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Phosphoprotein Phosphatase in the Rat

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A phosphatase acting on the natural phosphoproteins in frog eggs was discovered by Harris [6] in 1946. On account of the important position of phosphorus in the intermediary metabolism, an enzyme involved in protein-phosphorus transfer must be of considerable interest. The properties and occurrence of such an enzyme seem, however, to have been very little investigated.

In 1949 Feinstein and Volk [3] presented a study on phosphoprotein phosphatase in mammals. The evidence brought forward by Feinstein and Volk for the existence of this hydrolase as a catalyst sui generis is, however, unsatisfactory. They only state that more phosphorus is liberated when casein is added to a tissue homogenate incubated with excess of glycerophosphate. The independence of the phosphate liberation from non-protein nitrogen formation, *i. e.* from proteolytic activities, on the other hand, seems to be well established. Furthermore, Feinstein and Volk used ascorbic acid as an activator for the enzyme, whereas ascorbic acid, as shown below, is actually an inhibitor. There are thus several reasons for further investigations of the phosphoprotein phosphatase. The object of the present study was to establish the existence of phosphoprotein phosphatase in rat tissues as an independent enzyme, and to investigate its distribution in different organs. To this end a method of assay was first developed and some properties of the enzyme studied.

EXPERIMENTAL

Estimation of the enzyme activity

In preliminary experiments the technique of Feinstein and Volk [3] was followed, although the substrate was prepared from a commercial casein according to Hammarsten. It was, however, impossible to get even and reproducible results. Isolation of the liberated phosphate before the estimation was therefore tried. This was accomplished

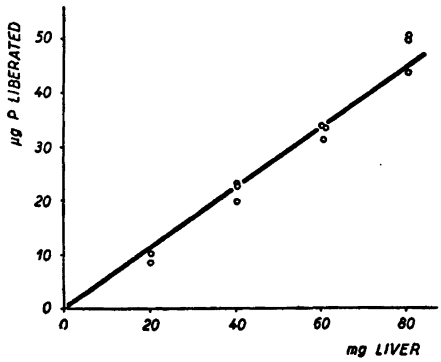

Fig. 1. Proportionality between enzyme quantity and amount of phosphorus liberated.

by precipitation with calcium (as hydroxyl apatite), slightly modified according to Fiske and Subbarow [4].

The following general technique was then used:

Four ml casein solution of pH 5.8 was preheated in a wide test-tube at the temperature of digestion, usually 37° C. One ml of the sample was added. Ten minutes later the reaction was stopped with 10 ml normal trichloracetic acid. Controls with casein and water were also made, and blank values with water and sample were run parallelly. Seven and a half ml of the protein-free filtrate was pipetted into a conical centrifuge tube. The aliquot was neutralized with 5 *N* and 0.5 *N* sodium hydroxide against brom thymol blue. Finally 2 ml 10 % calcium chloride in 0.5 *M* ammonium chloride buffer pH 9 was added. After 30 minutes the calcium phosphate was centrifuged down and washed once with 5 ml of a 1 : 5 dilution of the precipitating reagent. The precipitate was suspended in a few ml 0.05 *N* sulfuric acid and the phosphate determination with the Fiske-Subbarow reaction performed (see Norberg [11]). The amount of phosphorus was obtained from standard phosphate series precipitated in the same way.

The enzyme units are calculated as the number of micromols of phosphorus liberated per minute under the experimental conditions. The enzyme units were as a rule calculated per g of fresh tissue.

Source of enzyme. The organ to be studied was homogenized in 10 to 25 parts of 0.05 *M* acetate buffer of pH 5.8 with the technique of Potter and Elvehjem [14]. The opaque extract after centrifugation or filtration was used. All studies on the properties of the enzyme were made with fresh rat liver extracts.

Proportionality between the amount of enzyme and micrograms of phosphorus liberated was found to be satisfactory over a wide range. For liver up to 80 mg this emerges from Fig. 1. In other experiments proportionality was obtained up to 200 mg of liver. But sometimes the activities came out too low even with 80 mg of liver possibly due to influence of inorganic phosphate. It is therefore advisable to use the equivalent of about 50 mg liver or less. The standard deviation calculated from duplicates was 0.078 units per g liver. All values in this report are means of agreeing duplicates or triplicates. Thus their standard error is less than 0.055.

