The Effects of Inhibitors and Activators on the Activity of Acid Phosphatase in Seminal Fluid of Rabbits in the pH Range 5.1—6.5

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The phosphatase activity in the seminal fluid of the rabbit shows two separate pH maxima, situated at pH 5.5 and 6.3 (tris-maleate buffer). It is shown that some activators (Mg^{2+} and 1-butanol) and the inhibitor Zn^{2+} influence the activity in different ways at the two maxima. These facts support the idea that two different acid phosphatases with different pH maxima are present in the seminal fluid from the rabbit.

The existence of a prostatic acid phosphatase has already been shown by Kutscher and Wohlbergs,[1] who demonstrated high activity of this phosphomonoesterase also in human seminal fluid and prostate homogenate. The pH optimum of the enzyme was about 5. Interest in this enzyme grew when Gutman and Gutman [2] demonstrated an increased activity of acid phosphatase in blood serum from men with metastasizing prostatic cancer. However, Lundquist [3] mentions that an acid phosphatase which acts at a somewhat higher pH, 6.3, is possibly present in human seminal fluid. Later, many attempts to purify prostatic acid phosphatase have been made.[4-10] Hudson and Butler [11] isolated from prostatic extract a phosphatase with a pH optimum at 4.9. Unlike the initial material, this phosphatase lacked the capacity to hydrolyze phosphorylcholine, and it was demonstrated that this phosphatase activity was dependent on more than one enzyme. During the course of this study, two acid phosphatases were isolated, both from homogenized prostate and from seminal fluid from man.[9] The phosphatases of the seminal fluid had lower molecular weights than those from the prostate, and could probably not be derived from this gland. The presence of five antigens in prostate fluid from man was demonstrated by gel diffusion.[10] Four of these antigens showed phosphatase activity at pH 5.0.

The purpose of the present investigation was to elucidate whether the phosphatase activity of seminal fluid in the pH range 5.1 – 6.5 is dependent on more than one enzyme. This has been done by studying the effects of one inhibitor and some activators. As experimental material seminal fluid from rabbits was used throughout.

MATERIAL AND METHODS

Enzyme source. Semen was collected from rabbits of the Swedish Country Breed by means of an artificial vagina. Immediately after the ejaculation, the semen was placed in an ice-bath. The spermatozoa were removed by centrifugation ($+2°C$) at 2000 g for $15-20$ min. The seminal fluid was diluted with redistilled water (for degree of dilution, see Experiments and results), and the solution thus obtained was used as enzyme preparation. Usually, the analyses of the phosphatase activity were performed in close connection with the collection of semen. On some occasions, seminal fluid was cooled to $-20°C$ directly after the centrifugation and kept at this temperature until the starting of the experiments. This procedure did not influence the phosphatase activity.

Substrate. Disodium phenylphosphate was used as substrate. An aqueous stock solution of 0.3 M was stored, deep-frozen in small portions. The final concentration in the tests was 0.15 M, unless otherwise stated. In experiments with 1-butanol, this substance was introduced into the test samples by saturation of the substrate stock solution. This was shaken with butanol, the surplus of which was removed with a separation funnel.

Buffer solution. In most of the experiments Gomori's tris-maleate buffer,[12] mixed with an equal volume of substrate solution, was used. The final concentration of the buffer solution was 0.15 M, which gives maximum activity, according to Lundquist.[13] However, the substrate concentration in his experiments, performed at pH 6.0, was only 0.02 M. Otherwise, the experimental conditions were the same. Identical results were obtained when acetate buffer of the same concentration was used. McIlvain's phosphate-citrate buffer[14] was also used in some experiments. With this buffer, the maxima of activity appeared at a somewhat lower pH, 4.9 and 5.9, respectively.

The enzyme activity was determined by incubating 0.10 ml of enzyme preparation with 2.0 ml of buffer-substrate solution and 0.10 ml solution of inhibitor, activator, or buffer.[15] Control samples, in which the enzyme preparation (II) or the substrate-buffer solution (III) were replaced by a buffer solution, were also prepared (Table 1). Checks

Table 1. Composition of samples (ml).

Sample	I	II	III
Buffer	1.00	1.10	2.00
Substrate	1.00	1.00	–
Enzyme preparation	0.10	–	0.10

on the pH before and after incubation showed that it was not changed under the given circumstances. The phosphatase activity is expressed in King-Armstrong units (K.A., mg of phenol set free/100 ml of seminal fluid and hour).[13]

In order to determine the reliability of the method, the standard deviation of 21 paired determinations of extinction differences was calculated.[16] The standard deviation appeared to be 0.006 extinction unit (E) for extinction differences between 0.090 and 0.250 E (11 paired determinations), and 0.0016 E for greater extinction differences (10 paired determinations). The error is thus as low as $1-2$ % in the range of extinctions of $0.250-0.500$ E ($12-14$ K.A.). All the chemicals used were of analytical grade. All solutions were made up with water, distilled twice in a quartz apparatus.

