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THE EFFECT OF F^- IONS ON THE RATE OF SOME HYDROLYTIC ENZYME REACTIONS

by

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INTRODUCTION

The purpose of this investigation was to study the effect of fluoride ions on the rate of the hydrolysis catalyzed by some hydrolytic enzymes or enzyme preparations, as well as on some enzymes which may play a role in the degradation of the tooth in carious processes or of periodontal structure in periodontal disease. The pertinent literature lacks valid quantitative experimental data on this matter.

MATERIALS AND METHODS

Chemicals. All chemicals used in this study were supplied by E. Merck AG (Darmstadt, Germany) if not otherwise stated.

Commercial enzyme preparations. The following commercial enzyme preparations were used: Trypsine, (Trypure®, Novo Industri A/S, Copenhagen, Denmark); Papain, N. F. VIII, (E. Merck AG); Calculase, Fungal ML 443, (Mann Research Laboratories Inc., New York, N.Y., U.S.A.); Subtilopeptidase, Grade VIII, (Sigma Chemical Company, St. Louis, Mo., U.S.A.); Leucine aminopeptidase, Grade III, from hog intestinal mucosa, (Sigma

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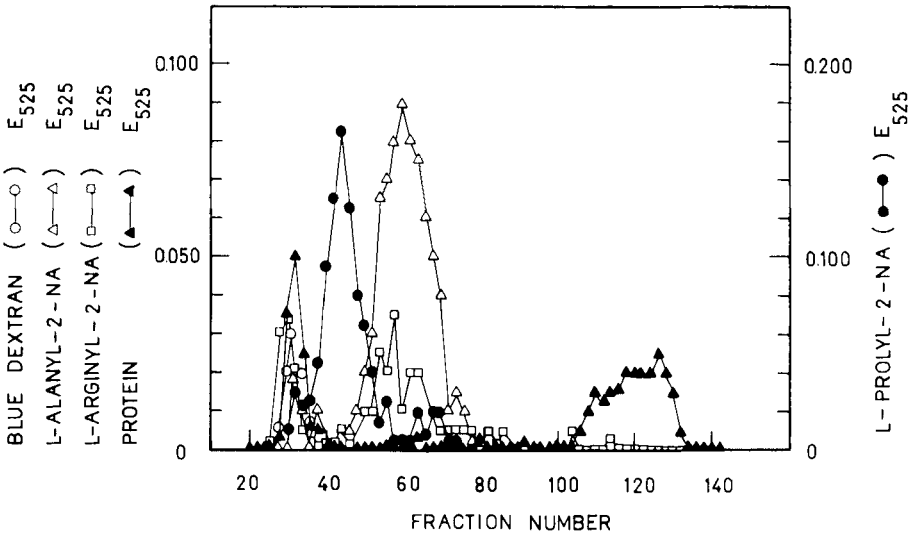

Fig. 1. Gel permeation chromatography performed on human dental plaque enzymes capable in hydrolysing N-L-arginyl-2-naphthylamine, N-L-alanyl-2-naphthylamine and N-L-prolyl-2-naphthylamine. Column: 55 cm × 2.5 cm; hydrostatic pressure: 30 cm; fraction size: 2.7 ml; flow rate: 3 ml/h; buffer: 0.01 M tris-HCl, pH 7.0; temperature: +2°C; 3 ml freeze dried enzyme solution. The fractions from 26 to 36 were pooled to obtain Pool (P I), and the fractions from 37 to 52 were pooled to obtain Pool II (P II), and the fractions from 52 to 71 were pooled to obtain Pool III (P III). Blue dextran®.

Chemical Company), α-Chymotrypsine, from bovine pancreas, type II (Sigma Chemical Company); Dextranase, neutral, NRRL 896, Penicillinum lilacinum (Swiss Ferment Co. Ltd., Basle, Switzerland); and Alkaline phosphatase, Type III S, E.coli (Sigma Chemical Company).

Dental plaque enzymes. Plaque material was obtained from persons coming to the clinic. The plaque (50 mg) was collected into 5 ml iced 0.154 M NaCl solution. The saline plaque mixture was then cold (+2°C) centrifuged for 20 min at 20.000 × g (Sorvall Superspeed Centrifuge, Model RC-2B). The supernatant obtained was collected and the pellicle was discarded. Enzyme preparations obtained from different persons were pooled. The pooled enzyme preparations were then subjected to gel partition chromatography on Sephadex® G-200. The enzyme were recovered after the chromatography, assaying the fractions with N-L-arginyl-, N-L-alanyl- and N-L-prolyl-2-naphthylamine. Three enzyme pools were obtained according to Fig. 1, and the enzyme preparations thus obtained were called P I, II nad III. For further details see Fig. 1.

