HUMAN PAROTID SALIVARY ACID PHOSPHOMONOESTERASE

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Human mixed saliva is known to contain phosphomonoesterases with both alkaline and acid pH optima[1]. A variety of hydrolytic enzymes has been found[2], and there was demonstrated an ability of whole saliva and cultures of oral microorganisms to hydrolyze monosodium β-naphthylphosphate at pH 4.8 and 9.1. The phosphomonoesterase of the parotid secretion, however, was active only in the acid range, the titer of which was of similar magnitude to that found in serum. Although the amounts of protein present were small, these studies indicated that a considerable portion of the protein was enzymic in nature. Since acid phosphatases were found to have relatively high concentrations among those enzymes found, a kinetic description of this enzyme is presented here in order to provide a comparison of its properties with other known acid phosphomonoesterases.

METHODS AND MATERIALS

Collection of saliva

Parotid saliva was obtained from normal males by cannulation of the parotid gland with a vacuum cap[3]. The orifice of Stensen's duct and the surrounding buccal mucosa were swabbed with 70% ethanol prior to fastening the cap. Secretion was stimulated by the chewing of flavored chicle. Samples were collected in tubes immersed in iced water. These were pooled and assayed within two hours after collection. Parotid saliva (pH 7.3–7.4) could be maintained at 4° C with little measurable loss of activity up to four days, although complete loss of phosphatase activity occurred in one hour at room temperature at physiological pH.

Phosphomonoesterase measurements

Phosphomonoesterase activity was determined by a modification of the method of SELIGMAN *et al.*[4]. The substrate was monosodium β-naphthylphosphate and incubation solution was 0.50 ml of parotid saliva buffered with 1.2 *M* acetate buffer at pH 4.5–4.6. The β-naphthol liberated was determined colorimetrically at pH 7.4–7.8 with tetrazotized diorthoanisidine at 540 mμ (Klett). Assays were made in triplicate and measured against blanks containing buffered substrate to which parotid saliva was added immediately before color development of the liberated β-naphthol with tetrazotized diorthoanisidine.

A group of 20 determinations made simultaneously under average experimental conditions with 0.5 ml of freshly secreted saliva incubated for one hour at 37° C and pH 4.58 in $4.05 \cdot 10^{-4}$ *M* (final concentration) of monosodium β-naphthylphosphate liberated 21.5 ± 1.0 μg/h (S.D.) of β-naphthol. The enzymic activity was found to be stable and reproducible, within the limits of precision given, below pH 5.0 at 37° C for a minimum of 18 hours. Under the same conditions,

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References p. 502.

using the LOWRY AND LOPEZ method[5], no measurable activity was determined using adenosine triphosphate and fructose-6-phosphate as substrates. Slight activity was observed using fructose 1,6-diphosphate and sodium pyrophosphate as substrates.

Phosphate liberated from sodium β-glycerophosphate was measured by the FISKE-SUBBAROW method[6]. Phosphomonoesterase activity was also determined employing disodium phenylphosphate[7] and disodium p-nitrophenylphosphate[8] as substrates.

RESULTS

Effect of pH

A well defined pH optimum was observed between pH 4.5–4.7 (Fig. 1) measured on successive days from a single pool of saliva. This was approximately 0.5 units lower than that observed for acid phosphomonoesterases of most sources[9], although several have been reported with optima very similar, *i.e.*, an erythrocyte phosphomonoesterase at pH 4.4–5.3[10], and a prostatic phosphomonoesterase at pH 4.5–5.0[11].

Enzyme substrate affinity

The influence of substrate at pH 4.56 and 37° C is demonstrated in Figs. 2 and 3. At the high concentrations of initial substrate (C_0) zero order kinetics were demonstrated (Fig. 2) whereas fractional and first order kinetics were observed as the substrate concentration was decreased. The rates were similarly altered by variations of saliva from 0.25 ml to 2.0 ml at fixed substrate concentrations. At the lowest

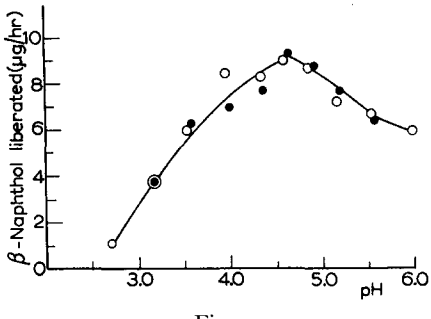

Fig. 1.

Fig. 1. The effect of pH on the reaction velocity at 37° C. The incubation mixture contained 0.5 ml saliva, 2.5 ml substrate (the final concentration was $4.10 \cdot 10^{-4} M$), and 2.5 ml of 1.2 M acetate buffer. (○) and (●) were determinations on successive days.

