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CHROMATOGRAPHIC FRACTIONATION AND CHARACTERIZATION OF RAT TESTICULAR ACID PHOSPHATASES

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SUMMARY

1. Acid phosphatases (acid orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) of rat testicular tissue were fractionated by DEAE-cellulose chromatography. Four enzymes were separated with distinct enzymic properties.

2. Enzyme I preferentially hydrolyzed *p*-nitrophenyl phosphate, followed by β-naphthyl phosphate and β-glycerophosphate, whereas the α forms were less readily hydrolyzed. Enzyme II was highly sensitive to fluoride and tartrate, but resistant to formaldehyde and heavy metal ions (Ag^+, Cd^{2+}, Cu^{2+}, Pb^{2+}). A tentative site within the lysosomes of the interstitial tissue is suggested.

3. Enzyme II hydrolyzed very well all the substrates tested. It was less sensitive to fluoride and tartrate than enzyme I and showed the highest resistance to thermal inactivation. Minor differences were observed in the effect of formaldehyde and heavy metal ions as compared with Enzyme I. Enzyme II may represent a membrane-bound lysosomal activity within the interstitial and also the tubular tissue.

4. Enzyme III hydrolyzed notably only *p*-nitrophenyl phosphate and β-naphthyl phosphate. It was less sensitive to fluoride and tartrate than Enzymes I and II, but was markedly inhibited by heavy metal ions (Ag^+, Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}) and formaldehyde. It was tentatively located within the seminiferous tubules.

5. Enzyme IV was resistant to fluoride and tartrate, but sensitive to heavy metal ions and formaldehyde. A special feature was the marked activation by some divalent metal ions (Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}). It showed the highest thermal inactivation and was rapidly inactivated in storage at 4°. It was a soluble enzyme and possibly entirely localized within the seminiferous tubules. A special function for this enzyme in spermatogenesis is possible.

6. All four enzymes showed slightly different pH optima and K_m values with *p*-nitrophenyl phosphate as substrate. Possibilities for their differential biochemical and histochemical demonstration are discussed.

INTRODUCTION

Studies on testicular homogenate and subcellular fractions revealed two main acid phosphatase (acid orthophosphoric monoester phosphohydrolase, EC 3.1.3.2)

activities in rat testicular tissue[1]. One, localized in the mitochondrial–lysosomal fraction, was inhibited by fluoride and tartrate. The other activity in the soluble fraction was suppressed by Cd^{2+}, Cu^{2+}, formaldehyde and glutaraldehyde. Acid phosphatase in the soluble fraction was also activated by Co^{2+}, Mn^{2+} and Ni^{2+}, a feature not previously described for any mammlian acid phosphatase. Testicular seminiferous tubules were suggested as a tentative site for the soluble enzyme[1]. Only testicular tissue contained appreciable amounts of this enzyme[2].

Since many other tissues contain multiple acid phosphatases with different substrate specificies and various sensitivities to inhibitors[3–8], the present work was intended to separate the rat testicular acid phosphatases by DEAE-cellulose chromatography and to study the modifier characteristics, pH-optima and substrate specificities of the separate activities. A special evaluation of possibilities for differential biochemical and histochemical demonstration of the distinct enzymes will be presented.

MATERIALS AND METHODS

Tissue preparation

Testicular tissue was obtained from adult male albino rats. The animals were killed by decapitation. Total homogenate was made in 0.02 M Tris–HCl buffer (pH 7.5), using initially a glass homogenizer with Teflon pestle. Subsequently the homogenate was sonicated with an MSE Ultrasonic Disintegrator for 30 sec. This and all subsequent procedures were carried out at 4°. Testicular tubules were isolated under a dissecting microscope in 0.25 M sucrose solution buffered to pH 7.5 with 0.02 M Tris–HCl. The tubular tissue was then homogenized in the same buffer and subjected to the same fractionation studies as the total testicular homogenate.

Chromatography

The enzyme sample was applied to a DEAE-cellulose (Whatman DE 23) column (2 cm × 30 cm), eluted with a linear NaCl gradient (0-0.35 M) in 0.02 M Tris–HCl buffer (pH 7.5). Fractions of 5 ml were collected. The protein content of the fractions were scanned by the absorbance at 280 nm with a Beckman DB spectrophotometer.

