The Inhibition of Seminal Acid Phosphatase by Macromolecular Compounds

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It has been reported previously that polymeric phosphates of phloretin and related compounds are potent inhibitors of several enzymes including alkaline phosphatase and hyaluronidase (Diczfalusy *et al.* 1953). Preliminary studies suggested that a similar inhibition of acid phosphatases may also take place (Diczfalusy *et al.* 1953).

This paper reports on the kinetics of human seminal acid phosphatase with special emphasis on the inhibition of this enzyme by different polymeric phosphates and other macromolecular compounds.

EXPERIMENTAL

Materials

Enzyme. Freshly obtained ejaculates were pooled from eight to ten subjects. The semen was kept for 4–6 hr. at 2°, the seminal plasma separated by centrifuging and diluted with 0.15M-acetate buffer, pH 5.5 (see below). In most experiments 0.1 ml. of a 1:2000 dilution of the seminal plasma/2.5 ml. of reaction mixture was used.

Substrate. A commercial preparation of the disodium salt of monophenyl phosphate (British Drug Houses Ltd.) was used without further purification.

Buffer. This was prepared by mixing 0.15M-sodium acetate with 0.15M-acetic acid containing 0.15M-sodium chloride as described by Lundquist (1947). The pH within the range 3.0–6.7 was measured before and after enzymic hydrolysis and the buffering capacity was found to be adequate. Distilled water was further purified by passing it through anionic- and cationic-exchange resins.

Inhibitors and other substances tested. Polyphloretin phosphate, polyphlorrhizin phosphate and polyhesperidin phosphate were synthesized as described by Diczfalusy *et al.* (1953). Polyestradiol phosphate was prepared according to Fernö *et al.* (1958) by the staff of AB Leo, Hälsingborg. Suramine (Germanine, Bayer 205) and protamine sulphate were commercial preparations.

Methods

Analytical. Phenol was determined by the colorimetric method of King & Armstrong (1934) as modified by Buch & Buch (1939) with a Beckman spectrophotometer, model DU. All pH determinations were made with a Radiometer (Copenhagen) pH meter (glass electrode).

Conditions of enzyme experiments. The reaction was started by the addition of 0.1 ml. of enzyme solution to 2.5 ml. of reaction mixture containing 2 mM-monophenyl phosphate and 0.15M-acetate buffer. Dilution of the enzyme was made immediately before the start of the

experiment. The substrate was dissolved in 0.1 ml. of buffer solution, pH 5.2, and the addition of this to any reaction mixture did not significantly change the pH. In those experiments where protamine sulphate was used, this was added in a small volume (0.1 ml.) and the total volume of the reaction mixture was kept constant. The rate of hydrolysis was studied by incubating the mixture for 8 min. at 37°, when 6–8% of the substrate was hydrolysed at the pH optimum (pH 5.5). The reaction was stopped by the addition of 1.4 ml. of 20% (w/v) trichloroacetic acid. Results are expressed as $\mu\text{g.}$ of phenol liberated/min./ml. of reaction mixture. All estimations were carried out in triplicate.

It was found that the acid phosphatase activity of the seminal fluid markedly decreased when stored in the cold room at 2°. Thus after storage for 3 weeks samples of seminal plasma gave only approx. 20% of their initial enzyme activity, when measured at the optimum pH. In dilute solution the inactivation was still more rapid. Therefore a fresh pool of seminal plasma was used for each experiment.

The enzyme activity of different pools of seminal plasma was found to be surprisingly constant. Eighteen pools collected in the course of 15 months exhibited a mean acid phosphatase activity of 2.42 ± 0.36 (S.D.) $\mu\text{g.}$ of phenol liberated by 0.001 ml. of seminal fluid/min./ml. of reaction mixture. The relatively small standard deviation (14.5%) indicates that results obtained with different pools of seminal fluids are comparable.

RESULTS

Effect of pH. The effect of pH on the rate of hydrolysis of phenyl phosphate by the enzyme was measured over the range pH 3.0–6.7 at a substrate concentration of 2 mM, with different concentrations of the enzyme. The results are shown in Fig. 1, and it can be seen that the optimum pH is near 5.5. This is in good agreement with values reported by previous investigators, including Lundquist (1947), Abul-Fadl & King (1949), Schönheyder (1952) and Boman & Westlund (1956). A well-defined pH optimum was invariably found when a relatively high enzyme concentration was employed. A lowering of the enzyme concentration resulted in a marked broadening of the pH curve. The same broad curve was obtained with enzyme preparations partially inactivated by storage in the cold room. When relatively high enzyme concentrations were employed (dilution

1:600 or less) a tendency to a second peak in the neighbourhood of pH 4.0 was frequently observed. This finding is in agreement with previously reported results on human erythrocyte acid phosphatase (Abul-Fadl & King, 1949) and rat-liver acid phosphatase (Goodlad & Mills, 1957).