EXPERIMENTS AND RESULTS

The phosphatase activity in the seminal fluid of the rabbit shows two clear maxima in the pH range $5.1-6.5$. These maxima are situated at pH 5.5 and 6.3, respectively (tris-maleate buffer, Fig. 1).
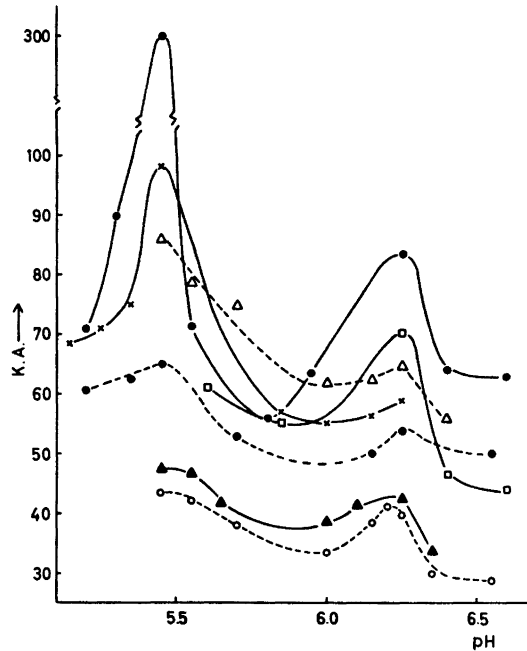
Fig. 1. The phosphatase activity in seminal fluid from the rabbit at different pH's. Tris-buffer. Different curves derive from separate rabbits. Seminal fluid diluted 1:2.

According to Abul-Fadl and King,[17] alkaline phosphatase is almost totally inhibited by 0.01 M cysteine, while the phosphatase activity at pH 5.5 is somewhat stimulated. Our experiments indicate a faint stimulation of the phosphatase activity at pH 6.3 in the presence of cysteine (Table 2), a stimulation which seems to be inversely related to the activity of the control samples.

Enzyme activities are often more or less specifically influenced by ions. An investigation of the effects of some ions on the phosphatase activity at different pH's may therefore elucidate the actual problem. Experiments with Mg^{2+} ions showed that the phosphatase activity at pH 5.5 is not appreciably

Table 2. The effect of cysteine on the phosphatase activity at pH 6.3. The seminal fluid was diluted 1:2. *a*, *b*, and *c*, experiments with ejaculates from different rabbits.

Experiment	Control, K.A.	0.05 M cysteine	Stimulation (%)
a	59.7	60.5	1.3
b	48.8	50.4	3.2
c	43.8	47.8	8.4

influenced by concentrations less than 0.15 M. On the other hand, a very strong activation appears at pH 6.3, at concentrations higher than 0.05 M (Fig. 2).
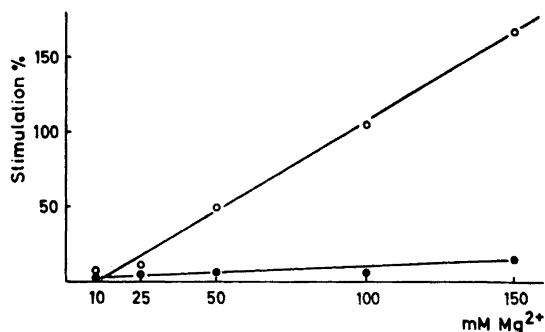

Fig. 2. The effect of Mg^{2+} on the phosphatase activity in seminal fluid. Stimulation expressed in per cent of activity of corresponding control samples (at pH 5.5, 107 K.A.; at pH 6.3, 82 K.A.). Seminal fluid diluted 1:2. Tris-buffer. Activity at pH 5.5, ●; activity at pH 6.3, O.

This means that at pH 6.3, the concentration of magnesium ions in the diluted seminal fluid of the control samples is sub-optimal.

Fluoride ions exert an inhibiting effect both at pH 5.5 and 6.3 (Table 3). Zinc ions influence the activity differently at the two pH optima. A concentration of 0.001 M effects a strong inhibition at pH 6.3, but only a relatively un-important inhibition at pH 5.5 (Table 4). Furthermore, with increasing con-

Table 3. The effect of 0.01 M NaF on the phosphatase activity. *a*, *b*, and *c*, separate experiments with ejaculates from different rabbits. Seminal fluid dilutes, 1:2.