Human alkaline phosphatase. Three different enzyme preparations (E I, II and III) were obtained from mineralizing bones of 8 to 10 weeks old

human foetuses. The alkaline phosphatase preparations were obtained by fractionation on DEAE-cellulose ionexchange columns as described by *Mäkinen & Paunio* (1969).

Human gingival enzymes. Both crude and fractioned enzyme preparations were used in this study. The fractionation of the enzyme preparation was obtained by gel permeation chromatography on Sephadex G-200 according to *Mäkinen & Paunio* (data to be published).

Pig dental pulp aminopeptidase (II). The enzyme was obtained by fractionation of pig dental pulp on DEAE cellulose ionexchange columns according to *Mäkinen et al.* (1970).

Hydrolytic enzymes of L-casei. The enzymes were obtained from a culture of L-casei by gel permeation chromatography on Sephadex G-200.

Substrates. The following N-L-aminoacid-2-naphthylamines were obtained from Mann Research Laboratories (Los Angeles, California, U.S.A.): N-L-arginyl-2-naphthylamine, N-L-arginyle-2-naphthylamine, N-L-benzoyl-2-naphthylamine, N-L-alanyl-2-naphthylamine, N-L-leucyl-2-naphthylamine, N-L-methionyl-2-naphthylamine and N-L-prolyl-2-naphthylamine. p-Nitrophenyl phosphate from Sigma Chemical Company (St. Louis, Mo., U.S.A.), L-djencolic acid from K & K Laboratories Inc. (Hollywood, California, U.S.A.) and Blue dextrane® from Pharmacia Fine Chemicals.

Determination of enzyme activity with synthetic substrates. The determination of the phosphorolytic activity with p-nitrophenyl phosphate as substrates was based on measurements of the color intensity produced by enzymatically liberated p-nitrophenyl anion at 410 nm as described by *Bessey et al.* (1946). The determination of N-L-aminoacyl-2-naphthylamine was determined as described by *Mäkinen* (1969). The results obtained are given as the velocities of the enzymatically liberated p-nitrophenyl anion or naphthylamine given as $Mmin^{-1}$.

Determination of keto acid forming enzymes. The method used in this study was described by *Mazelis et al.* (1967). The supernatant fluids were assayed for pyruvate colorimetrically by the total keto acid method of *Friedmand* and *Haugen* (1943). Pyruvic acid supplied by Fluka AG (Buchs SG, Switzerland) was used in this study as the standard.

Determination of transaminase activity. The activity of glutamate-pyruvate-transaminase and of glutamate-oxalacetate-transaminase was determined colorimetrically with 2.4-dinitrophenylhydrazine as described by *Bergmeyer* and *Bernt* (1963).

Determination of proteins. The proteins were determined according to the Folin-Ciocalteu method described by *Layne* (1963).

Determination of dextranase activity. Dextranase activity was determined

Table I

The innocuous effect of F^- ions on the rate of hydrolysis of three N-L-aminoacid-2-naphthylamines, blue dextran, L-djencolic acid and on transaminase activity by the listed enzyme preparations

Enzyme	Substrate	Buffer	Incubation time	pH	Activators
P I Dental plaque	N-L-arginyl-2-naphthylamine	0.05 M phosphate buffer	150 min.	7.2	
P II Dental plaque	N-L-methionyl-2-naphthylamine	0.05 M phosphate buffer	150 min.	7.2	
P III Dental plaque	N-L-methionyl-2-naphthylamine	0.05 M phosphate buffer	150 min.	7.2	
P II Swine dental pulp	N-L-arginyl-2-naphthylamine	0.05 M phosphate buffer	120 min.	7.2	0.4 M NaCl
Neutral dextranase mold	Blue dextran®	0.05 M $\beta\beta$-dimethylglutaric acid	60 min.	5.0	
Keto acid forming enzymes Lactob. casei	djencolic acid	0.1 M tris-HCl	180 min.	7.0	
Transaminase human gingiva	GOT	0.1 M phosphate buffer	60 min.	7.4	
	GPT	0.1 M phosphate buffer	90 min.	7.4	

Table II

The activating effect of F^- ions on the rate of hydrolysis p-nitrophenyl phosphate and two N-L-aminoacid-2-naphthylamides, tested at two substrate concentration levels (0.166×10^{-3} M and 0.071×10^{-3} M), when performed at 30°C by the listed hydrolytic enzymes. The activation is presented as an increase (in percentage) of the velocity (v) of the enzymic reaction

Enzyme	Substrate	Buffer	pH	Incub. time	Activation in % 0.166×10^{-2}M F^-
Alkaline phosphat. E. coli	p-nitrophenyl phosphate	0.05 M phosphate buffer	9.62	10 min.	10
Papain	N-L-lysyl-2-naphthylamine	0.05 M $\beta\beta$-dimethyl-glutarate	5.0	15 min.	25
P II Dental plaque	N-L-prolyl-2-naphthylamine	0.05 M phosphate buffer	7.2	150 min.	6

Table III.