Fig. 1. Effect of pH on the rate of hydrolysis of monophenyl phosphate at different concentrations of seminal acid phosphatase. Acetate buffer, 0.15M. Substrate concentration, 2 mM. Incubated for 8 min. at 37°. Diluted enzyme (0.1 ml.) was added to 2.5 ml. of reaction mixture. ○, Final enzyme dilution 1:15 000; ●, dilution 1:17 500; △, dilution 1:50 000; ▲, aged enzyme, stored for 3 weeks at 2°, dilution 1:15 000.

Fig. 2. Effect of varying substrate concentrations on seminal phosphatase activity at pH 5.5. Conditions were as in Fig. 1. ○, Plot of reaction velocity against substrate concentration; ●, the above data plotted according to the equation derived by Lineweaver & Burk (1934).

Effect of the substrate concentration. Fig. 2 shows the effect of varying substrate concentration on the reaction velocity at pH 5.5. The rate of hydrolysis increases with increasing substrate concentration, giving a typical Michaelis-Menten curve. Fig. 2 also shows the same data plotted according to the equation derived by Lineweaver & Burk (1934):

$$1/v = 1/V_{\max} + K_m/V_{\max} \cdot s,$$

where s is the substrate concentration, v and $V_{\max}$ are the observed and maximum velocities respectively and K_m is the Michaelis constant. It appears from this plot that the line, when extrapolated to meet the x axis according to the method described by Dixon (1953), gives a K_m value of 0.18 mM-mono-phenyl phosphate.

Determinations of K_m values at the optimum pH (5.5) were carried out on different occasions with pools of fresh seminal plasma. A value of 0.18 mM was obtained in five experiments and 0.17 mM in the sixth one. These values are in reasonable agreement with those reported by other workers (Tsuboi & Hudson, 1955). Determinations of K_m values were also carried out at different pH values. At pH 3.5 K_m was 0.3 mM, at pH 4.5, 0.25 mM, whereas at pH 6.5 K_m 0.5 mM was found.

The activity curve of Fig. 2 shows no inhibitory effect of the substrate in the concentration range 0.063–4.0 mM. Monophenyl phosphate was also tested at different pH values in concentrations as high as 34 mM and no inhibition of the enzyme was found. This suggested that in contrast with the hydrolysis of monophenyl phosphate by alkaline phosphatase (Morton, 1957) the optimum pH for hydrolysis of this substrate by seminal acid phosphatase may not be influenced by the concentration of the substrate. This is also shown in Fig. 3,

Fig. 3. Effect of pH on the hydrolysis of different concentrations of monophenyl phosphate by seminal acid phosphatase. Conditions were as in Fig. 1. Substrate concentrations (mM): ○, 2.0; ●, 1.0; △, 0.5; ▲, 0.25.

where the substrate concentration is varied at different pH values. It is obvious that the pH optimum was not displaced when the substrate concentration was increased.

Inhibition by polyoestradiol phosphate. In a preliminary report it has been shown that polyoestradiol phosphate is a potent inhibitor of human seminal acid phosphatase (Diczfalusy, 1954). The influence of pH on the inhibition of the enzyme by polyoestradiol phosphate is shown in Fig. 4, and it appears that the inhibition is mainly restricted to the acid side of the pH optimum and that it virtually disappears at pH 6-7. For characterizing the type of inhibition the equation of Hunter & Downs (1945) was used. In its general form this equation is

$$(I) \frac{\alpha}{1-\alpha} = K_i \left(1 + \frac{s}{K_m} \right) / 1 + \frac{K_i s}{K_1 K_m},$$

where I is the inhibitor concentration and α the fractional activity, i.e. the ratio of velocities of the reaction in the presence and the absence of the inhibitor at a given substrate concentration s . K_i is the dissociation constant of the enzyme-inhibitor complex, K_m the Michaelis constant and K_1 the constant of dissociation of the enzyme-substrate-inhibitor complex into enzyme-substrate and inhibitor. With non-competitive inhibition K_i equals K_1 , and the equation is simplified to:

$$(I) \alpha/(1-\alpha) = K_i.$$

Fig. 4. Effect of pH on the inhibition of seminal acid phosphatase by polyoestradiol phosphate. Experimental conditions were as in Fig. 1. O, Without inhibitor; ●, with polyoestradiol phosphate, 0.5 μ g./ml. of reaction mixture.