Measurement of enzymic activity

Acid phosphatase activity of the chromatographic fractions was determined by the methods given previously[1,2]. The following substrates were used for the phosphatase assay: *p*-nitrophenyl phosphate, *α*-naphthyl phosphate, *β*-naphthyl phosphate and naphthol AS-BI phosphate from Sigma Chem. Co., *α*-glycerophosphate from Fluka AG and *β*-glycerophosphate from Merck AG. The incubation medium contained equal amounts of 0.1 M acetate buffer (pH 5.0), enzyme solution and substrate solution. The substrate solution was 3 mM for *p*-nitrophenyl phosphate and naphthol substrates and 75 mM for glycerophosphates, if not indicated otherwise.

The enzymic activity is given as nmoles *p*-nitrophenol, naphthol or inorganic phosphate liberated per ml of enzyme solution per min.

Studies on enzyme characteristics

Various modifier substances of analytical grade were dissolved in 0.1 M acetate buffer (pH 5.0), and the enzyme was incubated with the modifier for 30 min at 37° before the addition of the substrate. The thermal inactivation was tested by incubating the enzyme in 0.1 M acetate buffer (pH 5.0) for 15 min at the temperature indicated. After the mixture had been cooled on ice, the substrate was added and the remaining enzymic activity was determined by incubation at 37° for 60 min.

In the determination of pH optima for the different enzymic activities a pooled sample from the chromatographic fractions was used as enzyme solution and a series of 0.2 M acetate buffers was used between pH 3 and 7.

The same pooled enzyme samples were used in the determination of Michaelis constants (K_m) with *p*-nitrophenyl phosphate as substrate at optimal pH for the separate enzymes.

RESULTS

Distribution of acid phosphatase in chromatography

When *p*-nitrophenyl phosphate was used as substrate four different areas of hydrolytic activity were obtained in DEAE-cellulose chromatography, namely: Enzyme I in Fractions 11–21; Enzyme II in Fractions 26–42; Enzyme III in Fractions 47–53 and Enzyme IV in Fractions 61–86 (Fig. 1A). The most active hydrolysis of α-naphthyl phosphate was observed in fractions for Enzyme II (Fig. 1B). Only very low activity was present in fractions for Enzymes I and III, and no hydrolysis at all in the area for Enzyme IV.

Fractions for Enzymes I, II and III were active in the hydrolysis of β-naphthyl phosphate (Fig. 1B), but no activity was present in fractions for Enzyme IV. The hydrolysis rate of naphthol AS-BI phosphate was almost equal to that of β-naphthyl phosphate in fractions for Enzyme II, only about half in those for Enzyme I and hardly any hydrolytic activity was present in areas for Enzymes III and IV (Fig. 1B).

The hydrolysis rate of both glycerophosphates was much lower than that of any of the other substrates. α-Glycerophosphate was not detectably hydrolyzed by Enzymes I and III, whereas Enzymes II and IV showed a clear hydrolysis (Fig. 1C). β-Glycerophosphate was hydrolyzed by fractions for Enzymes I, II and IV (Fig. 1C). Thus, neither glycerophosphate was hydrolyzed by fractions for Enzyme III.

The DEAE-cellulose chromatography of the isolated seminiferous tubules resulted in separation of three different enzymic activities hydrolyzing *p*-nitrophenyl phosphate (Fig. 2A). These activity peaks corresponded to those of Enzymes II, III and IV in fractionation of total testicular tissue; similar elution patterns together with substrate specificity confirmed their identity (Fig. 2B). Enzymes III and IV were clearly the most prominent in the tubular fractionation. This implied that these enzymes were localized within the seminiferous tubules. Enzyme II could be derived both from tubular and interstitial tissue. The major activity of Enzyme II may reside in the interstitial tissue, since its contribution in the tubular chromatography was rather weak and could also represent a contamination. Enzyme I clearly seems to be an interstitial cell enzyme, since no activity was present in fractionation of tubular tissue.

