the period of active photosynthesis and growth, and the enhanced level is maintained until the following spring. In the older trees, this progressive increase in tannin content with increase in age is balanced by increase in the dead and living bark elements, with the result that the level remains without apparent variation throughout the season. This suggests that the tannin undergoes further chemical changes in the dead elements of the outer bark.

3. The effect of ringing experiments on the normal distribution of tannin in the stembark of mature trees shows an accumulation of tannin above the girdle, suggesting that synthesis has taken place from phenolic precursors which are normally translocated downwards from the leaves.

4. Examination of the exudate obtained by tapping the sieve-tube system of growing trees shows the presence there of many of the phenolics found in the bark, including the pyrogallol precursors [(+)-gallocatechin and leucodelphinidin] of oak-bark tannin.

5. The evidence suggests that these pyrogallol phenols, which originate in the leaves, are translocated by the sieve-tube system to the cambium and undergo oxidation there, and the resulting phlobatannin is stored in the bark.

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Substrate Specificity of Phosphoprotein Phosphatase from Spleen

By T. A. SUNDARARAJAN AND P. S. SARMA
University Biochemical Laboratory, Madras-25, India

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Recent investigations from several Laboratories have revealed the presence, in mammalian tissues, of an enzyme system for the dephosphorylation of phosphoproteins, which appears to be distinct from phosphomonoesterases (Feinstein & Volk, 1949; Norberg, 1950; Mattenheimer, 1953; Thoai, Roche & Pin, 1954; Sundararajan & Sarma, 1954, 1957). Evidence for the specific nature of the enzyme has largely been based on its inability to split β -glycerophosphate, a typical phosphomonoester substrate. A detailed study of the substrate

specificity of this enzyme is warranted in view of the recent observation of Perlmann (1955) that phosphoproteins containing phosphomonoester linkages are susceptible to dephosphorylation by phosphomonoesterases. The present paper describes the results of an investigation on the specificity of highly purified preparations of phosphoprotein phosphatase from ox spleen towards a number of substrates representing different types of phosphorus linkages. As a parallel study, the specificity of a partially purified preparation of the enzyme

from rat spleen has also been investigated. These studies indicate that the mammalian enzyme possesses a wide substrate specificity, since it readily dephosphorylates a variety of compounds containing pyrophosphate linkages and also hydrolyses the phosphomonoesters of phenol and *p*-nitrophenol.

While the manuscript of this paper was in preparation, the attention of the authors was drawn to a publication of Hofman (1958), who reported essentially similar results with a partially purified enzyme from ox spleen. The present paper provides evidence of a more conclusive nature for the identical characteristics of the enzyme system dephosphorylating the various substrates.

MATERIALS AND METHODS

Enzymes. Purification of phosphoprotein phosphatase from ox spleen was effected by a modification of the procedure published earlier (Sundararajan & Sarma, 1954) and the details are given below. Rat-spleen phosphatase was prepared according to Sundararajan & Sarma (1957).

Protein substrates. Casein was prepared by the method of Van Slyke & Baker (1918). The preparation of β -casein was carried out according to Hipp, Groves, Custer & McMeekin (1952), with urea as fractionating agent. Phosvitin was prepared from egg yolk according to the method of Mecham & Olcott (1949). Phosphopeptone was prepared from β -casein by digestion of the protein with crystalline trypsin (Worthington Biochemical Co., N.J., U.S.A.) according to the method of Peterson, Nauman & McMeekin (1958). The preparation thus obtained appeared to be electrophoretically homogeneous.

Phosphomonoesters. Substrates employed for the detection of phosphomonoesterase activity were: β -glycerophosphate (British Drug Houses Ltd.); phenyl phosphate (disodium salt), *p*-nitrophenyl phosphate (disodium salt) and phenolphthalein phosphate (which were generous gifts of Sigma Chemical Co., St Louis, Mo., U.S.A.); *O*-phosphorylserine, kindly supplied by California Foundation for Biochemical Research, Los Angeles, U.S.A.; glucose 1-phosphate (dipotassium salt), glucose 6-phosphate (barium salt), fructose 6-phosphate (barium salt) and fructose 1:6-diphosphate (barium salt), all purchased from Schwarz Laboratories Inc., New York, U.S.A.