The activating effect of low F^- ion concentration levels and the inhibiting effect of high F^- ion concentration levels on the rate of hydrolysis of some N-L-aminoacid-2-naphthylamines tested at two substrate concentration levels (0.166×10^{-3} M and 0.071×10^{-3} M) by the listed enzyme preparations, when performed at 30°C. The activation and inhibition is presented as an increase or decrease (in percentage) of the velocity (v) of the enzymic reaction

Enzyme	Substrate	Buffer	pH	Incub. time	Activation at 0.83×10^{-4} M F^- concentr.	Inhibition at 0.16×610^{-2}M F^-concentr.
Trypure®	N-L-benzoylarginyl-2-naphthylamine	0.05 M phosphate	7.8	30 min.	15 %	15 %
Calculase	N-L-methionyl-2-naphthylamine	0.01 M tris-HCl	7.0	60 min.	3 %	16 %
α-Chymotrypsin	N-L-leucyl-2-naphthylamine	0.05 M phosphate	7.8	60 min.	3 %	18 %

Table IV.

The inhibiting effect of F^- ions on the rate of hydrolysis p-nitrophenyl phosphate and two N-L-aminoacid-2-naphthylamines, tested at two substrate concentration levels (0.166×10^{-3} M and 0.071×10^{-3} M) by the listed enzyme preparations, performed at 30°C. The calculated inhibition constants (K_i) for the F^- ion were obtained according to the plotting method of Dixon.

Enzyme	Substrate	Buffer	pH	Incub. time	K_i for F^-	$MgCl_2$ Enzyme affect
P III Dental plaque	N-L-alanyl-2-naphthylamine	0.05 M phosphate	7.2	150 min.	2.4×10^{-1} M	
Peptidase Hog intestine	N-L-leucyl-2-naphthylamine	0.05 M phosphate	7.0	60 min.	2.1×10^{-1} M	
Protease Subtilopeptidase	N-L-leucyl-2-naphthylamine	0.05 M boric acid borax	8.0	60 min.	1.8×10^{-1} M	
E I Alkaline phosphatase Human	p-nitrophenyl phosphate	0.05 M glycine-NaOH	9.62	210 min.	2.5×10^{-2} M	
E II »	»	»	»	»	4.5×10^{-2} M	
E III »	»	»	»	»	5.6×10^{-2} M	
E I »	»	»	»	90 min.	2.0×10^{-2} M	0.071×10^{-3} M
E II »	»	»	»	»	2.4×10^{-2} M	»
E III »	»	»	»	»	2.5×10^{-2} M	»