The value of K_i represents the amount of inhibitor required for 50% inhibition. When $\alpha/(1-\alpha)$ is plotted against s with two different inhibitor concentrations of polyoestradiol phosphate the graphs are straight lines which are parallel to the X axis (Fig. 5). This indicates that the inhibition is not influenced by the substrate concentration, i.e. it is of the non-competitive type. The value of K_i can be calculated from each of the two lines by multiplying the value of I corresponding to the line by the value of the intersection point obtained by extrapolating the line to the Y axis.

Reversion of inhibition by protamine. It has been reported previously that the inhibition of human seminal acid phosphatase by polyoestradiol phosphate can be counteracted by basic proteins such as protamine sulphate (Diczfalusy, 1954). We have confirmed and extended this observation in the present work and found that the addition of 5.0 μ g. of protamine sulphate/ml. of reaction mixture completely reversed the inhibitory action of 0.5 μ g. of polyoestradiol phosphate. In these experiments protamine sulphate and polyoestradiol phosphate were added to the reaction mixture before the enzyme. When the enzyme-substrate-inhibitor interaction was allowed to take place for 6 min., the addition of protamine sulphate still resulted in some reactivation of the enzyme, as shown in Fig. 6, the velocity of the reactivated enzyme approaching that of the uninhibited enzyme. Protamine sulphate alone did not activate the enzyme when tested within the range 0.5-10.0 μ g./ml. of reaction mixture.

Inhibition by other polymeric phosphates. It has been found that polymeric phosphates of phloretin, phlorrhizin and hesperidin also inhibited the

Fig. 5. Inhibition of seminal acid phosphatase by polyoestradiol phosphate at pH 5.5. Experimental conditions were as in Fig. 1 except that 0.1 ml. of inhibitor solution was added to the reaction mixture. The results are plotted according to the equation derived by Hunter & Downs (1945): $\alpha/(1-\alpha)$ against substrate concentration. Values of α are determined as the ratio of inhibited velocity to uninhibited velocity with the same concentration of the substrate. Inhibitor concentrations: O, 0.3 μ g./ml. (I_1); ●, 0.5 μ g./ml. (I_2).

enzyme although to a much lesser extent than polyoestradiol phosphate. The relative amounts of these three inhibitors required for a 50% inhibition of the enzyme at a substrate concentration of 2 mM and at pH 5.5 were estimated as 1.6, 5.0 and 17.0 $\mu\text{g./ml.}$ of reaction mixture respectively. With polyphloreitin phosphate the type of inhibition was also studied and found to be non-competitive. In Table 1 are shown estimates of the K_i values for the polymeric phosphates.

Inhibition by suramine. Since the trypanocide suramine is known to inhibit several enzymes (Wills & Wormall, 1950) it seemed to be of interest to investigate its action on seminal acid phosphatase. The results shown in Table 1 indicate that this compound is also a powerful inhibitor of the enzyme. The type of inhibition was found to be non-competitive. This is shown in Fig. 7, where the graph of $\alpha/(1-\alpha)$ against s is presented.

DISCUSSION

Since the results reported in this paper have been obtained by the use of a crude enzyme preparation, the significance of some of the kinetical data might be questioned. The hazards of using unpurified enzyme preparations for kinetic studies in general have been emphasized by several investigators, among others by Roy (1955) who pointed out that the presence of different activating or inhibiting substances in crude preparations might seriously interfere with the results. We think that the influence of possible impurities on the results obtained by the use of highly diluted human seminal plasma is probably negligible for the following reasons: in the present investigation identical K_m values were obtained when different pools of seminal plasma were used. Furthermore, in the work of Tsuboi & Hudson (1955) 40-fold and 300-fold purified preparations of human prostatic acid phosphatase were found to exhibit virtually the same K_m values as the crude enzyme. Finally our K_m values agree closely with those reported by these authors. The results on the inhibition of seminal acid phosphatase by polyoestradiol phosphate agree with those reported by Fernö *et al.* (1958), who also found that the inhibition was of the non-competitive type. It is of interest to note, however, that the inhibition of a purified alkaline phosphatase preparation by polyoestradiol phosphate was reported by these workers to be substrate-competitive. A similar situation seems to exist with the inhibition of various enzymes by suramine. The inhibition of seminal acid phosphatase by this compound is non-competitive, whereas the inhibition of urease was found to be competitive (Wills & Wormall, 1950).