Fig. 2. The influence of substrate concentration on the hydrolysis of β-naphthylphosphate by parotid saliva. C/C_0 = fraction of substrate remaining at time T. 2a. The effect of high S. The incubation mixture contained 0.5 ml of parotid saliva diluted 1:1 with water and 5.0 ml of buffered substrate at pH 4.56 and 37° C. The final substrate concentration × $10^{-3} M$/ml saliva was: ● —3.25, ○—2.43, ■ —2.04, □ —1.62, and ▲ —1.42.

2b. The effect of low S. The incubation mixture contained: ●— 1.0 ml of parotid saliva and 5.0 ml of buffered substrate (final concentration $3.09 \cdot 10^{-5} M$), and ○ —2.0 ml parotid saliva and 5.0 ml of buffered substrate (final concentration $2.27 \cdot 10^{-5} M$). The pH was 4.56 and T = 37° C.

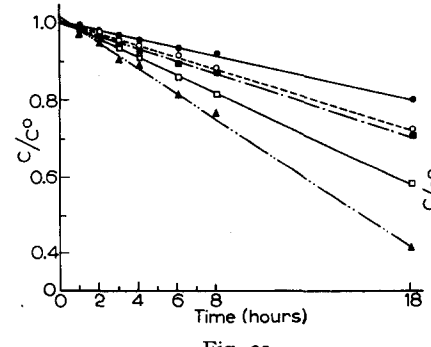

Fig. 2a.

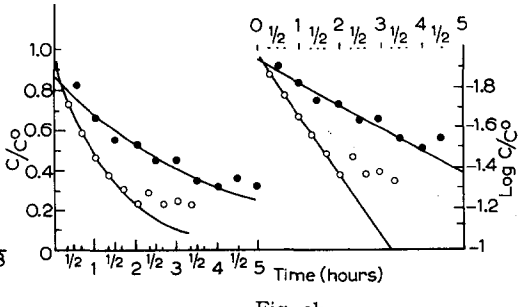

Fig. 2b.

References p. 502.

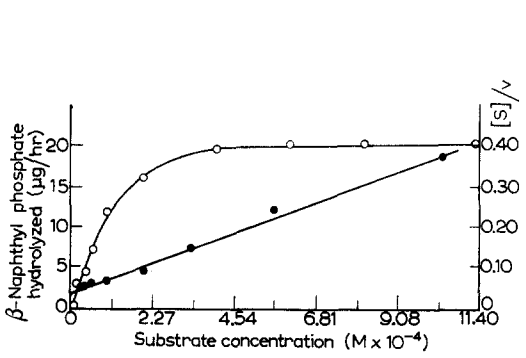

Fig. 3. The determination of K_m and V_{max} for parotid phosphomonoesterases. The initial velocities for eight substrate concentrations between $0.205 \cdot 10^{-4} M$ and $8.17 \cdot 10^{-4} M$ are shown. The upper curve gives the velocities in the first hour of the reactions. The lower curve gives the ratio of initial substrate to velocity for each of eight concentrations of substrate between $0.205 \cdot 10^{-4} M$ and $8.17 \cdot 10^{-4} M$. Parotid saliva (0.5 ml) was mixed with 5.0 ml of buffered substrate at pH 4.57.

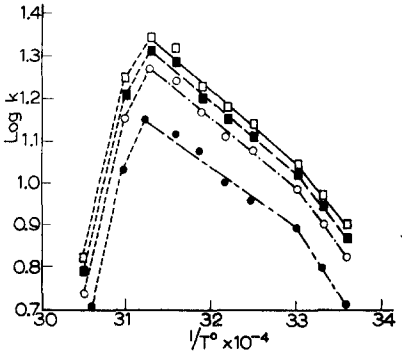

Fig. 4. Influence of temperature on parotid acid phosphomonoesterase. Log velocity sodium β-naphthylphosphate hydrolyzed (μg/h) *versus* $1/°T$. Mixtures containing 0.5 ml saliva and 5.0 ml buffered β-naphthylphosphate were incubated at pH 4.57 for two hours at ten temperatures. Final substrate concentrations ($M \times 10^{-4}$) were: ● 1.01, ○ 2.05, ■ 3.05, □ 4.05.