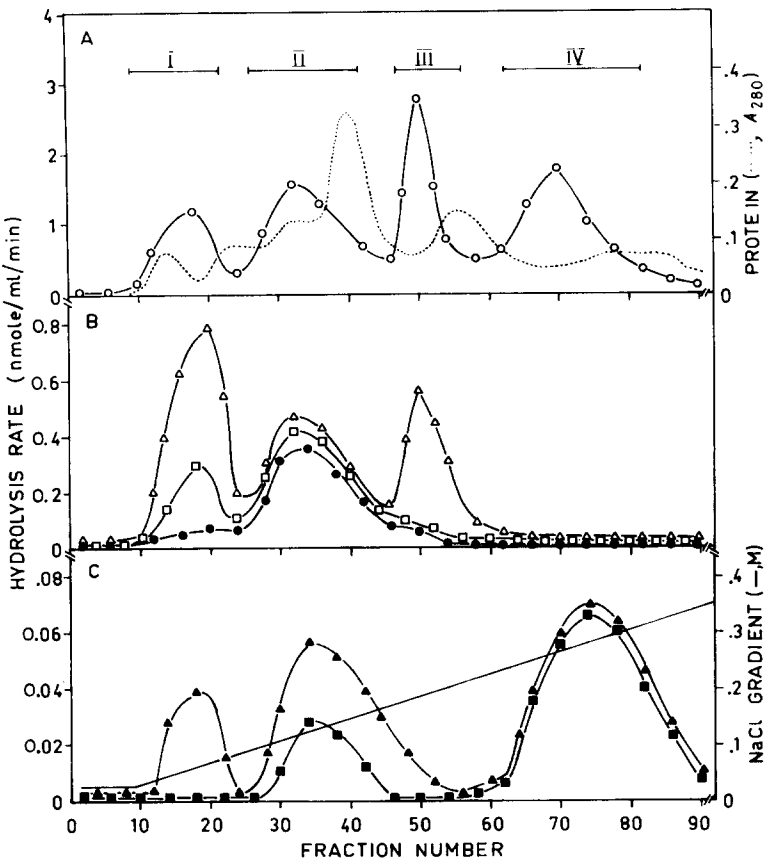


Fig. 1. Elution pattern of acid phosphatases from the DEAE-cellulose chromatography of total testicular homogenate. The column (2 cm × 30 cm) was eluted with a linear NaCl gradient (———). The protein contents of the 5-ml fractions were scanned by the absorbance at 280 nm (· · · · · ·). The hydrolysis rate of different substrates are given in nmoles/ml per min as follows: ○—○, *p*-nitrophenyl phosphate; ●—●, α-napthyl phosphate; △—△, β-napthyl phosphate; □—□, napthol AS-BI phosphate; ■—■, α-glycerophosphate; ▲—▲, β-glycerophosphate.

Optimal pH

The pH optima for the four enzymes separated by DEAE-cellulose chromatography of total testicular tissue were determined in 0.2 M acetate buffers from 3 to 7 with *p*-nitrophenyl phosphate as substrate. Fig. 3 shows the maximal hydrolysis for Enzyme I at pH 5, for Enzyme II at pH 5.7, for Enzyme III at pH 5.4 and for Enzyme IV at pH 6.0.

Modifier characteristics

Table I shows the percentage change of the enzymic activity in the presence of two concentrations of various modifier substances. Enzymes I–III showed closer similarities, whereas Enzyme IV was markedly different. Enzyme I was somewhat more sensitive to tartrate than was Enzyme II. The reverse held in the presence of Al^{3+}, Fe^{3+} and Mn^{2+}. Enzymes I and II differed from Enzyme III in their higher

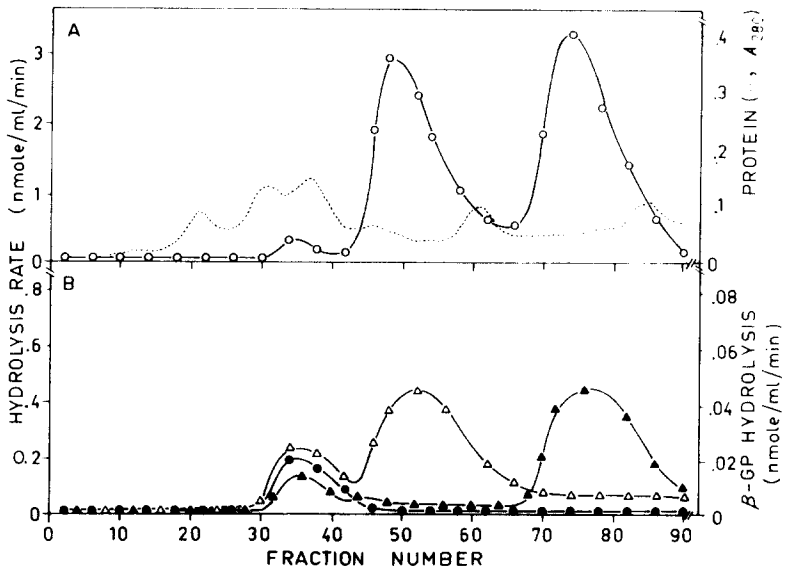


Fig. 2. Elution pattern of acid phosphatases from the DEAE-cellulose chromatography of homogenate prepared from isolated seminiferous tubules of the rat testis. The protein content of the fractions is indicated by the absorbance at 280 nm (· · · · · ·). The hydrolysis rates of different substrates are given in nmoles/ml per min as follows: ○—○, *p*-nitrophenyl phosphate; ●—●, *α*-napthyl phosphate; △—△, *β*-napthyl phosphate; ▲—▲, *β*-glycerophosphate (*β*-GP).