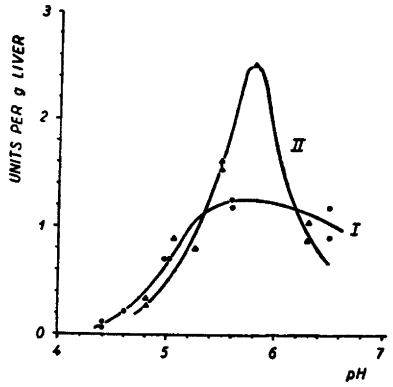

Fig. 2. Optimum pH for phosphoprotein phosphatase.

Properties of phosphoprotein phosphatase

Optimum pH was tested both with the method of Feinstein and Volk and with the new technique. The pH optimum is around pH 5.8, as is evident from Fig. 2. The peak was, however, not always so sharp as in the experiment reproduced in curve II.

Influence of the substrate. As standard substrate a commercial casein (Vitrum A. B., Stockholm) prepared according to Hammarsten was used. About 120 g of the air-dry substance was stirred with 800 ml redistilled water. About 25 ml normal sodium hydroxide was added and the pH adjusted to 5.8. This may be accomplished by dialysis against 0.05 *M* acetate buffer of pH 5.8 in the cold room, which is of advantage if the casein contains significant amounts of free phosphate. The organically bound phosphorus was estimated after precipitation with trichloracetic acid and digestion with sulfuric acid and nitric acid [13]. Finally, the substrate was diluted to an organic phosphorus content of 20 m*M*.

With this suspension the substrate concentration-velocity curve was studied. The results are shown in Fig. 3. Treatment of the values according to Lineweaver and Burk [8] gave a limiting velocity of 3 micromols of phosphorus liberated per minute per g liver and a Michealis-Menten constant of 3 m*M* casein phosphorus.

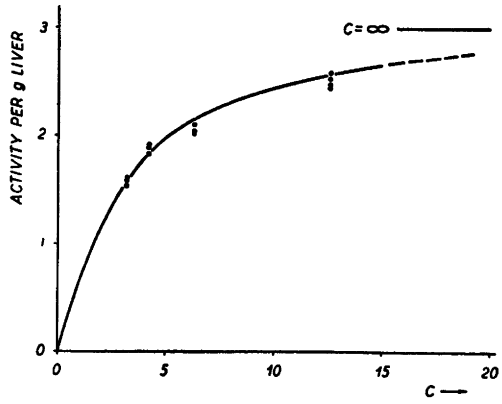

Fig. 3. Substrate concentration-velocity curve for phosphoprotein phosphatase with casein as substrate.

Table 1. Liberation of phosphorus from phosphovitin by phosphoprotein phosphatase from 50 mg liver at 37° C.

Phosphovitin phosphorus, m*M*	16	32	48	64
Phosphatase, units per g	0.715	1.15	1.16	1.15

From Fig. 3, however, it appears that the enzyme is not wholly saturated with substrate at casein concentrations corresponding to 15—20 m*M* protein phosphorus. Also the substrate is not homogeneous. It therefore appeared of interest to test phosphopeptones prepared from casein, as these are highly soluble. Calcium phosphopeptone according to Mellander [10] was dissolved in water and the calcium ion exchanged for sodium with zeocarb ion exchanger saturated with sodium. After dialysis against a 0.05 *M* acetate buffer of pH 5.8, the final concentration of phosphorus precipitable with trichloracetic acid was 7.5 m*M*. In eight experiments there was no liberation of phosphorus with phosphopeptone as substrate, whereas the activity towards casein was from 1.56 to 3.22 units per g liver. As traces of lead from the preparation or other toxic ions might disturb the enzyme activity, phenanthrolin to a final concentration of 0.4 m*M* was added in two experiments. No effect, however, was observed. Only in one experiment was phosphorus split off from phosphopeptone; but unfortunately there was a big difference between the two phosphorus values, and no conclusion can be drawn in this case. The overall results suggest that the enzyme can not attack casein after a profound proteolytic degradation.

As it seemed necessary to verify the ability of the enzyme to hydrolyze other phosphoproteins, phosphovitin was tested. Phosphovitin contains most of the protein-bound phosphorus in egg yolk. It was prepared according to Mecham and Olcott [9]. In the final dialyzed solution of phosphovitin the content of organically bound phosphorus was 54

Table 2. Activity of liver phosphoprotein phosphatase with different substrates in units per g liver. Protein phosphorus 20 mM in casein and 64 mM in phosphovitin (54 mM in preparation no. II).