Pyrophosphate esters. Sodium pyrophosphate (Bakers Analysed, J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.) was employed for detection of pyrophosphatase activity. Adenosine triphosphate (ATP) was obtained as the disodium salt from Pabst Laboratories, Milwaukee, U.S.A. Thiamine pyrophosphate hydrochloride was a product of Hoffman-La-Roche and Co., Basle, Switzerland.

Phosphoamides. Creatine phosphate and *N*-phosphoryl-DL-phenylalanine methyl ester were kindly supplied by Dr Si-Oh-Li.

Phosphodiester. Diphenyl phosphate, a generous gift from HM Chemical Co., Calif., U.S.A. and *p*-bis(nitrophenyl) phosphate (calcium salt), kindly supplied by Sigma Chemical Co., were used as phosphodiester substrates.

When the barium salts of substrates were used, the barium was quantitatively removed as barium sulphate.

All substrate solutions were adjusted to the pH of the incubation mixture before use.

Buffer. Sodium acetate-acetic acid buffer (0.05M) was used throughout the studies (Walpole, 1914).

Determination of phosphatase activity. Methods for the determination of phosphatase activity and definition of unit of enzyme activity are as described earlier (Sundararajan & Sarma, 1954). Phosphorus determinations were carried out according to the method of Fiske & Subbarow (1925). The method of Lowry & Lopez (1945) was used for measurement of phosphoamidase activity. Phosphodiesterase activity was determined by estimation of the liberated phenol according to King (1947).

Determination of protein nitrogen. This was determined by digestion of the protein with H_2SO_4 and estimating the ammonia titrimetrically (Ma & Zuazaga, 1942) or by the nessler reaction (Koch & McMeekin, 1924). The colorimetric method of Lowry, Rosebrough, Farr & Randall (1951) was used for measurement of low amounts of protein.

Purification of phosphoprotein phosphatase from ox spleen

The method of Sundararajan & Sarma (1954) was modified in several respects to get preparations of high specific activity. The use of a buffer at pH 5 for rendering the inactive proteins of spleen insoluble, and of manganese pyrophosphate gel for adsorption of the enzyme, constituted two of the important steps introduced in the following modified procedure.

Extraction of the enzyme. A weighed amount of minced spleen was homogenized with 2.5 vol. of 2.5% (w/v) NaCl soln., buffered at pH 5.0 with 0.2M-acetate buffer. The homogenate was centrifuged at 2000 rev./min. for 15 min. and the supernatant was filtered on a Büchner funnel, with 10g. of Celite L 665/l. as filter aid. (L 665 was obtained from Fisher Scientific Co., U.S.A.) (Table 1, stage 1.)

Fractionation with ammonium sulphate. The clear filtrate was treated with solid $(NH_4)_2SO_4$ to 0.5 saturation (the amount required for bringing solutions to any desired saturation level being calculated from the formula of Kunitz, 1952). After allowing the solution to stand overnight in the ice chest, it was centrifuged and the supernatant filtered through a layer of Celite on a Büchner funnel. The clear amber-coloured filtrate was treated with more $(NH_4)_2SO_4$ to 0.85 saturation. The solution, after storage at 0° overnight, was filtered on a large Büchner funnel. When the filter cake had been sucked almost dry, it was removed from the paper, suspended in water and dialysed at 5° against several changes of distilled water. After dialysis the precipitated protein was separated by centrifuging and extracted with 2.5% (w/v) NaCl soln. at pH 5.0 (stage 2).