sensitivity to Mo^{6+}, W^{6+}, fluoride and tartrate. On the contrary, Enzyme III was more sensitive to Ag^{+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, *p*-chloromercuribenzoate and formaldehyde than Enzymes I and II. Enzyme IV was markedly inhibited by Ag^{+}, Cd^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mo^{6+}, W^{6+}, *p*-chloromercuribenzoate and formaldehyde. A special feature for enzyme IV was, however, the marked stimulation by some divalent metal ions: Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+}.

During storage at 4°, enzyme IV was rapidly inactivated, but could be re-

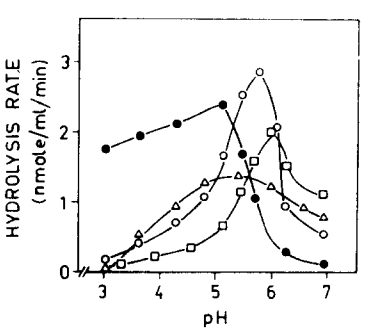


Fig. 3. Effect of pH on the hydrolysis of *p*-nitrophenyl phosphate by the four enzyme preparations separated by DEAE-cellulose chromatography. 0.2 M acetate buffers were used, and the actual pH was measured with a glass electrode before the addition of the aqueous substrate solution. Tracings for the different enzymes are: ●—●, Enzyme I; ○—○, Enzyme II; △—△, Enzyme III; □—□, Enzyme IV.

TABLE I

PERCENTAGE CHANGE OF THE *p*-NITROPHENYL PHOSPHATE HYDROLYSIS BY THE FOUR TESTICULAR ACID PHOSPHATASES IN THE PRESENCE OF DIFFERENT MODIFIERS

Aliquots of pooled enzyme samples were preincubated with the modifiers at two concentrations for 30 min at 37° before the addition of *p*-nitrophenyl phosphate (1 mM final concentration). The change in the hydrolysis is given as a percentage of the controls (100%). The two modifier concentrations were 0.5 mM (A) and 0.05 mM (B), if not otherwise indicated.

Modifier	*Enzyme I*		*Enzyme II*		*Enzyme III*		*Enzyme IV*	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Li	100	100	84	91	101	95	72	87
Mg	103	105	101	105	107	108	736	182
Al	95	92	35	92	30	100	37	66
Ca	94	95	97	93	101	96	71	86
Mn	98	100	63	67	34	83	379	426
Fe^{2+}	70	81	41	62	74	94	48	58
Fe^{3+}	62	71	18	24	16	26	20	70
Co	90	108	99	94	100	98	1494	566
Ni	103	100	98	91	95	101	749	310
Cu	20	70	70	85	2	10	18	34
Zn	78	98	93	99	67	100	1057	676
Mo	0	2	0	13	4	34	0	9
Ag	68	77	66	41	12	11	11	10
Cd	53	91	78	73	13	29	38	47
Sn	57	70	86	95	84	101	52	69
Sb	100	98	91	97	79	94	67	67
Te	78	105	47	53	10	39	44	51
Ba	84	101	100	89	89	98	52	87
La	65	100	97	91	79	96	60	70
W	0	0	0	0	1	43	0	8
Hg	43	56	45	43	12	10	8	20
Pb	76	93	98	83	20	79	32	40
Bi	92	100	84	88	61	86	70	76
NaF*	0	2	0	28	75	83	75	100
Tartrate*	0	0	63	71	91	95	85	91
EDTA	112	106	107	91	114	107	112	119
Cysteine	110	97	103	100	60	80	85	83
p-Chloromercuribenzoate	47	55	35	56	8	11	7	25
Acetone*	140	93	125	90	107	94	103	101
Formaldehyde**	91	108	93	97	29	64	51	69

* Concentrations: A = 5 mM (%); B = 0.5 mM (%).
** Concentrations: A = 0.05%; B = 0.005%.

activated by using the above-mentioned divalent metals, as shown in Fig. 4. The highest activation was obtained with all these metal ions at about 5 mM concentration in the decreasing order: Co^{2+}, Zn^{2+}, Ni^{2+}, Mg^{2+} and Mn^{2+}.