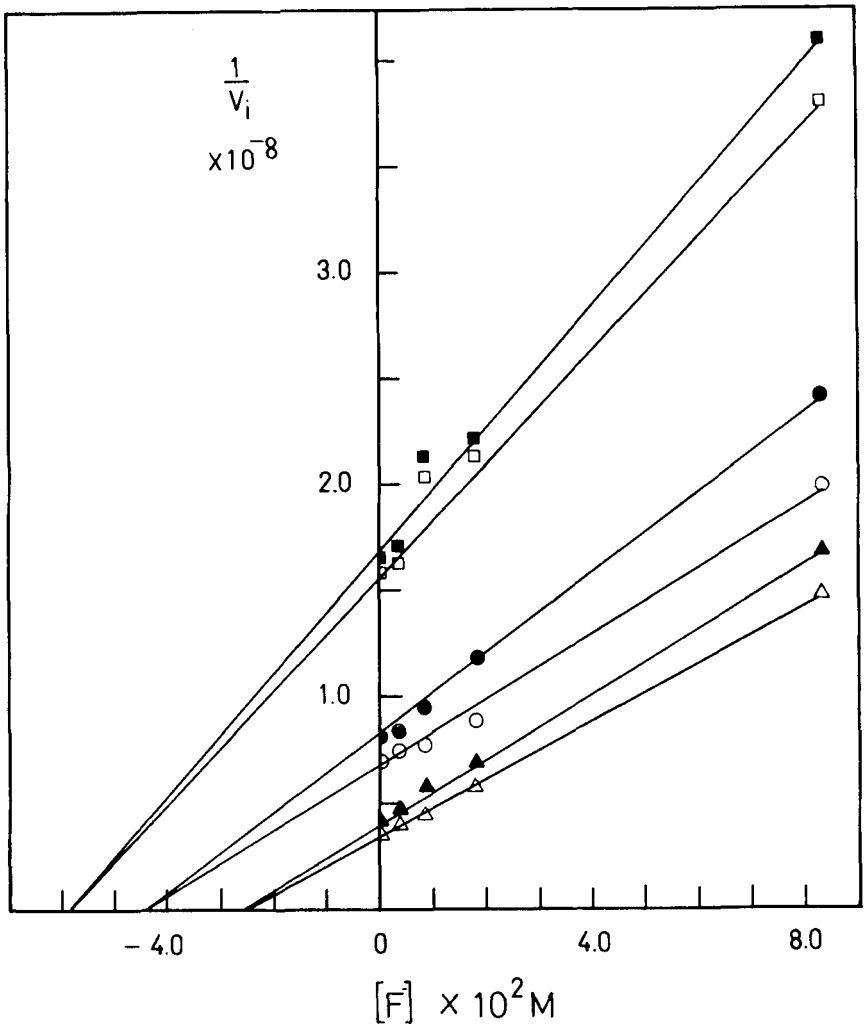

Fig. 2. The effect of F^-ions on the enzymic hydrolysis of 0.166×10^{-3} M (open marks) and 0.071×10^{-3} M (closed marks) p-nitrophenyl phosphate, produced by three different alkaline phosphatase preparations E I (△, ▲), E II (○, ●) and E III (□, ■) of mineralizing human foetal tissues. $1/v_i$ (v_i= the molar change of the enzymatically liberated p-nitrophenol anion in the reaction mixture per min.) is plotted against the F^-ion concentration according to Dixon. The incubation was performed at 30°C for 210 min.

according to the method described by *Mäkinen* and *Paunio* (1971) with Blue dextrane as substrate.

Determination of the inhibition constant K_i. The apparent inhibition constant K_i was determined in this study according to the plotting method

described by *Dixon* (1953). I.e. $1/v_1$ (v_1 = rate of reaction in presence of the inhibitor) was plotted as a function of the inhibitor concentration (I).

RESULTS

Fig. 1 shows the distribution and the way of pooling of human dental plaque enzymes used in this study for further investigation.

The results obtained in this study are presented in table form according to the effect F^- exerted on the respective enzymic reactions.

The enzymes listed in Table I were not affected by the presence of the F^- ions in the reaction mixtures. Table II presents the enzyme preparations being activated by fluoride, and Table III presents the enzymes being activated in presence of low fluoride concentrations, but the rate of enzymic reactions was decreased when higher concentrations of fluoride was present in the reaction mixtures. This Table reveals the readuction or increase of the rate of hydrolysis given in percentage. Table IV lists the enzyme preparations inhibited by the F^- ion, and reveals the graphically determined inhibition constants (K_i) for the tested enzymes. The K_i values of the human foetal alkaline phosphatase preparations obtained in experiments performed in presence of $MgCl_2$ were determined from only three different experimental values due to a nonlinearity of the experimental points. Fig. 2 reveals the distribution of the experimental values obtained with the same enzyme preparations but performed without $MgCl_2$, representing five different experimental values.

DISCUSSION

The purpose of this study was to elucidate the effect of F^- ions on a number of mainly hydrolytic enzyme activities using enzyme kinetic methods. Based on the results obtained in this study it could be said that fluoride ion does not exert any ecceptional effect on the tested enzyme activities. In some instances the fluoride ion produced an inhibition of the enzymatic reactions studied, but the inhibition was, however, not of any greater magnitude, which is based on the calculated rather high K_i values suggesting of a rather mild inhibition. Therefore it could be said that F^- ion exerts a similar effect (activation, inhibition and inocuous effect) on many enzymic reactions as the other halogenides, especially Cl^-.

The results obtained in this study do not reveal whether the inhibition or the activation of the enzymic hydrolysis reactions was produced by the F^- ions, or if the obtained changes were produced by secondary changes in the

ambient solution. Such changes could for instance be a change in the ionic strength due to an increased fluoride concentration. It is known that ionic strength changes may alter the activity of the important groups of the enzymes for the enzymic hydrolysis reactions. The slight alterations obtained in this study in the rate of reactions could probably be ascribed changes in the ambient solution rather than to an effect on the formation at the enzyme substrate (ES) complex by the F^- ion itself.