TABLE I

MICHAELIS CONSTANTS OF PAROTID SALIVA ACID PHOSPHATASE

Substrate	*Km* ($M \times 10^{-4}$)
Disodium *p*-nitrophenylphosphate	4.5
Sodium β-naphthylphosphate	3.2
Disodium phenylphosphate	0.17
Sodium β-glycerophosphate	Slow hydrolysis

Substrates disodium *p*-nitrophenylphosphate, disodium phenylphosphate, and sodium β-glycerophosphate were prepared in 0.2 M acetate buffer at pH 4.9. 0.5 ml parotid saliva + 5.0 ml buffered nitrophenylphosphate (final concentration range from $4.55 \cdot 10^{-3} M$ to $1.14 \cdot 10^{-3} M$) were incubated at 38° C for two hours. 0.5 ml of parotid saliva + 10 ml buffered phenylphosphate (final concentration range from $8.8 \cdot 10^{-3} M$ to $2.2 \cdot 10^{-3} M$) were incubated at 38° C for two hours. The initial velocities were determined from which Lineweaver and Burk plots gave the K_m values tabulated.

practical level of the enzyme substrate ratio (2.0 ml parotid saliva with $3.18 \cdot 10^{-5} M$ substrate) the reaction was clearly substrate-dependent. These relationships illustrate a typical Michaelis-Menten behavior of the parotid secretion as conditions leading to saturation of the enzyme at high S yielded velocities independent of S. The K_m and V_{max} were found by plotting the equation $S/v = (1/V)\, S \times K_m/V$, in which S/v was plotted against S, where v was the measured initial velocity and S the substrate concentration (Fig. 3). A maximum velocity (V_m) of 24.7 μg/h and a Michaelis-Menten constant (K_m) of $3.2 \cdot 10^{-4} M$ were obtained. The turnover number of the parotid saliva as obtained and without purification was calculated to be 0.094 moles substrate per 10^5 g of protein per minute at 37° C and pH 4.57. Nonenzymic hydrolysis of β-naphthylphosphate was zero under these conditions. Values obtained with *p*-nitrophenylphosphate and β-naphthylphosphate (Table I) demonstrated comparable enzyme-substrate affinities. The K_m values for the substrates employed cannot be

References p. 502.

correlated with the acid phosphomonoesterases of other sources such as prostatic, since considerable variations have been found[12]. It was not possible to calculate a value for parotid activity using sodium β-glycerophosphate. TSUBOI AND HUDSON[13] using a purified enzyme from prostate calculated for this substrate a K_m of $2.4 \cdot 10^{-3} M$. With sodium phenylphosphate they found a K_m of $1.5 \cdot 10^{-4} M$. This is approximately 9 times greater than the value obtained with the parotid enzyme and sodium phenylphosphate ($0.17 \cdot 10^{-4} M$). However, the nonidentity of the prostatic and parotid activities cannot be rigorously interpreted from K_m values calculated from enzymes obtained in widely varying degrees of purity. β-glycerophosphate proved to be unusable as a substrate because of relatively slow hydrolysis and because of turbidities from incomplete precipitation of the low levels of parotid secretion protein by trichloracetic acid.

Inhibition by ions

The influence of magnesium acetate, sodium fluoride and sodium tartrate on the reaction velocity was compared at several ionic concentrations of inhibitors (Table II). Sodium fluoride at a final concentration of $5.0 \cdot 10^{-4} M$ or greater produced 100% inhibition. Larger concentrations of magnesium were required for extensive inhibition (in the range $1 \cdot 10^{-2} M$ to $5 \cdot 10^{-2} M$). Sodium tartrate of $5.0 \cdot 10^{-3} M$ produced 100% inhibition.

TABLE II

INHIBITION OF PAROTID ACID PHOSPHOMONOESTERASE BY F^-, Mg^{++}, AND TARTRATE

Inhibitor added	*Final conc. M.*	*% Decrease in velocity*
NaF	$5.0 \cdot 10^{-4}$	97.0
	$1.0 \cdot 10^{-4}$	61.0
	$5.0 \cdot 10^{-5}$	23.0
	$1.0 \cdot 10^{-5}$	0.0
$Mg(AC)_2$	$5.0 \cdot 10^{-2}$	32.0
	$1.0 \cdot 10^{-2}$	16.0
	$5.0 \cdot 10^{-3}$	6.0
	$1.0 \cdot 10^{-3}$	6.0
Sodium L-tartrate	$5 \cdot 10^{-3}$	100
	$5 \cdot 10^{-4}$	81.4
	$1 \cdot 10^{-4}$	51.5
	$5 \cdot 10^{-5}$	46.9
	$1 \cdot 10^{-5}$	24.5
	$1 \cdot 10^{-6}$	0.0

Incubation mixtures contained 0.5 ml saliva, 2.5 ml β-naphthylphosphate, 2.5 ml of $0.2 M$ acetate buffer, at pH 4.90, and 1.0 ml of inhibitor at the final concentration given. The final concentration of β-naphthylphosphate was $3.42 \cdot 10^{-4} M$. A solution of sodium L-tartrate at pH 4.9 was prepared by titrating a sufficient amount of (L) tartaric acid with 10 N NaOH to make a stock solution $0.65 M$ which was diluted further in acetate buffer.