Different concentrations of fluoride (Fig. 5), tartrate (Fig. 6), Cu^{2+} (Fig. 7) and formaldehyde (Fig. 8) were tested on the hydrolysis of *p*-nitrophenyl phosphate by different enzymic species. These studies showed that Enzymes I and II were rather sensitive to NaF and tartrate, whereas Enzymes III and IV appeared resistant. In contrast, the latter enzymes were markedly inhibited by Cu^{2+} and formaldehyde, which had a much lower suppressive effect on Enzymes I and II.

Thermal inactivation

Pooled enzyme preparations were incubated for 15 min at different temperatures

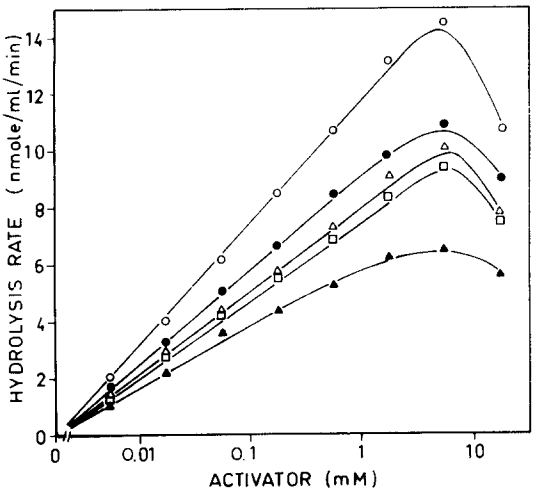


Fig. 4. Reactivation of Enzyme IV with divalent metal ions after 5 days of storage at 4°. The substrate solution of *p*-nitrophenyl phosphate was added to the incubation medium after 30 min preincubation with different concentrations of the following divalent ions: ○—○, Co^{2+}; ●—●, Zn^{2+}; △—△, Ni^{2+}; □—□, Mg^{2+}; ▲—▲, Mn^{2+}.

from 40 to 70°, and subsequently the hydrolysis of *p*-nitrophenyl phosphate was tested. Fig. 9 shows the percentage activity remaining from different enzymes after the treatment as compared with the untreated control. Enzymes I and III showed closely similar thermal inhibitions. Enzyme II was the most resistant enzyme, whereas Enzyme IV was the most sensitive. In the presence of Ni^{2+} this enzyme was slightly protected from inactivation and the resulting curve was almost superimposable on those of Enzymes I and III.

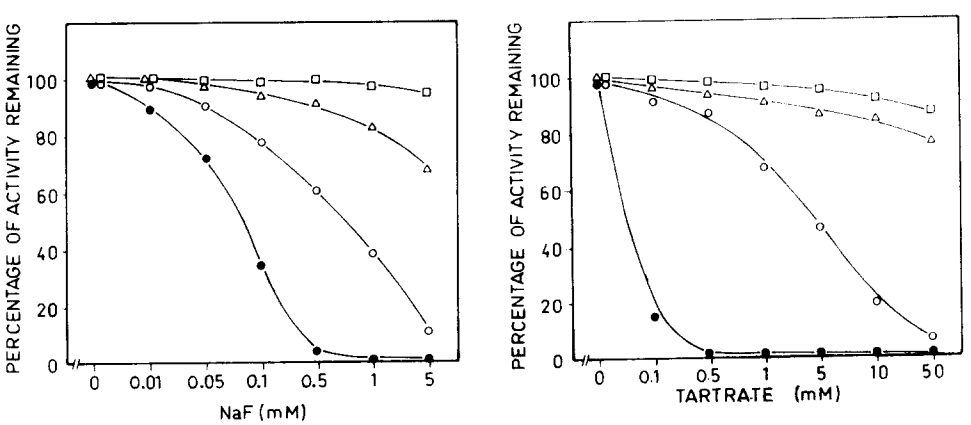
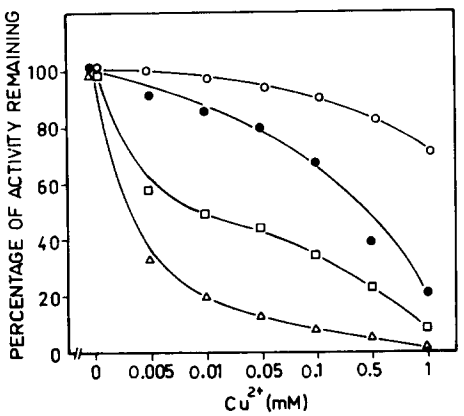


Fig. 5. Effect of NaF on the *p*-nitrophenyl phosphate hydrolysis by the four different enzyme preparations. A 30-min preincubation of the enzyme with the inhibitor at concentrations indicated preceded the addition of the substrate. Tracings for the different enzymes are similar to those given in Fig. 3.