Adsorption on manganese pyrophosphate. The clear extract obtained from the previous step was adsorbed on manganese pyrophosphate gel. The adsorbent was prepared by the gradual addition of 0.1M-sodium pyrophosphate to 2 vol. of 0.1M-manganous sulphate which was kept well stirred during the addition. The ppt. was allowed to settle and the supernatant removed by decantation. The gel was repeatedly washed with 1% (w/v) NaCl soln. and finally filtered on a Büchner funnel without applying suction. The ppt. was removed and suspended uniformly in water. Only freshly prepared gels were used since those allowed to age at room temperature or in the ice chest were inefficient as adsorbents. For adsorption studies, 1 vol. of the enzyme

solution containing about 10 mg. of protein/ml. was mixed with an equal volume of the gel (6.5 mg. of total solids/ml.). Enough water was added to bring down the concentration of NaCl to 1%. The suspension was stirred for 2 min. and centrifuged. The gel was washed once with 1% NaCl at pH 5. Elution of the enzyme was carried out by taking up the residue in 0.2 saturated $(\text{NH}_4)_2\text{SO}_4$, and after gentle stirring for 5 min. at room temperature the suspension was centrifuged (stage 3).

Refractionation with ammonium sulphate. The eluate from manganese pyrophosphate gel was subjected to refractionation with $(\text{NH}_4)_2\text{SO}_4$. The ppt. separating between 0.58 and 0.85 saturation was collected by centrifuging and was extracted with NaCl soln. (stage 4).

Fractionation with acetone. The extract from the previous step was subjected to fractionation with acetone, according to the procedure outlined earlier (Sundararajan & Sarma, 1954). The active fraction, separating between 50 and 66% (v/v) concentration of acetone, was separated and extracted with saline (stage 5).

The purification achieved in a typical fractionation experiment is outlined in Table 1. The final fraction, which represented a 600-fold purification over the initial spleen extract, was used as source of enzyme in specificity studies.

RESULTS

Substrate specificity of phosphoprotein phosphatase

The action of purified preparations of the enzyme from spleen on several phosphate esters is summarized in Table 2. The action of the enzyme on each substrate was studied at various pH values ranging from 4 to 6. The values given in the table represent the hydrolysis observed at the optimum pH for each substrate.

Dephosphorylation of phosphomonoesters. Aliphatic phosphate esters were not hydrolysed to any

Table 1. *Purification of phosphoprotein phosphatase from ox spleen*

Activities were tested with casein (5 $\mu\text{g. atoms of P/ml.}$) as substrate and thioglycollic acid (mm) as activator. Incubations were carried out at 37° and at pH 5.8 for a period of 15 min. Details of stages of purification are given in the text.

Stage no.	Volume (ml.)	Activity (units/ml.)	Protein N (mg./ml.)	Specific activity (units/mg. of N)	Yield (%)
1	1790	14.7	4.30	3.4	100
2	60	322	1.34	240	74
3	80	140	0.14	1000	43
4	20	224	0.14	1600	17
5	20	217	0.11	2000	16

Table 2. *Substrate specificity of spleen-phosphoprotein phosphatase*

The substrates, at the indicated concentrations, were incubated with the enzyme preparation for 60 min. at 37° with thioglycollic acid (mm) as activator. Activities were tested at pH 5.8 for casein and at pH 5.0 for the other substrates, except that with ox-spleen phosphatase acting on phenyl phosphate, the pH of the medium was 4.5.