Fig. 6. Effect of protamine sulphate on the inhibition of the enzyme by polyoestradiol phosphate at pH 5.5. Conditions were as in Fig. 1 except that the time of incubation was varied. ●, Without inhibitor (controls); ○, with polyoestradiol phosphate, 0.5 $\mu\text{g./ml.}$; △, tubes in which 2.5 $\mu\text{g.}$ of protamine sulphate/ml. was added to the enzyme-inhibitor-substrate mixture after 6 min. of incubation.

Table 1. K_i values of three different non-competitive inhibitors of seminal acid phosphatase

Values of K_i were calculated as described in Fig. 5. For each inhibitor two values of K_i are shown, calculated from the results of two experiments, with different inhibitor concentrations.

Inhibitor	Concn. of inhibitor ($\mu\text{g./ml.}$)	Calculated K_i ($\mu\text{g./ml.}$)
Polyoestradiol phosphate	0.3	0.6
	0.5	0.5
Polyphloreitin phosphate	3.0	1.5
	6.0	1.6
Suramine	2.5	2.3
	5.0	2.5

Fig. 7. Inhibition of seminal acid phosphatase by suramine at pH 5.5. Experimental conditions were as in Fig. 1. Inhibitor concentrations: ○, 2.5 $\mu\text{g./ml.}$; ●, 5.0 $\mu\text{g./ml.}$ For further details see Fig. 5.

Finally the present results indicate that polyphloretin phosphate, polyphlorrhizin phosphate and polyhesperidin phosphate as well as polyoestradiol phosphate inhibit seminal acid phosphatase. These polymeric phosphates inhibit other enzymes as well (Diczfalusy *et al.* 1953, Fernö *et al.* 1953, 1958). It is thus very likely that the inhibition of acid phosphatase by these compounds is just an example of a widespread and non-specific action of high molecular-weight polyelectrolytes on several enzymes, as suggested by Spensley & Rogers (1954). The reversibility of the enzyme inhibition also suggests a macro-anion-macro-cation interrelationship similar to that postulated by these authors for the interaction between polyphloretin phosphate and protamine on hyaluronidase or between heparin and polylysine on pepsin.

SUMMARY

1. The hydrolysis of monophenyl phosphate by unpurified human seminal acid phosphatase has been investigated.

2. The optimum pH was found to lie between 5.4 and 5.7 and was not influenced by the substrate concentration.

3. The Michaelis constant was estimated at several pH values. At the optimum pH a K_m value of 0.18 mM was repeatedly found. High substrate concentrations did not inhibit the enzyme activity.

4. Polyoestradiol phosphate was found to be a powerful, non-competitive inhibitor of seminal acid phosphatase. The inhibitory effect was strongest on the acid side of the pH optimum. The inhibition could be reversed by small amounts of protamine sulphate.

5. The enzyme was also inhibited by polymeric phosphates of phloretin, phlorrhizin and hesperidin.

The inhibition by polyphloretin phosphate was non-competitive.

6. The trypanocide suramine proved to be a non-competitive inhibitor of seminal acid phosphatase.

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Transport of some Mono- and Di-Saccharides into Yeast Cells

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The mechanism of transport of sugars into yeast cells is still subject to discussion. Rothstein (1956) maintains that the transport of fermentable sugars into the cells of *Saccharomyces cerevisiae* is effected by phosphorylating reactions, but other authors, e.g. Sols (1956), assume that this transport in another yeast strain is independent of phosphorylating processes.

In this paper experiments on the transport of some sugars, fermentable and non-fermentable, into yeast cells (*S. cerevisiae* R XII, *Saccharomyces fragilis*, *Saccharomyces carlilis*) are reported. It has been shown that under certain experimental conditions a considerable amount of apparently free sugars is found in the cells. The results of some kinetic studies of the fluxes of D-galactose and