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Isodynamic Pyrophosphatases in Rat Liver

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Since the investigations on the liver pyrophosphatases of pig and man by Bamann and Gall [1] in 1937 the existence of three isodynamic pyrophosphatases in human and animal tissues seems to have been generally accepted. In experiments on the pH-activity correlation in rat liver homogenates, however, I repeatedly found four peaks. A closer investigation was therefore carried out. The experiments which are presented in this report demonstrate the existence of a fourth pyrophosphatase in rat liver.

To begin with the assay was performed essentially according to the method of Gall [6] where the orthophosphate produced by the enzyme is determined directly on an aliquot of the deproteinized digestion mixture by means of the ceruleomolybdate reaction of Fiske and Subbarow (see *e. g.* Norberg [12]). Later on this technique was modified in many respects, and for the most acid enzymes the final method involves precipitation of the liberated orthophosphate.

As source of enzyme a homogenate (see *e. g.* Potter [13]) of rat liver in glass-redistilled water was used. It was always employed as soon as possible after the death of the animal.

EXPERIMENTAL

First method of assay

The substrate was prepared from 0.1 *M* sodium pyrophosphate, adjusted to the proper pH, by dilution 1 : 100 with 0.05 or 0.1 *M* buffer of the same pH. $MgSO_4$ at a final concentration of 0.002 *M* was also included. Five ml of the substrate were warmed to 37° C and 0.20 ml of the homogenate added. The digestion was allowed to proceed until 10 to 50 micrograms of phosphorus had been liberated, usually 15 to 30 minutes. Then the process was stopped by addition of 1.8 ml normal trichloracetic acid. 3.5 ml of the proteinfree filtrate were used for phosphate estimation. As pyrophosphate interferes with the Fiske-Subbarow reaction the micrograms of phosphorus were read from a calibration curve prepared with similar amounts of pyrophosphate as were present in the

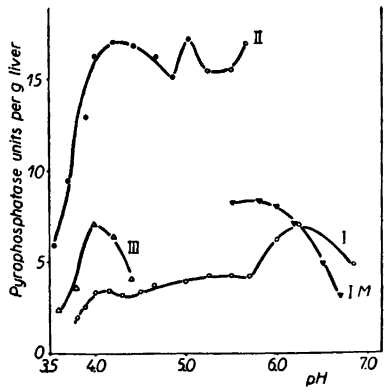

Figure 1. Activity-pH correlation for rat liver pyrophosphatases.
Curve I: with phthalate buffer.
» *I M: same homogenate with maleic acid buffer.*
» *II: with acetate buffer.*
» *III:* » » » *and second method of assay.*

samples. Duplicates or triplicates were run with each sample, and also duplicate blanks (homogenate added to substrate + trichloracetic acid at the moment of stopping the action of the enzyme). *The enzyme units* are the number of micromols of phosphorus liberated per minute under the experimental conditions used. In this paper all values are given in units per g of fresh liver.

The buffers used were: 0.05 *M* phthalate for pH 3.8—6.8, 0.1 *M* acetate for pH 3.5 to 6, 0.1 *M* maleic acid (Smits [19]) for pH 5.8—6.7, 0.1 *M* collidine (Gomori [7]) for pH 7—8, 0.1 *M* ammonium chloride-ammonia for pH 7.6—10, and 0.0028 *M* veronal-acetate (Michaelis [9]) for pH 4.4—9.2. All pH-values were controlled by the glass electrode.

The pH-activity curve. Figs. 1 and 2 show some of these experiments. In all curves there are indications of optima at pH 4—4.2, around pH 5, at pH 5.8—7, and on the alkaline side at pH 8—8.4. However due to the influence of the different ions in the buffers the peaks are more or less pronounced, and the pH optima may also be displaced. Thus phthalate clearly is more toxic to the pH 4 and 5 enzymes than is acetate. Also veronal seems to inhibit the pH 6 pyrophosphatase very strongly at the same time displacing its optimum towards pH 7. Maleic acid buffer on the other hand tends to displace the optimum of this enzyme to pH 5.8.

Table 1. Units of alkaline pyrophosphatase per g rat liver calculated from experiments with different digestion periods.