The experiments performed with human alkaline phosphatase preparations, on the other hand, raised questions about the action of fluoride ions. It has been suggested by *Hellung-Larsen* and *Klenow* (1969) that the fluoride inhibition of the hydrolysis reaction produced by alkaline phosphatase would be Mg^{2+} dependent due to a complex formed between Mg^{2+}, F^- and the phosphate. The results obtained in this study suggest that the inhibition would not be Mg^{2+} dependent. This view is supported by the rather similar K_i calculated from the experimental values obtained with and without $MgCl_2$. The calculated K_i values are, however, highly subjective, especially with $MgCl_2$ present in the incubation media, while only three experimental points were used for the calculations.

In the experiments performed in this study and in corresponding studies the fluoride ion concentration has been kept rather high, ranging from 10^{-4} M to 5 M. The lowest F^-ion concentration in this study produced very little reduction or increase in the rate of enzymic hydrolysis of the substrate, though a greater decrease was first produced at high fluoride ion concentration levels. When these F^-ion concentration values are compared with those found in blood serum, same question rise about F^-ion being an enzyme inhibitor under *in vivo* conditions. Human serum probably contains two types of fluoride. The observation that 80—90 % of the total serum fluoride is not available as exchangeable fluoride until after ashing suggests that it is bound to serum proteins (*Taves*, 1968, 1969). The existence of two types of fluoride in the serum reduces the amount of fluoride available for different enzyme reactions. The fluoride concentration in serum seems to be rather constant (between 0.10—0.20 ppm) when fluoride ingestion is kept reasonably low, e.g. below 2.5 ppm in the drinking water (*Singer & Armstrong*, 1960). If 80—90 % of the total fluoride were in some way bound to the serum proteins, then only 0.02—0.04 ppm would be available for the different exchange reactions. Approximately 2×10^{-6} M F^-ion concentration would then be available. When this value is compared with those tested in this study, and other corresponding studies, it could be said that the values obtained in serum do not produce any measurable changes in the enzymic hydrolysis reactions.

SUMMARY

The effect of F^- ions on a number of mainly hydrolytic enzyme preparations was studied. Enzyme kinetic methods were used to elucidate the effect of the tested ions. In a total of 23 different enzyme activities were studied. The results revealed that fluoride ions produced some inactivation of some enzymic reactions by reducing the enzymic reaction velocities. The calculated K_i values for the fluoride ions were, however, rather high. A number of the tested enzyme preparations were completely unaffected, and some enzymic reactions were activated by the fluoride ion. The role of F^- ion as strong enzyme inhibitor *in vivo* conditions discussed.

RÉSUMÉ

ACTION DES IONS F^- SUR LA VITESSE DE QUELQUES RÉACTIONS ENZYMATIQUES D'HYDROLYSE

Une étude a été faite sur l'action des ions F^- sur plusieurs préparations enzymatiques, surtout hydrolytiques. Des méthodes cinétiques enzymatiques ont été utilisées pour mettre en lumière l'action des ions testés. Au total 23 activités enzymatiques différentes ont été étudiées. Les résultats ont montré que les ions fluorure déterminaient une certaine inactivation de quelques réactions enzymatiques en réduisant les vitesses de réaction. Les valeurs K_i calculées pour les ions fluorure étaient cependant assez élevées. Plusieurs des préparations enzymatiques testées n'étaient absolument pas affectées, et quelques réactions enzymatiques se trouvaient activées par l'ion fluorure. L'auteur discute le rôle de l'ion F^- comme puissant inhibiteur enzymatique dans les conditions *in vivo*.

ZUSAMMENFASSUNG

DIE WIRKUNG VON FLUOR AUF DIE REAKTIONSGESCHWINDIGKEIT EINIGER HYDROLYTISCHEN ENZYME

In dieser Untersuchung wurden hauptsächlich hydrolytische Enzyme verwendet. Enzymkinetische Methoden wurden verwendet um die Wirkung von F^- zu ermitteln. Insgesamt wurden 23 verschiedene Enzymaktivitäten untersucht. Die Ergebnisse zeigten F^- Abnahme bei einigen enzymatischen Reaktionsgeschwindigkeiten. Die gerechneten K_i-Werte für Fluor waren jedoch verhältnismässig hoch. Die Ergebnisse zeigten weiter, dass F^- bei

einigen Enzymen die Zunahme von enzymatischen Reaktionsgeschwindigkeiten verursachte und in anderen Fällen Fluor gar keine Wirkung hatte. In der Arbeit diskutiert man über die Wirkung von Fluor bei den Verhältnissen *in vivo*.

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