Fig. 6. Effect of various concentrations of sodium tartrate on the *p*-nitrophenyl phosphate hydrolysis by the four different enzyme preparations. Tracings for the enzymes are given in Fig. 3.



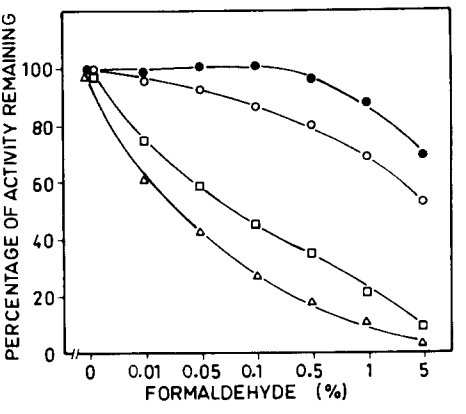


Fig. 7. Effect of various concentrations of Cu^{2+} on the *p*-nitrophenyl phosphate hydrolysis by the four different enzyme preparations. Tracings for the enzymes are given in Fig. 3.

Fig. 8. Effect of various concentrations of formaldehyde on the *p*-nitrophenyl phosphate hydrolysis by the four different enzyme preparations. Tracings for the enzymes are given in Fig. 3.

K_m values

The K_m was determined at optimal pH for each enzyme: pH 5.0 for Enzyme I, pH 5.7 for Enzyme II, pH 5.4 for Enzyme III and pH 6.0 for Enzyme IV. The incubation medium for Enzyme IV additionally contained 1.5 mM Co^{2+}. The K_m values for different enzymes were as follows: Enzyme I, 0.38 mM; Enzyme II, 0.50 mM; Enzyme III, 0.43; Enzyme IV, 0.22 mM.

DISCUSSION

Identity of different acid phosphatases

The four different enzymes separated by chromatographic fractionation showed

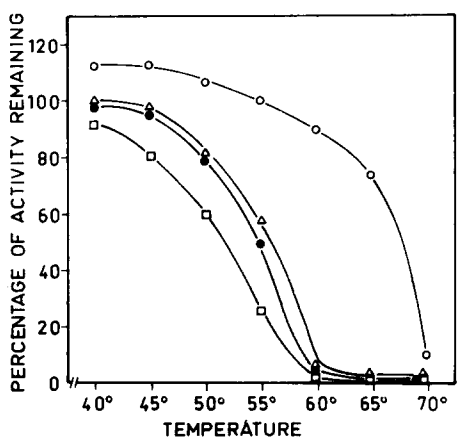


Fig. 9. Thermal inactivation of the four testicular acid phosphatases. The enzyme was incubated in 0.1 M acetate buffer (pH 5.0) for 15 min at the indicated temperature. After the mixture had been cooled on ice, *p*-nitrophenyl phosphate was added, and the remaining enzyme activity was determined. The results are given as percentages of the unincubated control value for each enzyme. Tracings for the enzymes are given in Fig. 3.

an acid pH optimum and were able to hydrolyze aryl phosphates. On this basis they can all be defined as non-specific acid phosphohydrolases until there is further evidence that they have a specific function in testicular tissue. Their ability to hydrolyze various physiological substrates will possibly reveal some special function to each of them. Until then we have to consider whether they are separate enzymic entities or represent only isozymic varieties of the classical lysosomal acid hydrolase similar to those of prostatic acid phosphatases.[9]

The present study showed differences in many enzymic properties of the four activities. These included differences in substrate specificity, pH optima, inhibitor and activator characteristics and thermal inhibition as well as K_m values. Enzyme I was characterized by a high hydrolysis of *p*-nitrophenyl phosphate, β-naphthyl phosphate, naphthol AS-BI phosphate and β-glycerophosphate, but relatively low or no hydrolysis was observed with α-naphthyl phosphate or α-glycerophosphate as substrates. This observation indicated that this enzyme preferred the hydrolysis of phosphate groups in the 2-position of the aromatic ring and aliphatic structure. The high sensitivity of this enzyme to fluoride and tartrate was clearly established.