Digestion period in minutes	Amount of fresh liver used		
	22 mg	33 mg	66 mg
2	4.4	3.1	3.0
4	2.7	1.8	1.8
6	1.7	1.6	1.4
8	1.3	1.4	1.3

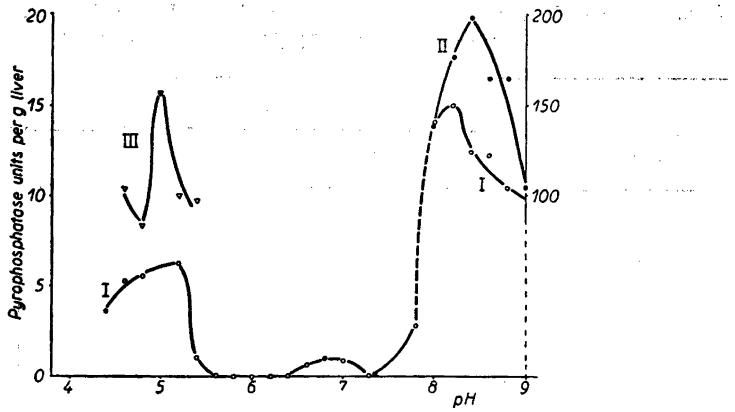

Figure 2. Activity-pH correlation for rat liver pyrophosphatases.
Curve I: with diethylbarbituric acid buffer. Five rats were used in order to get the same time from killing to assay in all points. Note that the alkaline pyrophosphatase is about ten times as active (right hand ordinate) as the pH 5 pyrophosphatase.
Curve II: with ammonium chloride buffer.
» III: » acetate buffer and second method of assay.

In order to study the other optimum conditions for these enzymes the three acid ones were investigated with acetate buffers at pH 4.0, 5.0, and 5.8, and the alkaline pyrophosphatase at pH 8.4 in ammonium chloride-ammonia buffer.

Time of digestion was next studied with the alkaline pyrophosphatase. It turned out that the enzymatic activity undergoes a rapid decay with time so that there is very poor proportionality between amount of enzyme and quantity of phosphorus liberated. This is evident from Table 1.

It was decided to try a method of assay with the shortest possible time of digestion. For practical reasons this time was fixed at 120 seconds.

Second method of assay

In order to enable an exact timing of the reaction the substrate was divided into two reagents, 1) the activating solution, and 2) the substrate proper. For the alkaline enzyme the *activating reagent* is a 0.1 *M* ammonium chloride buffer containing magnesium ions in the concentration stated as optimum, initially 0.002 *M*, later 0.2 *M* (see below). The *substrate* is a pyrophosphate solution adjusted to the same pH as that of the activating reagent and 1.7 times the intended final concentration of pyrophosphate.

For every sample one tube with 2.0 ml of the activating reagent and one with 3.0 ml of the substrate are placed in the water thermostat. To the activating reagent tubes 0.20 ml of the samples are added. The enzymatic process is started by pouring the substrate into the tube with the sample. When a number of reactions are started (5 seconds interval) the empty substrate tubes get 1.8 ml normal trichloracetic acid each. The digestion is stopped by pouring the trichloracetic acid in the digests and mixing back and forth between the tubes. With this technique 12 pairs of tubes can be handled at a time.

Table 2. Change in liver pyrophosphatase units with varying substrate concentration. Activation a) 4 mM Mg, b) 200 mM Mg.

$Na_4P_2O_7$ concentration m*M*	Assay at pH					
	4.0	5.0	5.8		8.4	
			a	b	a	b
0.25			10.5	73	16	131
1			15.5	75	5.6	148
2	7.2— 8.4					137
3		20.6		57		
4	11.4—13		21.7		1.1	
6		22.5		64		
8	7.5— 7.8		20.3			
9		25		52		
12		21.2				
16	2— 5	9	16.2			

Blanks are run in the same way pouring the substrate into the sample only after the addition of trichloracetic acid.

Due to the small volume adhering to the walls of the substrate tube the substrate concentration in the digestion mixture will be a little less than calculated. This does not seem to be of any significance, however.