Substrate	Concn. of substrate ($\mu\text{g. atoms of P/ml.}$)	Phosphorus released ($\mu\text{g. atoms of P/ml.}$)	
		Rat-spleen phosphatase	Ox-spleen phosphatase
Casein	5.0	1.75	1.25
Phosphomonoesters			
β -Glycerophosphate	5.0	Nil	Nil
O-Phosphorylserine	5.0	Nil	Nil
Glucose 1-phosphate	2.5	0.01	Nil
Glucose 6-phosphate	2.5	0.02	0.02
Fructose 1-phosphate	2.5	0.03	0.01
Fructose 1:6-diphosphate	2.5	0.06	0.02
Phenyl phosphate	5.0	1.56	1.43
p-Nitrophenyl phosphate	5.0	1.76	1.93
Phenolphthalein phosphate	5.0	0.09	0.09
Phosphoamides			
N-Phosphorylphenylalanine	1.6	*	0.53
Phosphocreatine	1.6	*	0.13
Pyrophosphates and phosphodiester			
Inorganic pyrophosphate	2.5	2.03	2.10
Adenosine triphosphate	2.5†	2.28	2.20
Thiamine pyrophosphate	2.5	*	1.20
Diphenyl phosphate	2.5	Nil	Nil

* Not tested.

† Concentration given corresponds to acid-labile phosphorus.

appreciable extent by the enzyme. In marked contrast, the phosphomonoesters of phenol and *p*-nitrophenol were dephosphorylated at a rapid rate. Maximum hydrolysis of phenyl phosphate occurred at pH 4.0–4.5 (Fig. 1), and there was a marked fall in activity below pH 4.0. Phenolphthalein phosphate, though an aromatic ester, was dephosphorylated at a much slower rate.

Dephosphorylation of phosphoamides. *N*-Phosphorylphenylalanine and phosphocreatine were dephosphorylated to an appreciable extent by the purified enzyme from ox spleen, indicating the presence of phosphoamidase activity in the

enzyme preparation. This finding agrees with that of Singer & Fruton (1957), who demonstrated the identical nature of phosphoprotein phosphatase and phosphoamidase of ox spleen.

Dephosphorylation of pyrophosphate and phosphodiester substrates. Compounds containing pyrophosphate linkages appeared to be very good substrates for the enzyme. Inorganic pyrophosphate and ATP were hydrolysed at comparable rates. Both the acid-labile phosphate groups of ATP were cleaved by the enzyme, whereas the α -phosphorus atom appeared to be resistant to enzymic action. This is in conformity with the finding of Hofman (1958) that ox-spleen phosphatase does not dephosphorylate adenosine 5'-phosphate. Thiamine pyrophosphate was also readily dephosphorylated, only the terminal phosphate being split off by the action of the enzyme. Maximum hydrolysis of the pyrophosphate substrates was observed at pH 5.0 (Fig. 1). The enzyme had no action on diphenyl phosphate, a diester substrate, even after prolonged incubation.

The close similarity in specificity characteristics, observed with enzyme preparations of different states of purity and derived from entirely different sources, suggested that the hydrolysis of the various substrates was mediated by a single enzyme or by a system of enzymes with similar properties. This was strikingly borne out by the observation that the ratio of the activities towards the phosphoprotein and pyrophosphate substrates remained more or less constant in the course of purification of the enzyme from ox spleen (Table 3). Singer & Fruton (1957) have, by a similar criterion, shown that the phenylphosphatase and phosphoamidase of ox spleen possess properties similar to those of phosphoprotein phosphatase. Evidence for the identical nature of the enzymes was also obtained from heat-denaturation and adsorption experiments as well as from studies on the behaviour of these enzymes towards several activators and inhibitors. Details of these studies are given below.

Fig. 1. Effect of pH on the dephosphorylation of ATP (x), pyrophosphate (O) and phenyl phosphate (●) by ox-spleen phosphoprotein phosphatase.

Table 3. *Hydrolysis of casein and pyrophosphates by active fractions from ox spleen*

Active fractions obtained at various steps during purification of phosphoprotein phosphatase were tested for their ability to dephosphorylate casein (20 μ g.atoms of protein P/ml.), inorganic pyrophosphate (20 μ g.atoms of P/ml.) and ATP (10 μ g.atoms of acid-labile P/ml.). Incubations were at 37° for 15 min. with thioglycolic acid (mM) as activator. Stages of purification of the enzyme are as in Table 1. One unit is the amount of enzyme catalysing the splitting of 1 μ g. of inorganic P/min. at 37°.