Phosphovitin	Casein	Phosphovitin	Activity with casein / Do. with phosphovitin	Liver no.
No. I	2.31	1.64	1.41	25
	1.55	0.996	1.56	37
	1.94	1.20	1.62	38
	2.20	1.53	1.44	37 R
	1.91	1.16	1.65	38 R
No. II	3.03	2.66	1.14	47
	3.01	2.42	1.24	73
	3.11	2.64	1.18	74

Table 3. Course of digestion. Activity in units per g fresh liver.

Digestion time minutes	Extract 17 at 30° C	Extract 24 at 40° C
5	2.89	1.62
10	2.97	1.66
15	—	1.54
20	1.86	1.52
30	1.50	1.16

Table 4. Stability of phosphoprotein phosphatase from two liver samples at different pH values. Activities in units per g fresh liver.

Time from excision of liver, minutes	pH of homogenate			pH of extract		
	5.0	5.85	7.25	5.0	6.10	6.50
30	2.28	2.50	2.30	0	1.90	2.38
90	2.04	2.52	3.03		2.30	2.05
240	1.72	2.81	2.52	0	1.20	0

Table 5. Heat inactivation of two phosphoprotein phosphatase extracts. Assay at 37 C.°

Time of heating, minutes	At 37°		At 60°	
	Units/g	% inactivation	Units/g	% inactivation
0	2.35		2.00	
2	2.31	2	0.81	59
8	2.19	7	0.45	77
16	2.08	11.5	0.43	78
32	2.12	9.5		

and 64 m*M*. The nitrogen/phosphorus quotient should be 1.23 [9]. In the two preparations used it was 1.62 and 1.75, however, indicating a nitrogenous impurity. The solutions were tried, however, without further purification. As shown in Table 1, the enzyme seemed to be saturated with substrate at 32 m*M* of protein phosphorus. The activity was, however, always lower than with casein. This was tested on a number of different liver extracts. From table 2 it is evident that the activity was about 50 percent higher with casein as substrate than with phosphovitin for the first preparation and about 20 percent higher for the second preparation.

Table 6. Influence of different substances on the activity of phosphoprotein phosphatase. The figures denote the average percentual deviation from the activities under standard conditions.

Substance	Concentration in digest					
	0.01	0.1	1	5	10	m*M*
Ascorbic acid			− 2.5	− 12	− 32	
Iodoacetic »			− 5		− 70	
Na_2S		+ 8	+ 18		0	
Cystein. HCl			− 16		− 38	
$CuSO_4$	− 17	− 80	− 71		− 88	
Tocopheryl phosphate	+ 6	− 15	+ 6			
		+ 87	+ 40			
	0.02	0.2	2	20	m*M*	
KCN	0	+ 10	+ 7	− 40		
NaF	− 7	− 9	− 53	− 87		
$MgSO_4$			− 6	− 4		
Phenanthrolin		+ 9	+ 6			
	0.1	0.2	0.4	m*M*		
KH_2PO_4	− 8	− 27	− 40			
	1.0	6.7	10.0	26.7	mg per liter	
Heparin	− 8		+ 57			
Bayer 205	− 25		− 30			
Protamine sulfate		+ 2		+ 2		

The course of digestion was studied at two temperatures with several extracts. As examplified in Table 3, the decrease in reaction velocity was insignificant for the first ten to fifteen minutes. Digestion for ten minutes was therefore adopted as the standard period.

The stability of the enzyme was studied at different pH values. Homogenates were prepared in 0.1 *M* buffers in the cold, and samples taken for assay at different times after excision of the liver. As appears from Table 4, there is good stability between pH 5.8 and neutral reaction, whereas at pH 5 a marked drop in activity occurs after a time. When these experiments were repeated with extracts the activity was zero at pH 5 at once, at pH 6 to 7 full activity was retained for 90 minutes, but at 4 hours there was no

enzyme action in the neutral extract although about 50 % of the original activity was left in the pH 6 extract. In order to minimize spontaneous inactivation, all extracts were therefore made with cold 0.05 *M* acetate buffer of pH 5.7, and were used within half an hour. The pH of such extracts was 6.0 to 6.2.

Heat inactivation was studied at 60° and 37° C at pH 6. As appears from Table 5, the activity is little influenced at 37°. At 60°, however, the inactivation is fairly strong. After 2 minutes, when the temperature in the extract only attained 56°, as much as 50 % inactivation had occurred. After 4 minutes only about one fourth of the activity was left, but this amount of the enzyme seems to remain active even after a quarter of an hour at 60°.