Experiment	pH	Activity of control sample, K.A.	Inhibition %
a	5.5	64.4	37.5
	6.3	55.6	40.6
b	5.5	49.0	80.0
	6.3	45.9	69.7
c	5.5	35.1	46.5
	6.3	34.4	51.8

centration of zinc ions, the inhibition is greatly enhanced at pH 5.5, but decreases and even turns into a faint but definite activation at pH 6.3 (Fig. 3).

For the experiments with butanol, a phosphate-citrate buffer was used. The effect of 1-butanol on the phosphatase activity at pH 4.9 was relatively unimportant at both high and low substrate concentrations (Fig. 4*a*). At pH 5.9 (Fig. 4*b*), the butanol caused a strong activation.

Table 4. The effect of 0.001 M Zn^{2+} on the phosphatase activity. *a, b,* and *c,* separate experiments with ejaculates from different rabbits. K.A. = King-Armstrong units.

Experiment	Dilution of seminal fluid	pH	Activity of control sample, K.A.	Inhibition (%) in the presence of 0.001 M Zn^{2+}
a	1:1	5.5	51.6	6.0
		6.3	43.8	37.4
b	1:2	5.5	54.9	12.8
		6.3	47.1	29.5
c	1:1.5	5.5	67.5	14.6
		6.3	51.2	28.2

In another type of experiment it was intended to compare at pH 5.9 the effects of 1-butanol when introduced into the test sample by saturating the buffer solution or the substrate solution with butanol, both of which enter into the test sample in the same proportion (*cf.* Table 1).

As appears from Table 5, butanol is considerably more effective when added by saturating the substrate solution. This is especially apparent at the lower
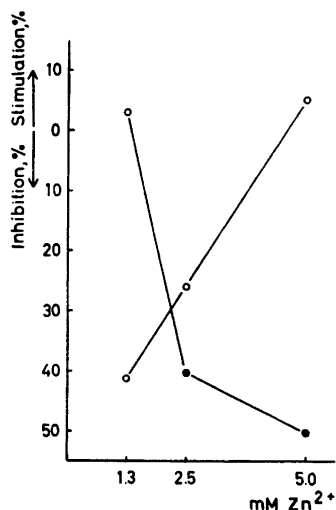

Fig. 3. The effect of Zn^{2+} on the phosphatase activity at pH 5.5, ●; and at pH 6.3, ○. Stimulation and inhibition expressed in per cent of activity of control samples (at pH 5.5, 61 K.A.; at pH 6.3, 44 K.A.). Seminal fluid diluted 1:2.

substrate concentration. 1-Butanol was the only one among a number of non-polar or weakly polar substances tried (benzene, isooctane, chloroform, ethyl ether, 1-pentanol, and 1-butanol), which stimulated the phosphatase activity.
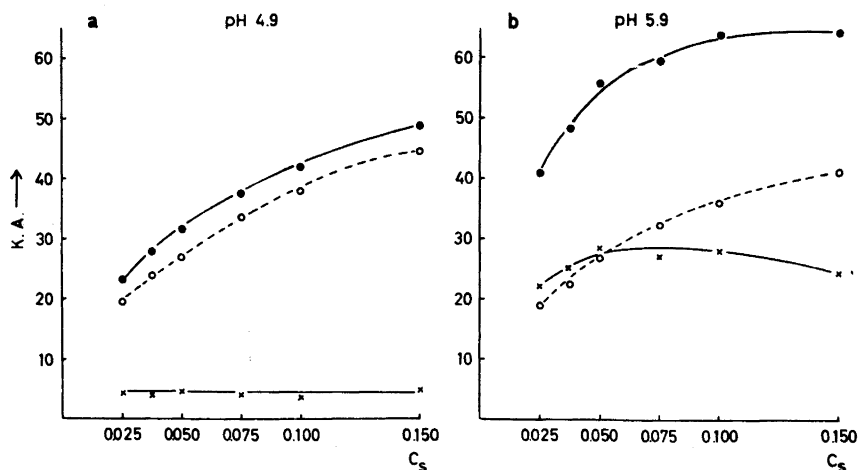
Fig. 4. The effect of 1-butanol on the phosphatase activity, (*a*) at pH 4.9, and (*b*) at pH 5.9, at different substrate concentrations. Phosphate-citrate buffer. O, control samples; ●, samples with butanol. ×, difference between samples with and without butanol, at different concentrations of substrate. Seminal fluid diluted 1:2.

Table 5. The effect of butanol on the phosphatase activity when buffer solution or substrate solution were saturated with 1-butanol. Phosphate-citrate buffer, pH 5.9. Seminal fluid diluted, 1:2. K.A. = King-Armstrong units.