Effect of temperature

The influence of temperature on the reaction velocities was determined for four concentrations of substrate (Fig. 4) in which the incubation mixture was heated to the temperature indicated. Application of the Arrhenius equation, log K =

References p. 502.

$-\mu/2.303R\,(1/T + C)$. where K equals the velocity constant, μ the activation energy, R the gas constant = 1.987 calories per degree and T = °K the absolute temperature, yielded linear plots of log velocity versus the reciprocal of the absolute temperature over the temperature intervals 298–320° K. No significant change in slope with concentration of substrate is apparent. Hence, it is probable that the estimated activation energies reflect changes only in the rate of decomposition of the enzyme substrate complex (at moderately high S) and are relatively free of the influence of substrate[14]. The activation energies were calculated from the slopes ($-\mu/4.58$). Above 303° K the average of the slopes of the four curves gave a value of 7,300 cal/mole. A transition temperature was observed at 303° K below which a value of 12,400 cal/mole was estimated. Inactivation appeared precipitously above 323° K. The magnitude of the activation energy above 303° K was considerably lower than that given by LUNDQUIST[12] who found values of 11,300 cal/mole for β-glycerophosphate, 11,800 cal/mole for phenylphosphate, and 11,700 cal/mole for phosphorylcholine using phosphatases of human semen. A value of 9,940 cal/mole was found by BODANSKY[15] for cat and man bone phosphatase with β-glycerophosphate as substrate. These authors found no transition temperature, which apparently is peculiar to the parotid enzyme for the substrate (β-naphthylphosphate) used. However, the estimated value below 303° K in this paper is in good agreement with the other reported values.

Inactivation energies were obtained under optimum substrate and pH conditions by heating the parotid secretion buffered at pH 4.57 for periods up to one hour for temperatures between 37° C and 55° C followed by incubation with substrate at 37° C for two hours. The rates (Fig. 5) followed first order kinetics from which the log slope *vs* $1/T$ plot (Fig. 6) yielded an energy of inactivation of 88,300 calories/mole.

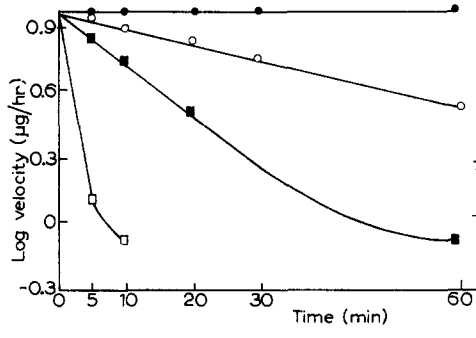

Fig. 5. Inactivation of parotid acid phosphomonoesterase by heat. Log velocity = µg β-naphthylphosphate/hour. 0.5 ml saliva was heated for the specified intervals (abscissa) followed by incubation with $2.05 \cdot 10^{-4} M$ (final concentration) substrate for two hours at 37° C and pH 4.57. The temperature (°K) = ● 310, ○ 320, ■ 323, □ 328.

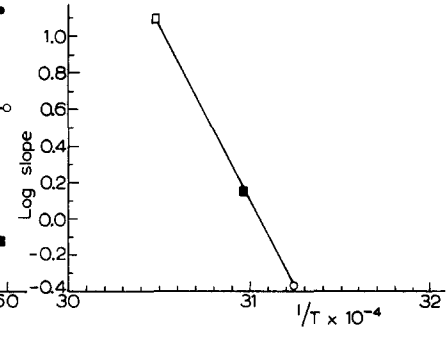

Fig. 6. Inactivation energy of heated acid phosphomonoesterase. Log velocity of inactivated enzyme (µg sodium β-naphthylphosphate hydrolyzed/h) *vs.* $1/T°$ absolute. Other conditions as in Fig. 5.

DISCUSSION

The non-specific character of phosphomonoesterases exhibiting optimal activity in acid environment renders difficult the correlation of enzyme activities of various

References p. 502.