Stage no.	Activity (units/ml.)			Ratios	
	Casein	Pyrophosphate	ATP	Casein/pyrophosphate	Casein/ATP
1	14.7	40	23.3	0.4	0.6
2	322	547	287	0.6	1.1
3	140	420	200	0.3	0.7
4	224	760	200	0.3	1.1
5	217	533	145	0.4	1.5

Effect of heat-treatment on phosphatase activity. Samples of the purified enzyme from ox spleen were held in a water bath at 80° for various periods. At the end of the indicated time interval, the sample was chilled in ice and the activity estimated with casein and pyrophosphate as substrates. The loss in activity towards both the substrates was observed to be of the same order of magnitude (Table 4). The heat-treated preparations were also found to be active towards ATP and phenyl phosphate.

Adsorption studies. Calcium phosphate gel was found to be a poor adsorbent for the enzyme. The supernatant solution obtained after treatment with calcium phosphate exhibited activity against casein as well as pyrophosphate. Selective adsorption of phosphoprotein phosphatase on casein was tried by mixing a portion (0.2 ml.) of the enzyme from ox spleen with 2 ml. of 10% (w/v) solution of casein and precipitating the casein by addition of dilute acid (0.1N-HCl) to pH 4.8. The supernatant obtained by centrifuging was, however, found to be inactive towards all the four substrates. Similar results were obtained with manganese pyrophosphate gel, which adsorbed the protein phosphatase as well as pyrophosphatase. The eluates obtained after treatment of such gels with 0.2 saturated ammonium sulphate exhibited activity towards casein, pyrophosphate, ATP and phenyl phosphate.

Activation and inhibition studies. Thioglycolic acid at mM-concentration had a marked activating effect on the hydrolysis of the various substrates by the purified enzyme from ox spleen (Table 5). Sodium tungstate at a concentration of 10 μ M completely suppressed the hydrolysis of the various substrates. L-Tartrate at a concentration of 10 mM did not inhibit the hydrolysis of any of these substrates. Incidentally, this observation serves to differentiate the enzyme from the acid phosphatase of spleen which is sensitive to tartrate (Abul-Fadl & King, 1949). Enzyme preparations allowed to stand at pH 9.0 at room temperature for a period of 1 hr. were completely inactive against all the substrates.

Evidence for the participation of a single enzyme in the hydrolysis of the various substrates was also provided by the observation that the simultaneous presence of any two of the substrates in the incubation mixture caused mutual inhibition of their enzymic dephosphorylation. This is evident from the results presented in Table 6, which shows that *p*-nitrophenyl phosphate inhibits the hydrolysis of pyrophosphate and of phosvitin in a reciprocal manner. The dephosphorylation of the

Table 4. *Effect of heat-treatment on phosphoprotein phosphatase and pyrophosphatase from ox spleen*

Portions of the purified enzyme from ox spleen were held in a water bath at 80° for the indicated periods and the activity was determined against casein (20 μ g.atoms of P/ml.) and inorganic pyrophosphate (20 μ g.atoms of P/ml.). Incubation was at 37° for 30 min. and at pH 5.8 with thioglycolic acid (mM) as activator.

Period of heating (min.)	Phosphorus released (μ g.atoms/ml.)	
	With casein	With pyrophosphate
0	1.85	2.10
5	0.90	1.60
10	0.73	0.97
15	0.73	0.97

Table 5. *Effect of thioglycolic acid on hydrolysis of various substrates by phosphoprotein phosphatase*

Dephosphorylation of the indicated substrates by purified enzyme preparation from ox spleen was followed in the presence and in the absence of thioglycolic acid (mM-concn., when present). Incubation was carried out at pH 5.8 for 30 min. at 37°.

