4. Albumin 'tailing' under the conditions described amounts to 3-5 % of the total albumin of normal serum.

5. A staining procedure is described which gives a linear relationship between protein and dye concentration.

6. The relationship of dye binding capacity to other properties of proteins commonly used to measure concentration varies more than twofold among different proteins.

7. Normal values for serum protein fractions measured by the technique described, and the results of a study of the reproducibility of the method under conditions of routine use, are presented.

The authors wish to thank Mr D. G. Krc for his assistance with scanning, and Mrs M. J. Neff for the nitrogen determinations.

REFERENCES

- Armstrong, S. H., Budka, M. J. E. & Morrison, K. C. (1947). *J. Amer. chem. Soc.* **69**, 416.
- Armstrong, S. H., Budka, M. J. E., Morrison, K. C. & Hasson, M. (1947). *J. Amer. chem. Soc.* **69**, 1747.
- Block, R. J., Durrum, E. L. & Zweig, G. (1955). *A Laboratory Manual of Paper Chromatography and Paper Electrophoresis*. New York: Academic Press.
- Brand, E. (1947). *Ann. N.Y. Acad. Sci.* **47**, 216.
- Conn, H. O. & Klatzkin, G. (1954). *Amer. J. Med.* **16**, 822.
- Cremer, H. D. & Tiselius, A. (1950). *Biochem. Z.* **320**, 273.
- Crook, E. M., Harris, H., Hassan, F. & Warren, F. L. (1954). *Biochem. J.* **56**, 434.
- Durrum, E. L. (1951). *J. Amer. chem. Soc.* **73**, 4875.
- Durrum, E. L. & Gilford, S. R. (1954). *Rev. Sci. Instrum.* **26**, 51.
- Durrum, E. L., Paul, M. H. & Smith, E. R. B. (1952). *Science*, **116**, 428.
- Esser, H., Heinzler, Fr., Kazmeier, F. & Scholtan, W. (1952). *Arzt. Forsch.* **6**, 156.
- Flynn, F. V. & de Mayo, P. (1951). *Lancet*, **2**, 235.
- Franglen, G. T. & Martin, N. H. (1954). *Biochem. J.* **57**, 626.
- Gabrieli, E. R., Goulian, D., Kinersly, T. & Collet, R. (1954). *J. clin. Invest.* **33**, 136.
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
- Grassmann, W. & Hannig, K. (1952). *Hoppe-Seyl. Z.* **290**, 1.
- Hardwicke, J. (1954). *Biochem. J.* **57**, 166.
- Köiw, E., Wallenius, G. & Grönwall, A. (1952). *Scand. J. clin. Lab. Invest.* **4**, 47.
- Kunkel, H. G. & Tiselius, A. (1951). *J. gen. Physiol.* **35**, 89.
- Latner, A. L., Molyneux, L. & Rose, J. D. (1954). *J. Lab. clin. Med.* **43**, 157.
- Levin, B. & Oberholzer, V. G. (1953). *Amer. J. clin. Path.* **23**, 205.
- Lindgren, F. T., Elliott, H. A. & Gofman, J. W. (1951). *J. phys. Chem.* **55**, 80.
- Mackay, I. R., Volwiler, W. & Goldsworthy, P. D. (1954). *J. clin. Invest.* **33**, 855.
- Merklen, F. P. & Masseyeff, R. (1952). *C.R. Soc. Biol., Paris*, **146**, 1905.
- Pezold, F. A. & Kofes, A. (1954). *Klin. Wschr.* **32**, 504.
- Pezold, F. A. & Peiser, U. (1953). *Klin. Wschr.* **31**, 982.
- Plückthun, H. & Götting, H. (1951). *Klin. Wschr.* **29**, 415.
- Röttger, H. (1952). *Naturwissenschaften*, **39**, 451.
- Sommerfelt, S. C. (1952). *Scand. J. clin. Lab. Invest.* **4**, 307.
- Sommerfelt, S. C. (1953). *Scand. J. clin. Lab. Invest.* **5**, 299.
- Stein, W. & Moore, S. (1949). *J. biol. Chem.* **178**, 79.
- Van Os, G. A. J. (1953). *Chem. Weekblad.* **49**, 242.
- Williams, Pickels & Durrum (1955). *Science* (in the Press).