Enzyme II was able to hydrolyze all the substrates used and thus could be called the "most non-specific" acid phosphatase in testicular tissue. Differences in the hydrolysis rates of various substrates were, however, observed: the enzyme seemed to prefer the β forms over the α forms of both naphthol and glycerophosphate substrates. This enzyme was sensitive to fluoride and tartrate, although clearly more resistant than Enzyme I.

Enzyme III hydrolyzed only *p*-nitrophenyl phosphate and β-naphthyl phosphate while the other substrates tested were split minimally or not at all. A more purified enzyme preparation would be necessary to show whether it is capable of hydrolyzing other substrates and whether the low activity in the fractions of this enzyme only represents contamination from the other enzymes. This enzyme showed much lower sensitivity to fluoride and tartrate than Enzymes I and II, but was highly sensitive to many heavy metal ions and formaldehyde.

Enzyme IV showed the highest pH optimum. It did not hydrolyze the naphthol substrates to any appreciable degree. In contrast, *p*-nitrophenyl phosphate and both glycerophosphates were hydrolyzed very well. It was not inhibited by fluoride or tartrate, while sensitivity to heavy metal ions, formaldehyde and thermal inactivation were high. A distinct feature for Enzyme IV as compared with the others was the marked activation by some divalent metal ions. The most active in this respect was Co^{2+}, followed by Zn^{2+}, Ni^{2+}, Mg^{2+} and Mn^{2+}. The present as well as previous studies[1,2] indicated that this activity was present in the soluble fraction. All these properties were markedly different from the classical properties of the particle-bound lysosomal enzymic activity[10]. The previous observation[1], that only testicular tissue, particularly the seminiferous tubules, contained this enzyme, would make its value more important in the male reproduction.

All these findings indicated that the four testicular acid phosphatases separated were distinct enzymes and not only separate isozymic forms of one enzyme. Further studies with a variety of substrates may confirm a specific function for each of them.

Correlation with enzymes from other tissues

Although there are many reports of the presence of at least three distinct

enzymic species in different mammalian tissues their mutual relationship and differences have not been worked out completely[3,6–8]. Nor is it obvious from these previous studies which of the three acid phosphatases represents the classical lysosomal activity or those possibly derived from other subcellular sites like endoplasmic reticulum and Golgi apparatus. In addition, two distinct lysosomal acid phosphatases have been described, one soluble and heat labile and the other membrane-associated and thermostable[11]. Both are inhibited by fluoride and tartrate[11]. Enzymes I and II could represent similar enzymes in testicular tissue. An enzyme less specific for β-glycerophosphate than for p-nitrophenyl phosphate was reported in the renal medulla, and a distinct localization from lysosomes was suspected[12]. From the three placental enzymes reported by DiPietro and Zengerle[7], Enzymes I and II are also potential candidates as lysosomal enzymes in this tissue. On the basis of inhibitor studies, testicular Enzymes I and II were closely related to the placental ones in reverse order. The sequence of elution depends on the different fractionation methods used in the present study (DEAE-cellulose chromatography) and that of the placental enzymes (gel filtration on Sephadex G-200)[7].

Placental[7] Enzyme III was sensitive to SH reagents. Similar acid phosphatases have also been demonstrated in other tissues[13,14]. Also the low affinity for α-naphthyl phosphate has been noticed previously[7]. β-Naphthyl phosphate was reasonably well hydrolyzed, whereas glycerophosphates were not hydrolyzed at all. This indicated that Enzyme III had a narrow substrate specificity with the requirement for an aromatic ring. The inhibition of the placental Enzyme III by pyridoxine 5'-phosphate was suggested to be due to an allosteric inhibition by the ring structure[7].

It is appropriate to consider the possible identity of Enzyme III and phosphoprotein phosphatase. This latter enzyme has been shown to be SH activated, able to hydrolyze p-nitrophenyl phosphate, but unable to hydrolyze β-glycerophosphate[15]. These similarities are opposed by the relative heat stability of bovine spleen phosphoprotein phosphatase[15] as compared with testicular Enzyme III. Further studies are therefore necessary to define any specific character for this enzyme. The relationship of this enzyme to some soluble[4,5] and microsomal[4,16] acid phosphatases in liver tissue remains to be evaluated.