Substrate concentration. This had to be investigated since fairly different concentrations have been used, from 1 m*M* by Gall [6] and others up to 20 m*M* by Desruisseaux [4], and because increasing pyrophosphate concentration may inhibit both pyrophosphatase and other enzymes, as shown inter alia by Schmidt and Thanhauser [18] and by Naganna and Menon [11].

Studies with the first method of assay had indicated different substrate optima at the four pH-optima. This supporting evidence for the existence of four pyrophosphatases was therefore followed up. The results differ somewhat in different experiments, but on the whole the enzymes of pH 8.4 and 5.8 have their optimum at about 1 m*M* pyrophosphate, whereas the pH 5 enzyme shows optimum activity at 10 m*M* pyrophosphate, and the pH 4 enzyme is most active at 1—4 m*M* P_2O_7. Table 2.

After these experiments the substrate reagents were prepared at such concentrations that the final pyrophosphate levels were 1 m*M* at pH 5.8 and 8.4, 10 m*M* at pH 5 and 4 m*M* at pH 4.

Activation and inhibition. The strong activation of alkaline pyrophosphatase by magnesium is well known (see *e. g.* Bamann and Gall [1]) whereas the pH 4 pyrophosphatase is not influenced by this ion (Bamann and Gall) or even inhibited (Roche, Thoai and Durand [15], Chevillard [2]).

With magnesium fairly weak concentrations are generally used, 1—10 m*M*, although Chevillard [2] used as much as 100 m*M* without stating, however, if this is an experimentally found optimum condition. With erythrocyte pyrophosphatase Naganna and Menon [11] find the optimum range to be 20—50 m*M* magnesium.

Table 3. Activation of pyrophosphatases by magnesium ion at 1 mM substrate concentration.

Concentration of $MgSO_4$, m*M*	Activity at pH 5.8	Activation in %	Activity at pH 8.4	Activation in %
0	29		3.7	
2	31	7	4.7	27
20	39	35	12.7	240
50	81	180	15.5	320
100	99.5	240	65.0	1 660
200	112	290	151	4 000
400	98	240	56.5	1 430
800			18	390

In our experiments, however, both the alkaline and the pH 5.8 pyropshophatase required 0.2 *M* $MgSO_4$ in the activating reagent for full activation. Some experiments are reported in Table 3.

The pyrophosphatase activity at pH 4 is not at all or only very slightly influenced by magnesium ions, the highest observed activation being 30 % at 20 m*M* $MgSO_4$. The pH 5 pyrophosphatase, on the other hand, has shown activation up to 345 %, so a real effect can hardly be doubted. In other experiments, however, magnesium had no effect. The behaviour of this enzyme is thus very irregular. Examples are given in Table 4.

Combination of 10^{-3} *M* Mg and 10^{-4} Zn, shown by Roche and Thoai [16] to activate intestinal pyrophosphatase, was tried at pH 5. The response to this combination was also very irregular, varying between no influence and activation by 150 to 250 %.

In this connection some other factors were investigated. Potassium cyanide which at higher concentrations inhibits certain phosphomonoesterases (Cloetens [3]) and erythrocyte pyrophosphatase activates the pH 5 liver pyrophosphatase slightly from 0.02 to 2 m*M* . 2 m*M* phenanthroline has the same effect. This substance according to Dumazert, Lévy and Marszak [5] also increases the activity of the alkaline pyrophosphatase from fntestine. Sodium fluoride is a potent inhibitor for the activity at pH 5. At 0.02 m*M* luoride, only about fifty per cent of the activity is left.

Table 4. Influence of magnesium on pyrophosphatase activity at pH 5.

Concentration of $MgSO_4$, m*M*	Assay with second method	Assay with third method		Activation in b) in %
		a	b	
0	3.19	4.05	1.59	
2	3.19	4.01	2.31	45
20	3.19		7.08	345

Table 5. Apparent stability of the rat liver pyrophosphatases.