Substrate	Phosphorus released (μ g.atoms/ml.)	
	With activator	Without activator
Casein	2.0	0.3
Inorganic pyrophosphate	1.6	0.4
ATP	1.5	0.4
Phenyl phosphate	1.3	0.5

Table 6. *Mutual inhibition of hydrolysis of p-nitrophenyl phosphate, pyrophosphate and of phosvitin*

Purified enzyme preparation from rat spleen was incubated with the indicated substrates at concentrations corresponding to 5 μ g.atoms of P/ml. at 37° for 15 min. and at pH 5.0 with thioglycolic acid (mM) as activator.

Substrate	Phosphorus released (μ g.atoms/ml.) from		
	<i>p</i> -Nitrophenyl phosphate	Pyrophosphate	Phosvitin
<i>p</i> -Nitrophenyl phosphate	0.93	—	—
Pyrophosphate	—	1.40	—
Phosvitin	—	—	0.28
<i>p</i> -Nitrophenyl phosphate + phosvitin*	0.50	—	0.10
<i>p</i> -Nitrophenyl phosphate + pyrophosphate*	0.68	0.70	—

* Phosphorus contributed by *p*-nitrophenyl phosphate was calculated from the amount of *p*-nitrophenol formed, as estimated according to Sinsheimer & Koerner (1952). This value was deducted from the total phosphorus to give the amount of phosphorus formed from the other substrate.

Table 7. *Effect of phosphodiester substrate on hydrolysis of β -casein by spleen phosphatase*

The influence of *p*-bis(nitrophenyl) phosphate on hydrolysis of β -casein and β -casein phosphopeptone by rat-spleen phosphatase was studied. The substrates at a concentration corresponding to 1.25 μ g.atoms of P/ml. were incubated with the enzyme at 37° for 15 min. with thioglycollic acid (mM) as activator. The pH of the medium was 5.8 for casein and 5.0 for the peptone.

Substrate	Concn. of diester (μ g.atoms of P/ml.)	Phosphorus released (μ g.atoms/ml.)	Inhibition (%)
β -Casein	Nil	0.25	—
β -Casein	1.25	0.25	Nil
β -Casein	5.0	0.18	28
β -Casein peptone	Nil	0.13	—
β -Casein peptone	5.0	0.13	Nil
β -Casein peptone*	Nil	0.55	—
β -Casein peptone*	5.0	0.55	Nil

* Incubation time 1 hr.

Table 8. *Hydrolysis of phosphopeptone from β -casein by spleen phosphatase*

Purified enzyme from rat spleen was incubated with the substrates at a concentration corresponding to 1.25 μ g.atoms of P/ml. for 60 min. at 37° with thioglycollic acid (mM) as activator. Values for phosvitin are given for purpose of comparison.

Substrate	pH of medium	Dephos-phorylation (%)
β -Casein	5.0	17
	5.8	76
β -Casein peptone	4.5	45
	5.0	27
	5.8	2
Phosvitin	4.5	27
	5.0	45
	5.8	57

last-named substrates was not affected by the presence of *p*-nitrophenol in the medium. The results show that these substrates inhibit each other by competition for the same active centre on the enzyme surface.

*Dephosphorylation of β -casein by spleen phosphatase**

Perlmann (1954*a*) has recently achieved a step-wise dephosphorylation of β -casein by incubation of the protein with phosphodiesterase from snake venom followed by treatment with a phosphomonoesterase. In view of the non-specific nature of spleen phosphatase it was of interest to investigate the mechanism of dephosphorylation of β -casein by this enzyme with reference to the possible participation of a phosphodiesterase in this reaction. Substrate-specificity studies indicated, however, the virtual absence of phosphodiesterase activity in

* These investigations were carried out recently during the tenure of a Post-Doctoral National Research Fellowship awarded to one of us (T. A. S.) by the Ministry of Education, Government of India.

purified phosphatase preparations from spleen (Table 2). Evidence against the participation of diesterase in this reaction was also provided by the observation that the dephosphorylation of β -casein was not appreciably affected by the presence of a large excess of a phosphodiesterase substrate in the incubation mixture (Table 7). Similar results were obtained when β -casein was replaced by a phosphopeptone from β -casein which represents, according to Peterson *et al.* (1958), the phosphorus-containing core of the protein.