Enzyme IV was entirely different from all the other acid phosphatases. It revealed some similarities with the erythrocytic, Mg^{2+}-activated acid phosphatase, which is also heat labile and inhibited by formaldehyde and Cu^{2+} [17]. The erythrocytic enzyme hydrolyzes only α-glycerophosphate[17], whereas the testicular enzyme attacks equally well both α-glycerophosphate and β-glycerophosphate. The testicular enzyme also differed from the latter in the plurality of activating divalent metal ions. The testicular tissue was carefully cleaned of all major blood vessels, and since the isolated seminiferous tubules contained this activity, a contamination from the erythrocytes did not seem possible. Neither did other more vascular tissues with appreciable amounts of retained blood contain a similar activity[2]. This suggests a specific function of this enzyme in rat testicular seminiferous tubules.

Another type of Mg^{2+}-activated acid phosphatase has been demonstrated in baker's yeast[17], but other divalent ions did not activate it. Lingcod muscle contains five acid phosphatases with p-nitrophenyl phosphate as substrate[18]. Two of these (A, E) were activated by Zn^{2+}, but neither of them was activated by Co^{2+} or Ni^{2+}. These may, however, be metal enzymes closely related to the testicular enzyme IV.

Biochemical determination of different enzymes

The differential estimation of the activity of various acid phosphatases in total testicular homogenate would be useful in the evaluation of their physiological significance in testicular tissue. None of these enzymes had a substrate that was not hydrolyzed by any other activity. Enzyme II seemed to be the only one able to hydrolyze α-naphthyl phosphate; consequently this is an appropriate substrate for its quantitative assay. A low level of sodium tartrate (0.1 mM), which totally inactivates the hydrolysis by Enzyme I without any effect on Enzyme II, would make the assay more specific.

The best substrate for Enzyme I would be either β-naphthyl phosphate or naphthol AS-BI phosphate. In physiological experiments the variability in the changes between α-naphthyl phosphate and naphthol AS-BI phosphate hydrolysis could reflect the activity of Enzyme I. The tartrate (0.1 mM) sensitive hydrolysis of β-naphthyl phosphate or naphthol AS-BI phosphate could most accurately indicate Enzyme I activity.

Enzyme III only hydrolyzed *p*-nitrophenyl phosphate and β-naphthyl phosphate to any appreciable degree. Its relative insensitivity to NaF (0.5 mM) could serve to differentiate its activity from Enzymes I and II with β-naphthyl phosphate as substrate. With this substrate Enzyme IV did not interfere nor did NaF totally abolish Enzyme II activity at this concentration, which makes the quantitation of Enzyme III biased.

Enzyme IV can be determined by using *p*-nitrophenyl phosphate as substrate with specific activators. From this value the contribution of other enzymes must be subtracted. Tentatively this may be possible by including NaF (5 mM) in the same incubation medium. With α-glycerophosphate as substrate and NaF (5 mM) as inhibitor for Enzymes I and II the activity of Enzyme IV could also be assayed in the presence of any of the divalent metal activators.

Further studies may reveal more specific substrates and assay conditions for each of these enzymes, which would facilitate their differential quantitative estimation.

Histochemical demonstration of different enzymes

Although biochemical studies do not always give compatible answers to all the problems of the histochemical methods for enzyme localization, a few obvious conclusions can be based on the present observations.

The conventional GOMORI[19] technique for the histochemical acid phosphatase demonstration utilized glycerophosphates as substrates in the presence of Pb^{2+} in the incubation medium. The present study showed that Enzymes I and II were resistant to Pb^{2+} and able to hydrolyze α-glycerophosphate and/or β-glycerophosphate. Enzymes III and IV were highly sensitive to Pb^{2+} as well as to the conventional fixatives, formaldehyde and glutaraldehyde[1]. Cold acetone appeared to be better for the preservation of these enzymes.

Since *p*-nitrophenyl phosphate cannot be used as a histochemical substrate, the following preference for the differential histochemical demonstration of the four enzymes can be chosen: naphthol AS-BI phosphate shows Enzyme I, and its reaction is abolished by low tartrate concentration (0.1 mM) in the incubation medium. The activity left after tartrate inhibition would show the site of Enzyme II activity.

α-Naphthyl phosphate without any inhibitor would also be specific for the demonstration of Enzyme II. The NaF (0.5 mM) resistant activity hydrolyzing β-naphthyl phosphate would mainly show the activity of Enzyme III. The only possibility for the histochemical demonstration of Enzyme IV would be to use either glycerophosphate as substrate in the presence of a divalent activator ion and NaF (5 mM) as an inhibitor for Enzymes I and II. However, Pb^{2+} cannot be used to react with the liberated phosphate ion and thus another complexing ion must be chosen. Acetone would be the only possible fixative in the preservation of this soluble enzyme.

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