Time from excision of liver to assay	At pH 4	At pH 5	At pH 5.8	At pH 8.4
5 minutes	7.3	25.2	75.8	134
10 »	3.55	10.8	67.5	—
15 »	5.8	7.2	68.1	129
20 »	4.0	4.1	66.2	122
25 »	3.12	7.4	61.7	111
30 »	4.3	6.4	61.1	121

As amino acids and some of their derivatives are activators for certain monophosphatases some such substances were tried with all four pyrophosphatases. In 0.5 m*M* concentration, however, alanine, betain hydrochloride, choline chloride, DL-alanylglycin, DL-leucylglycin, and DL-leucylglycylglycin showed no significant effect. The inhibitory effect of diethylbarbituric acid mentioned above was also tested with all four enzymes. It was found to be different for each enzyme. Thus at pH 4 a slight activation of about 20 % over acetate is observed whereas at pH 5 a moderate inhibition of the same order is found. At pH 5.8 the inhibition is very strong leaving but 10 % of the activity with acetate. The alkaline pyrophosphatase shows a moderate inhibition ranging from 15 to 45 % compared with ammonium chloride buffer. Inhibition of erythrocyte pyrophosphatase by veronal at pH 7.6 has been observed by Naganna [10].

Stability. The activity of the four pyrophosphatases was investigated at different times after the excision of the liver under the optimum conditions arrived at by means of the foregoing experiments. At pH 8.4 and 5.8 there is a small decrease in activity during the first 30 minutes which may reach 20 %. It is therefore advisable to perform the digestion within 15 minutes, when the activity seems to be between 90 and 100 % of the maximum measurable. The activities at pH 4 and 5 show much faster decay. Some of these results are presented in Table 5.

From the rapid change in activity at pH 4 and 5 it is clear that an adequate estimation of these enzymes is impossible. Attempts to stabilize the enzymes by the addition of $MgSO_4$ to the homogenate did not result in any improvement. Preparation of the homogenates in different buffers (pH 4.3, 5.0 and 6.1) gave even a somewhat lower assay than the homogenate in redistilled water. Obviously a new method of assay had to be found.

Third method of assay

During studies on another hydrolytic enzyme it was found convenient to precipitate the orthophosphate before applying the colour reaction. This method also proved useful for the pH 4—5 pyrophosphatases. With this modification no fall in activity either during the digestion period (up to 10 minutes) or with time after excision of the liver (within 30 minutes) could be detected. The rapid formation of an *inhibitor for the cerulemolybdate reaction* is therefore assumed.

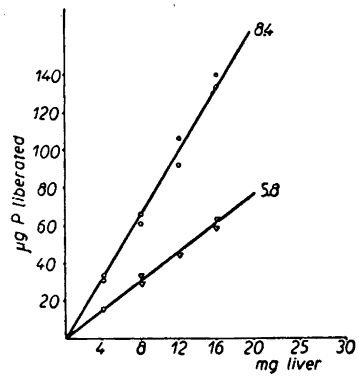

Figure 3. Proportionality for the alkaline and the pH 5.8 pyrophosphatases. Second method of assay.

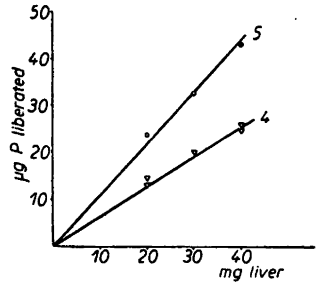

Figure 4. Proportionality for the acid pyrophosphatases assayed according to the third method.

The third method of assay, used at pH 5 and 4, consists of digesting the samples as in method 2 but for 10 minutes. The pyrophosphate concentration is kept at 1—4 m*M*. An aliquot of the trichloracetic acid filtrate is neutralised and the phosphate precipitated with 0.2 volumes of 10 % $CaCl_2$ containing sufficient $Ca(OH)_2$ to bring the solution to pH 9. This technique is adapted from the method for "true phosphate" given in the manual of Umbreit, Burris and Stauffer [22]. The precipitate is centrifuged down and washed twice with 2 % $CaCl_2$ containing $Ca(OH)_2$. Finally the precipitate is dissolved in a few ml of 0.1 N HCl and the Fiske-Subbarow reaction applied.