Conc. of substrate M	Control sample K.A.	Buffer solution saturated with 1-butanol		Substrate solution saturated with 1-butanol	
		K.A.	Stimulation, %	K.A.	Stimulation, %
0.025	17.6	20.0	6.5	30.2	72.2
0.150	39.0	43.2	10.6	51.0	30.6

DISCUSSION

The phosphatase activity in the seminal fluid of rabbits shows two separate pH maxima in the pH interval 5.1 − 6.5, *viz.* at pH 5.5 and 6.3 (tris-maleate buffer). This may be due to (*a*) the presence of two separate acid phosphatases; (*b*) one phosphatase having two apparent pH maxima because of the presence of an ampholytic inhibitor, as postulated for potato invertase by Rorem and Schwimmer;[18] or (*c*) a common range of activity of acid and alkaline phosphatase. Measurable activity of alkaline phosphatase may be recognized at a pH as low as 6.0.[19] However, the possibility that the higher pH optimum depends on a common activity range for acid and alkaline phosphatase is rejected, since this pH optimum persisted, in spite of the addition of cysteine, which more or less completely inhibits the alkaline phosphatase.[17]

Acid phosphatases have been classified in two groups, A II and A IV.[20] Phosphatases belonging to A II are characterized mainly by the fact that their maxima of activity are situated between pH 4.5 and 5.0, and that they are not activated by Mg^{2+}. The enzymes of group A IV have a maximum of activity about pH 6, and are activated by Mg^{2+}.

As appears from Fig. 2, the phosphatase activity is stimulated at pH 6.3 by Mg^{2+}, which, however, has no effect at pH 5.5. The solubility product of $Mg(OH)_2$ was not exceeded, even at the highest concentration of Mg^{2+}, *i.e.* 0.150 M, at pH 6.3. Thus, the actual concentration of this ion was equal to that calculated from the added amount. With regard to the pH optima and the results obtained with Mg^{2+} in the present experiments, one of the phosphatases may be referred to group A II, and the other to group A IV.

Kutscher and Wörner [21] showed that acid prostatic phosphatase, like most acid phosphatases, is inhibited by fluoride and suggested that this might be due to the presence of Mg^{2+} in the enzyme molecule, since fluoride forms a non-dissociable complex with Mg^{2+}. However, analyses of ashed samples did not verify this hypothesis.[22] Furthermore, it was found that, at pH 4.9, MgF_2 and NaF exert the same degree of inhibition, and the conclusion reached that the effect of fluoride is not caused by the binding of metal ions necessary for the enzyme activity.[23] Also zinc ions influence the phosphatase activity in different ways at the two pH optima (Fig. 3), causing with increasing concentration an increasing inhibition at pH 5.5 and a decreasing inhibition at pH 6.3. The actual concentrations of zinc ions in the different samples are those calculated from the added amount, as the solubility product of $Zn(OH)_2$ is not exceeded in any case.

The relative stimulation of the enzyme activity caused by 1-butanol (Fig. 4) is not only different at the two pH optima, but is also dependent on the substrate concentration. Thus, at pH 4.9, the stimulation is about 18 % at a substrate concentration of 0.025 M and only 11 % at a substrate concentration of 0.100 M. At pH 5.9, the corresponding figures are 118 and 55, respectively. The relative stimulation is thus in both cases smaller at high than at low substrate concentrations.

Acid phosphatases are known to originate from lysosomes, and we have to reckon the presence of lysosomes in the seminal fluid, as this contains particulate matter derived from desquamated cells.[24] It is thus most reasonable to assume that 1-butanol stimulates the phosphatase activity by labilizing lysosomal membranes and releasing the enzymes. However, if this were the actual mechanism, then some of the other non-polar or weakly polar substances tried (*cf.* p. 447) should have exerted the same effect. Furthermore, the portion of the activity which is brought into existence by the presence of butanol demonstrates another correlation between rate and substrate concentration than that of the original activity (*cf.* Fig. 4).

The increase in activity induced by 1-butanol may be due to a conformational change in the enzyme molecule. What seems most important in relation to the present problem is that this hypothetical conformational change brings about a much stronger stimulation at pH 5.9 than at pH 4.9.

The stimulation of the phosphatase activity is much more efficient when 1-butanol is introduced into the samples by saturating the substrate solution

than by saturating the buffer solution (Table 5). The reason for this has not been analyzed so far. Probably the concentration of 1-butanol after saturation is higher in the substrate than in the buffer solution because of association between the molecules of 1-butanol and disodium phenylphosphate.

The very different effects of the applied inhibitor and the activators at the two pH optima exclude the possibility of one enzyme having two apparent optima (point *b*, p. 448) and support strongly the idea that the two pH optima in the range of pH from 5.1 to 6.5 are due to two different phosphatases.

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