	60
$MgCl_2$	1.0	0	+ 40	+ 90
	0.5	0	+ 20	+ 70
$MnCl_2$	1.0	0	0	+ 40
	0.5	0	0	+ 40
NaF	1.0	0	80	20
	0.5	0	30	10
Diethyl-*p*-nitrophenyl phosphate	1.0	0	0	100
	0.5	0	0	100
Diisoprolylfluorophosphate	1.0	0	0	100
	0.5	0	0	100
p-chloromercuribenzoate	1.0	0	100	0
	0.5	0	100	0
N-ethylmaleimide	1.0	0	100	0
	0.5	0	70	0
EDTA	1.0	0	30	100
	0.5	0	20	100

In Fig. 5 b is shown the pH dependence curves of dog saliva sediment enzyme(s), hydrolyzing 6-bromo-2-naphthyl sulphate. The pH optimum of the enzyme preparation DEAE I and DEAE II was at pH 7.4.

Fig. 6 a shows that the enzymes hydrolyzing l-naphthyl phosphate obtained by DEAE-cellulose chromatography from dog saliva supernatant fluid had their optimum temperature at +35—40°C. Three hours incubation at +60°C totally destroyed the enzyme activity. The hydrolysis of 6-bromo-2-naphthyl sulphate catalyzed by DEAE I enzyme preparation from dog saliva sediment had its temperature optimum at +35°C and DEAE II at +40°C.

Table II

The effects of various chemical compounds on the rate of hydrolysis of 6-bromo-2-naphthyl sulphate (0.166 mM) by enzyme preparations obtained with DEAE-cellulose chromatography from dog saliva sediment. The effect is given in percentage inhibition as compared to the effect observed without added affector (water instead). 0 signifies that no effect was observed

Compound	Concentration (mM)	DEAE I	DEAE II
$CaCl_2$	1.0	0	0
	0.5	0	0
$CuCl_2$	1.0	0	0
	0.5	0	0
$HgCl_2$	1.0	70	100
	0.5	30	100
$MgCl_2$	1.0	0	0
	0.5	0	0
$MnCl_2$	1.0	0	0
	0.5	0	0
NaF	1.0	100	70
	0.5	100	40
EDTA	1.0	0	0
	0.5	0	0
Diethyl-*p*-nitrophenyl phosphate	1.0	0	0
	0.5	0	0
Diisopropylfluorophosphate	1.0	0	0
	0.5	0	0
p-chloromercuribenzoate	1.0	0	30
	0.5	0	0
N-ethylmaleimide	1.0	0	40
	0.5	0	20

(Fig. 6 b). Incubation for 30 min at +60°C totally destroyed the enzyme activity.

The effect of some enzyme affectors on the hydrolysis of l-naphthyl phosphate is seen in Table I. Of the compounds studied only Hg^{2+} ions (0.05 mM) totally inhibited the DEAE I enzyme. N-ethylmaleimide (1.0 mM) and *p*-chloromercuribenzoate (0.5 mM) inhibited totally, and Hg^{2+} ions (1.0 mM) and F^- ions (1.0 mM), inhibited roughly 80 % of the DEAE II enzyme activity. The DEAE III was totally inhibited by diethyl-*p*-nitrophenyl phosphate (0.5 mM), by diisopropylfluorophosphate (0.5 mM), and by EDTA (0.5 mM). Marked activation of DEAE II and DEAE III phosphatase

activity was obtained with Mg^{2+} ions (0.5 mM); DEAE III was additionally activated by Ca^{2+} and Mn^{2+} ions, both at a concentration of 0.5 mM in the reaction mixture.

Most of the chemical compounds studied had no effect on the hydrolysis of 6-bromo-2-naphthyl sulphate. Hg^{2+} ions (1 mM) and F^- ions (1.0 mM) inhibited strongly or totally the enzyme preparations DEAE I and DEAE II, *p*-chloromercuribenzoate and N-ethylmaleimide inhibited slightly the preparation DEAE II.

DISCUSSION

The enzymes described in the present investigation, especially those catalyzing the hydrolysis of 6-bromo-2-naphthylsulphate, were probably largely produced by oral microorganisms, because (1) the specific activity was in general higher in the sediment fraction, (2) the sediment fraction was only 1/10 of the volume of the supernatant. Thus the main source of these enzymes in mixed saliva seemed to be the material of the sediment, i.e. microorganisms, epithelial cells, food debris etc.

The observation that dog saliva does not possess amylase activity was confirmed in this study. Additionally, the rather low activity against 6-bromo-2-naphthyl-α-D glucoside, probably indicating low α-glucosidase activity (α-D-Glucoside glucohydrolase, EC 3.2.1.20) would indicate that the carbohydrate metabolism in the oral cavity of dog differs from that of human. In dog saliva there seems to be no hydrolysis of α-1,4-glucan links in polysaccharides and only a few enzymes hydrolyzing α-D-glucosidic linkages. The rate of the hydrolysis of these linkages by human salivary enzymes would be rather high (*Mäkinen*, 1966 c). The significance of this observation in the different attitude of dog to caries, however, could not be established in this study.

The hydrolysis of carboxylic acid ester by dog salivary enzymes was relatively high. The rate of hydrolysis of various carboxylic acids seemed to be similar to that of man: the 1- and or 2-naphthol esters of longer chain carboxylic acids were hydrolyzed at a lower rate than those of shorter chains. Also the fractionation patterns of these enzymes with DEAE-cellulose chromatography resembled that obtained with man (*Mäkinen*, 1966 c) because several different peaks hydrolyzing l-naphthyl acetate and 2-naphthyl laurate could be observed.

Chayncey et al. (1963) were not able to demonstrate alkaline phosphatase activity in dog parotid or submaxillary saliva. The present findings revealed

that the highest rate of hydrolysis of 1-naphthyl phosphate by dog mixed saliva was obtained on the alkaline side. The pH dependence curves obtained with pooled enzyme preparations after DEAE-cellulose chromatography showed that one enzyme peak had its pH optimum on the alkaline side (DEAE II). It was activated by Mg^{2+} ions and by some other divalent cations. Further, diethyl-*p*-nitrophenyl phosphate and diisopropylfluorophosphate, compounds known to inhibit enzymes which require the action of an active serine residue, inhibited this enzyme preparation. All these results would indicate the presence of an alkaline phosphatase in dog mixed saliva (*Morton*, 1961).

On the other hand the enzyme preparation DEAE II was identified as an acid phosphatase, because (1) pH optimum, (2) N-ethylmaleimide and *p*-chloromercuri benzoate, compounds known to inhibit SH enzymes, inhibited it and (3) Mg^{2+} ions slightly activated it. All these enzymic properties would indicate the presence of acid phosphatase in dog saliva (*Morton*, 1961).

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