The temperature coefficient for the hydrolytic action was determined through assay at 30 and 40° C or 27 and 37° C. The average value of ten experiments was 1.70 ± 0.09.

Inhibitors. Feinstein and Volk[3] found moderate activation through Mg, Ba, Ca, and Mn ions. But with 0.01 *M* ascorbic acid they had a considerable activation of + 50 to + 240 %. The ascorbic acid was, moreover, necessary to get proportionality in their assay. In my experiments, on the contrary, ascorbic acid was clearly an inhibitor, as is evident from Table 6. Also with respect to cyanide and cystein I found inhibition when Feinstein and Volk had activation. At low concentrations, however, cyanide and also sodium sulfide and hydroxylamine were slightly activating. Iodoacetate was found to be a strong inhibitor. In agreement with Feinstein and Volk, copper and fluoride ions were found to be very strong inhibitors. In conformity with the inhibitory effect of copper a slight activation resulted from the addition of complex-forming substances like phenanthrolin and diethyldithiocarbamate. Magnesium sulfate had no activating effect.

A possible inhibition through one of the split products was tested by addition of small amounts of potassium phosphate to the substrate. The inhibitory effect is obvious from the table.

Although the phosphoprotein phosphatase seems to act without any connection with proteolytic enzymes, which according to Zierler, Grob and Lilienthal[16] are inhibited by alpha tocopheryl phosphate, the latter substance was tested. As appears in the table, no effect was obtained in some series but a fairly strong activation with other extracts. This different behaviour might depend on a phenomenon similar to the balance between inhibition of alkaline phosphomonoesterase by estradiol diphosphate and restoration of its activity by tocopheryl phosphate demonstrated by Aldman, Diczfalusy, Ingelman-Sundberg, and Rosenberg[1]. If the activation, on the other hand, depends on inhibition of proteolytic activity through the tocopheryl phosphate other inhibitors of proteolysis might activate the phosphoprotein phosphatase as well. This was tested with heparin which, in fact, was activating in some experiments. Bayer 205 which as a polysulfonic acid substance has anticoagulant activity was also tested. No activation occurred, however. Finally, the heparin antagonist protamin was tested. It had no effect.

Distribution of phosphoprotein phosphatase

Liver homogenate at pH 6 was centrifuged for 5 minutes at 1400 times gravity. The opalescent extract and a suspension of the precipitate (without washing) were assayed at pH 5.8 for phosphomonoesterase with phenyl phosphate and magnesium activation, and for phosphoprotein phosphatase. The results are given in Table 7. Obviously, the phosphoprotein phosphatase is more soluble than the phosphomonoesterase, which fol-

Table 7. Distribution of phosphatases between solution (extract) and granulate matter that settles at 1 200—1 400 g in 5 minutes at pH 6—6.2 (precipitate). Units per g liver.

Date April		14	17	18	20	20 : 2
Phosphoprotein phosphatase	Precipitate	0.56	0.83	0	0	0.32
	Extract	2.60	2.91	1.87	3.52	1.84
Phosphomono-esterase	Precipitate	3.90	6.45	2.72	4.80	2.94
	Extract	5.17	4.77	3.47	3.88	2.37

lows the precipitate to a considerable extent. The phosphoprotein phosphatase activities on a homogenate and on the extract prepared therefrom were identical. Therefore the investigations on different organs were all made with extracts. The results are collected in Table 8.

The highest activities were found in the spleen. The relative activity was, however, lower than in the experiments of Feinstein and Volk [3]. Thus, as compared with the relative activity of liver taken as 100, the activity of spleen varied from 50 to 214 with a mean value of 126, whereas the values of Feinstein and Volk correspond to over 300.

Table 8. Distribution of phosphoprotein phosphatase in different organs. Digestion at 37° C for 10 minutes.