Proportionality. When analyzing a rat liver homogenate for pyrophosphatases at pH 8.4 and 5.8 with method 2 and at pH 4 and 5 with method 3 a reasonably good proportionality is obtained. This is evident from Figs. 3 and 4.

The standard deviations were calculated from duplicates. At pH 5.8 the standard deviation on the mean of duplicates is 3.5 %. At pH 8.4 the error is less. At pH 5 the calculated standard deviation for the mean value of duplicates is 3.9 %. The same applies for the more acid enzyme.

DISCUSSION

According to the results presented above there seems to be no doubt about the presence of four different isodynamic pyrophosphatases in rat liver. Two of them have their optima at distinctly acid pH values, about 4 and 5 respectively. One has an optimum pH between 5.8 and 7 depending on the buffer and may be referred to as the neutral pyrophosphatase. Finnaly there is the alkaline pyrophosphatase with optimum activity at pH of about 8. The acid pyrophosphatases differ in respect to optimum substrate concentration and the action of diethylbarbituric acid. They both are apparently unstable so that their activities may easily be overlooked. This is the case particularly

with the pH 5 enzyme. The pH 4 pyrophosphatase seems to be quite insensitive to magnesium ions whereas the pH 5 enzyme is sometimes considerably activated.

The neutral and alkaline pyrophosphatases are consistently and strongly activated by magnesium. They have similar substrate optima but the alkaline enzyme is only moderately inhibited by veronal whereas the neutral one is almost completely inhibited.

The pertinent data to the four pyrophosphatases are collected in the adjacent table:

Optimum pH	4	5	5.8—7	8—8.4
Substrate optimum	4	10	1	1 m*M*
Mg^{++} optimum	0	0—20	200	200 m*M*
Effect of diethylbarbituric acid	Weak stimulation	Slight inhibition	Strong inhibition	Moderate inhibition

In earlier investigations on the pyrophosphatases much longer digestion periods of 30 minutes to 24 hours have been used. The preliminary treatment of the enzyme may also seriously affect the results and even change the optimum pH as was found for the pyrophosphatases of basidiomycetes by Thoai [20]. Finally the natural occurrence or formation of inhibitors (Thoai [21]) makes comparison with the present results difficult.

Nevertheless the alkaline pyrophosphatase with the optimum pH at 7.4—9.4 is certainly the same enzyme or group of enzymes in all investigations. The acid pH 4 pyrophosphatase also has a fairly well characterized counterpart in most earlier investigations, where the optimum pH has been stated to be from 3.6 to 4.2. The interval from pH 5 to 7 is more difficult to judge. Bamann and Gall [1], Roche [14], Roche, Thoai and Marcelet [17], Desruisseaux [4], and others find an optimum around pH 6 (5.5—6.4). This enzyme is activated by magnesium. In leucocytes Chevillard [2] finds a plateau at pH 5 to 5.8, but on addition of magnesium ions the optimum is displaced to pH 6.3. Simultaneously an activation of several hundred percent takes place. It seems reasonable to assume that this enzyme belongs to one and the same group as the neutral pyrophosphatase of the present study.

In some investigations only two pyrophosphatases have been found, *e. g.* by Kroon, Neuman and Veerkamp [8]. In the past never more than three pyrophosphatases seem to have been observed, although Chevillard [2] in bone marrow found optima at pH 3.6, 4.4 and 8.7 plus a hint in the region where magnesium activates at an optimum of pH 5.2. This last enzyme should correspond to the »neutral» pyrophosphatase. Then the pH 4.4 enzyme of Chevillard would match the pH 5 pyrophosphatase of the present study.

The above considerations suggest that the pH 5 pyrophosphatase found in rat liver has previously been overlooked.

SUMMARY

The pyrophosphatase activity of rat liver homogenate has been studied with the necessary modifications in the methods of assay. Four distinct pH optima have been found, *viz.* at pH 4, 5, 5.8—7 ("neutral pyrophosphatase"), and about 8 (alkaline pyrophosphatase). The corresponding enzymes have been characterized by their differences reagarding substrate optima and activators or inhibitors. The pH 5 enzyme seems previously to have been overlooked.

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