The action of phosphoprotein phosphatase on phosphopeptone is shown in Table 8. Earlier experiments, carried out with non-homogeneous preparations of the peptone, indicated that this substrate was resistant to dephosphorylation at pH 6.0 (Sundararajan & Sarma, 1954). The results with β -casein peptone confirm this observation and show besides that the peptone has a lower pH optimum as compared with the parent protein. The values obtained with β -casein and its degradation product are not strictly comparable in view of the limited solubility of the protein substrate below pH 5.5. It was hence considered of interest to investigate the true pH optimum for protein-dephosphorylation with phosvitin as substrate since this protein remains soluble over the entire range of pH used in these studies. From the results presented in Table 8 it is evident that phosvitin is more akin to casein than to phosphopeptone in its dephosphorylation behaviour. The reason for the shift in pH optimum observed with the peptone is not clear.

DISCUSSION

The results of the present investigation establish beyond question the non-specific nature of phosphoprotein phosphatase of mammalian origin. Thus the enzyme preparations exhibit high activity against several other substrates besides phosphoprotein, and this specificity pattern remains un-

altered even after extensive purification of the enzyme. Attempts to eliminate the pyrophosphatase and phenylphosphatase activity from the preparation through various techniques have proved uniformly unsuccessful. The results obtained in the course of these experiments indicate on the contrary that the dephosphorylation of the various substrates is mediated by a single enzyme. In view of these findings the enzyme must be considered to be distinct from the phosphoprotein phosphatases of frog eggs and of chick embryo, which appear to be strictly specific for phosphoprotein substrates (Harris, 1946; Foote & Kind, 1953).

The unusual specificity characteristics exhibited by spleen phosphatase poses a problem as to its classification in the scheme of Folley & Kay (1936). There appears to be little justification for classifying the enzyme as a phosphomonoesterase since it hydrolyses pyrophosphate esters as readily as it does phenyl phosphate and, besides, the two substrates appear to combine with the enzyme at a common active centre. Further, the enzyme differs from the common acid phosphatases in showing a high degree of specificity towards phenyl phosphate among the phosphomonoester substrates. Similar results have been reported recently by Singer & Fruton (1957) and by Hofman (1958). Judging from the specificity pattern of spleen phosphatase, it might appear that the phosphorus in phosphoproteins is linked to the tyrosine hydroxyl groups of the protein rather than to the aliphatic hydroxyl group. However, the presence of such linkages in casein was ruled out by the observation that the removal of phosphorus from the protein did not lead to any increase in the free phenolic groups of the protein, as tested with phenol reagent (Sundararajan, 1956). The virtual absence of tyrosine in phosvitin, a protein with the highest phosphorus content, may also be taken as an indication of the non-involvement of a tyrosine hydroxyl group in the binding of phosphorus in phosphoproteins.

The presence, in proteins, of phosphorus linkages other than the monoester type was first suggested by Perlmann (1954*a*) from her experiments on the enzymic dephosphorylation of β -casein. This protein was dephosphorylated by prostatic phosphatase only after a preliminary treatment of the protein with phosphodiesterase prepared from snake venom. It was hence suggested that the phosphorus in this protein was present exclusively as diesterified phosphate. The results of our experiments with β -casein do not, however, support this conclusion (Table 7). The presence of diester linkage in casein is made less likely by the observation that phosphatase preparations derived from other sources and essentially free from diesterase activity are also effective in bringing about ex-

tensive dephosphorylation of the protein (Mecham & Olcott, 1949; Sampath Kumar, 1958).