Tissue	Enzyme units per g fresh weight							Average	Relative activity
Liver	1.87	2.18	2.37	2.64	2.91	3.10	3.74	2.69	100
Adrenals	1.55	1.12	1.82	2.17			3.08	1.95	75
Blood clot	0.32			0.82			0.45	0.53	20
Bone marrow	0	1.62		1.08				0.90	36
Brain		1.55		1.16	1.88			1.53	60
Gastric mucosa			1.67	1.28			2.28	1.74	60
Heart muscle				0.71		1.42	1.70	1.28	39
Skeletal „		0		1.32	1.76			1.02	38
Kidney		1.33	1.94	2.25		4.21		2.43	96
Lungs		0.51	1.28			3.30	2.36	1.86	62
Lymph nodes	0	1.80		1.71				1.17	49
Mammary gland		0.94	0.84	1.15				0.98	41
Ovary				2.64	1.60		1.95	2.06	69
Pancreas	0.82		0.75			0.72		0.76	32
Salivary gland	0.58	0	1.83	3.86		0.47		1.35	54
Spleen	0.93	4.67	2.65	4.09	2.80	5.48	2.84	3.35	126
Testicle		2.16	1.90					2.03	90
Thymus			2.56	2.29		2.58		2.48	93

Other lymphoid organs have lower activity, although thymus is fairly high. With a view to the possible connection between phosphoprotein phosphatase and protein formation, the activity in organs which produce enzymes or other proteins is of great interest. As is evident from Table 8, the values were relatively low in salivary glands, gastric mucosa, pancreas, and mammary glands from lactating rats, whereas the liver had a high activity. Some samples of salivary glands, however, had a considerable activity. This might be due to differences in the stimulation. In two experiments, therefore, pilocarpine in a dose of 1 and 2 mg per kg was injected subcutaneously. The rats were killed 4 and 20 hours after the injection. The relative phosphoprotein phosphatase activity was about 70 for salivary glands, gastric mucosa, and pancreas, indicating an increased activity after secretory stimulation.

In brain and adrenals, as well as the sex glands and kidneys, the activity was found to be fairly high. Low activity was found in heart and skeletal muscle, as well as in red bone marrow and erythrocytes. Blood serum has practically no activity. Thus on the whole the phosphoprotein phosphatase was found to have a very wide distribution. The distribution as such therefore gives no hint as to the specific functions of this enzyme.

DISCUSSION

In the pH-optimum region of the phosphoprotein phosphatase also other enzymes are active which liberate inorganic phosphorus. The phosphomonoesterases I and II in the scheme of Folley and Kay [5], the adenylpyrophosphatase of Jacobsen [7], neutral pyrophosphatase [12], and glucose-6-phosphatase [2, 15] must be considered. The phosphoprotein phosphatase is, however, different from these enzymes in certain respects. This will be discussed presently with regard to liver, where ambiguity is most likely to occur.

Phosphomonoesterases active at pH 5.8—6 are rapidly destroyed by heat at this pH [7], whereas phosphoprotein phosphatase is comparatively resistant, Table 5. Moreover, the pH-optima are so different, and the extractability and precipitability are in such contrast (Table 7), that separate enzymes must be assumed.

Neutral pyrophosphatase is also less heat-resistant [7] than phosphoprotein phosphatase. Moreover, this enzyme is strongly activated by magnesium [12], which has no effect on the phosphoprotein phosphatase.

Adenyl pyrophosphatase has a very flat pH-optimum with the peak at pH 7.2. This enzyme is also most stable in the faintly alkaline region. But even at pH 6 it is fairly resistant, retaining about 50 % activity after 20 minutes at 55° C. The different pH-optimum and the greater stability at pH 6 than at pH 7 suggest that the phosphoprotein phosphatase is different from Jacobsen's enzyme.

Glucose-6-phosphatase has recently been investigated by de Duve, Berthet, Hers, and Dupret [2] and by Swanson [15]. They found the pH-optimum at pH 6 or possibly lower. The enzyme is, however, very labile in acid medium and

more stable at pH 8. The heat lability and pH sensitivity differentiates this enzyme from phosphoprotein phosphatase. Furthermore, glucose-6-phosphatase activity has only been found in liver, kidney and intestine [2], whereas phosphoprotein phosphatase is present in most tissues, Table 8.

The evidence put forward in the present study together with known properties of phosphatases seem to justify the assumption that the phosphoprotein phosphatase activity belongs to a specific enzyme. The wide distribution of this enzyme suggests general metabolic functions.

SUMMARY

Phosphoprotein phosphatase is an enzyme which liberates inorganic phosphorus from casein and other phosphoproteins. In the present study a method of assay has been developed. The properties and the distribution of the enzyme in rat tissues have been investigated. Comparison with known properties of other phosphatases shows that phosphoprotein phosphatase is an enzyme sui generis.

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