TABLE III

COMPARATIVE PROPERTIES OF ACID PHOSPHOMONOESTERASES

Source (human)	*pH optima*	K_m-β-GPA *(moles)*	$K_m PhPO_4$ *(moles)*	*Inhibition %*			*Activation energy (cal/mole)*
				Mg++	*F−*	*L-Tartrate*	
Parotid*	4.57	none	$0.17 \cdot 10^{-4}$	32.0 ($5 \cdot 10^{-2} M$)	97.0 ($5 \cdot 10^{-4} M$)	98.0 ($5 \cdot 10^{-3} M$)	6,600–7,600 (β-N P)
Prostate	4.5–5.0	$2.4 \cdot 10^{-3}$ (13)	$1.5 \cdot 10^{-4}$ (13)	Slight ($1 \cdot 10^{-2} M$)	95 ($1 \cdot 10^{-2} M$)	90 ($1 \cdot 10^{-2} M$)	11,800 ($PhPO_4$)
Erythrocyte	5.0–5.7 (10)	none	high	inhibition above	none or slight	none	—
	5.5 (16)	(10)	(16)	0.1 *M*	($1 \cdot 10^{-2} M$) (10)	(10)	

Numbers in parenthesis indicate references.
* This paper.

tissues. It is apparent from the constants and properties derived for the parotid acid phosphomonoesterase activity that it does not coincide exactly with measured characteristics of the more common types. Admitting the oversimplification of tabular comparisons among enzymes which vary appreciably as to conditions, nature of substrate, buffers, and ionic composition of media, they are nevertheless helpful in describing the nature of the parotid species of enzyme (Table III). Cautious interpretation suggests a similarity of the parotid enzyme with that of prostatic origin. Although many similarities exist among the phosphatases of various sources it is reasonably well established that prostatic and blood (erythrocyte and plasma or serum) are definitely different in character. From the tabulation it is evident that the parotid enzyme more closely resembles the prostatic variety in pH optima, reactivity to phenyl phosphate and inhibitory phenomena. Although a labile enzyme of transient character has been repeatedly shown[10,17] in erythrocytes with a similar pH peak (4.3–4.8) the stable activity of cells and plasma is optimally active above pH 5.0. The substrate specificity of the parotid with regard to phenylphosphate and β-glycerophosphate is less conclusive. In fact it suggests closer similarity to erythrocytic phosphatase since neither react with β-glycerophosphate and both react with phenyl phosphate. However, the K_m of prostatic phosphatase is comparable to that obtained with the parotid secretion. Inhibition by Mg^{++} again seems to favor the erythrocytic variety but the activity of the various enzymes to Mg^{++} has been too little characterized to be highly significant. Inhibition by fluoride and tartrate affords clear differentiation of the parotid from the blood phosphatases and definitely relates the former to the prostatic kind. Although the conditions are only qualitatively comparable, the magnitude of inhibition justifies noting the resemblance. It would seem, therefore, that the parotid gland secretes an acid phosphomonoesterase with properties intermediate between those of blood and prostatic origin, but closer in similarity to the latter.

The technique of measurement of parotid activity using sodium β-naphthylphosphate as substrate offers advantages of color development of a non-physiological derivative (β-naphthol). Its extraction as a colored complex allows for good quanti-

References p. 502.

tation and freedom from turbidities often encountered in alternate procedures using acid-soluble tissue extracts. The typical Michaelis-Menten characteristics and kinetics, the orders of which may be manipulated as either zero or first order attest to the relatively uncomplicated action of the enzyme and substrate. However, first order kinetics were not ordinarily easily demonstrated since more than 3.0 ml of parotid saliva were required for the lowest practical amounts of substrate.

SUMMARY

The acid phosphomonoesterase of the human parotid was studied with sodium β-naphthylphosphate as substrate. The pH optimum was well defined between 4.5–4.7. The enzyme exhibited typical Michaelis-Menten behavior over a wide range of substrate concentrations. The K_m value for β-naphthylphosphate was $3.2 \cdot 10^{-4} M$, for sodium *p*-nitrophenyl phosphate was $4.5 \cdot 10^{-4} M$, and for disodium phenyl phosphate was $0.17 \cdot 10^{-4} M$. The enzyme was readily inhibited by fluoride, and L-tartrate, and less so by magnesium ion. Average values for four substrate concentrations gave an average activation energy of 7,300 cal/mole above 30° C. Below this transition temperature a value of 12,400 cal/mole was estimated. The energy of inactivation was 88,300 cal/mole. The properties of the parotid acid phosphomonoesterase resemble both the phosphomonoesterases of blood and prostate but with greater similarity to the latter.

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RIBONUCLEASE ACTIVITY AND CELLULAR GROWTH*

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INTRODUCTION

In a previous study on the nuclease activities in the human placenta a close correlation was found between the growing state of the tissue and the desoxyribonuclease activity in the homogenate[1,2]. This activity showed a considerable decrease during

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References p. 506.