The high pyrophosphatase activity exhibited by spleen phosphatase is of interest in view of the recent report by Perlmann (1954*b*) on the presence of pyrophosphate linkage in α -casein. Employing crystalline yeast pyrophosphatase, she showed that about 20% of the phosphorus in α -casein was present as pyrophosphate. It is thus possible that the rapid dephosphorylation of casein by phosphoprotein phosphatase depends, in part, on the pyrophosphatase activity inherent in these preparations. In view of the non-specific nature of spleen phosphatase the results of the present studies cannot be considered as providing conclusive proof for the presence of pyrophosphate linkages in proteins. The use of purified enzymes strictly specific for phosphomonoester linkage may, however, be expected to throw more light on the possible occurrence of these types of phosphorus linkages in proteins. The recent work on the isolation and characterization of such enzymes from a variety of sources (Morton, 1955; Tsuboi & Hudson, 1955, 1956) appears encouraging in this respect.

SUMMARY

1. A method is described for preparing phosphoprotein phosphatase of high specific activity from ox spleen. The enzyme was purified 600-fold with a yield of 16%.

2. The enzyme was non-specific in its action. A number of substrates containing pyrophosphate linkage were readily dephosphorylated as also were the phosphomonoesters of phenol and *p*-nitrophenol.

3. Attempts to separate an enzyme specific for phosphoproteins proved unsuccessful. The evidence obtained indicated that a single enzyme was responsible for the dephosphorylation of the various substrates.

4. The purified enzyme from spleen had no diesterase activity and dephosphorylated β -casein readily even in the presence of an excess of phosphodiester. These results suggest the absence of phosphodiester linkages in β -casein.

5. The significance of these findings in relation to the nature of phosphorus linkages in casein is discussed.

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Nephelometric Determination of Elastase Activity and Method for Elastoproteolytic Measurements

BY ILONA BANGA, J. BALÓ AND MAGDOLNA HORVÁTH

*The First Department of Pathological Anatomy and Experimental Cancer Research
Medical University, Budapest*

(Received 15 April 1958)

Measurements on the pancreatic elastase activity are performed by different methods in different laboratories. Baló & Banga (1950) and Banga (1952) suggested a gravimetric method in which the fraction of insoluble elastin that has not been dissolved by the enzyme is determined. The same method was used by Lewis, Williams & Brink (1956). Hall (1955) and Hall & Gardiner (1955) used the biuret reagent described by Sols (1947) in determining the elastin dissolved by the elastase. The Folin-phenol reagent (Lowry, Rosebrough, Farr & Randall, 1951) was applied by Grant & Robbins (1957) to measure the proteolytic dissolution of elastin. Sachar, Winter, Sicher & Frankel (1955) measured the colour liberated during the elastolytic action of elastase on elastin which was previously stained with orcein. Robert & Samuel (1957) measured the elastolytic dissolution of 'azoelastin' by a spectrophotometric method and gave some data for the kinetics of elastolysis.

In this paper we describe a method by which the activity of different elastase preparations obtained in different laboratories may be compared. The

need for such a method is emphasized by the fact that the mechanism of elastolysis is far from being uniform and well understood.

The enzymic dissolution of elastin, i.e. elastolysis, is not a mere proteolysis. Banga (1951) stressed that no free amino groups are demonstrable by the formol method or the Van Slyke determination during the dissolution process. Hall, Reed & Tunbridge (1952) and Hall (1953) believe that a polysaccharide and a sulphate group are liberated. Hall & Gardiner (1955) performed quantitative measurements on the amount of liberated acid during elastolysis and considered the acid, in accordance with the original assumption of Hall *et al.* (1952), to be an organically bound sulphate. Since they held the view that the sulphate ion would be bound to mucopolysaccharide, they conceived the elastolysis to be mucolysis. Partridge & Davis (1955), on the other hand, demonstrated the formation of free α -amino groups with the fluorodinitrobenzene technique. This led them to suppose that elastase or one of its components is a proteolytic enzyme. These authors found no more