Potato Pyrophosphatases

By B. NAGANNA,* A. RAMAN, B. VENUGOPAL AND C. E. SRIPATHI
Department of Chemistry, Madras Veterinary College, Madras 7, India

(Received 28 June 1954)

The early studies with malt sprouts (Lüers, Zychlinski & Bengtsson, 1931), takadiastase and rice bran (Kurata, 1931; Uzawa, 1932; Munemura, 1933) have established the existence of acid pyrophosphatases in higher plants. Pfankuch (1936) prepared by ethanol precipitation a highly active extract from potato and sugar beet which showed phosphatase and pyrophosphatase (PP-ase) activities optimally at the pH of the press juice, viz. pH 5.8-5.9. Giri (1937) also prepared such active extracts from germinated soya beans and found that they hydrolysed pyrophosphate, glycerophosphate, and hexose diphosphate at an optimum pH

* Present address: Department of Biochemistry, Andhra Medical College, Visakhapatnam, India.

of 5.1-5.5. Fleury & Courtois (1937) made a comparative study of phosphatase and pyrophosphatase activities of a number of vegetable tissues and found that pyrophosphate was hydrolysed at an optimum pH of 5.6-5.8 by all the tissues; takadiastase, however, required a more acid medium for optimum activity.

Until recently it was generally believed that vegetable tissues contain PP-ases that are active only in acid medium, but in 1951, one of us (Naganna, 1951a) reported the occurrence of a potent alkaline PP-ase in fresh extracts of potatoes. This enzyme requires the presence of Mg^{2+} , without which it is inactive. In this respect it resembles those specific PP-ases which are reported to be present in yeast

(Bauer, 1937; Bailey & Webb, 1944; Kunitz, 1952), liver (Lohmann, 1933; Norberg, 1950; Naganna, 1951*b*; Swanson, 1952), muscle (Lohmann, 1933; Naganna, 1951*b*; Gilmour & Calaby, 1953), erythrocytes (Naganna & Menon, 1947, 1948), brain and adrenals (Gordon, 1950, 1952; Naganna, 1951*b*), heart, lung, kidney, spleen, intestine, prostate, and testes (Naganna, 1951*b*), and firefly lanterns (McElroy, Coulombre & Hays, 1951) and which are characterized by their absolute dependence on added Mg^{2+} for their activity. These enzymes should therefore be considered as a class by themselves, that is, PP-ases active only in the presence of added Mg^{2+} .

In this paper the results of a detailed study on the potato alkaline PP-ase are presented. Acid PP-ase and acid phosphatase were also studied simultaneously and the results are given.

EXPERIMENTAL

Materials

Crude enzyme solution. Ordinary potatoes (*Solanum tuberosum*), obtained from the market, were peeled, cooled in ice for 30 min. and cut into small slices, which were quickly macerated in porcelain mortars, usually with their own weight of cold, double-distilled water. The extracts were centrifuged while still cold and the supernatants stored in an ice chest. Keeping these crude extracts at room temperature for a day produced marked deterioration of the alkaline PP-ase activity.

Purified enzyme solution. The crude enzyme solution (100 ml.) was treated with 22.4 g. of solid $(NH_4)_2SO_4$ (British Drug Houses Ltd., AnalaR) and, after storing in the ice chest for 30 min., the precipitated proteins were removed by centrifuging. The supernatant was treated with another 22.4 g. of $(NH_4)_2SO_4$ and again placed in the ice chest for 30 min.; the precipitated enzyme was isolated by centrifuging, and the precipitate dissolved in 100 ml. of water to give a clear solution.

Substrates. (i) Neutralized sodium pyrophosphate (0.01*M*) prepared by diluting 10 ml. of a stock solution of 0.1*M*- $Na_4P_2O_7$ to 100 ml. after adding 9 ml. of 0.1*N*-HCl. (ii) Unneutralized sodium pyrophosphate (0.01*M*). (iii) Sodium β -glycerophosphate (0.1*M*).

Buffers. 0.028*M* Veronal-acetate buffers (Michaelis, 1931).

Activator. *M* and 0.1*M*- $MgCl_2$ (B.D.H.).

Methods

Acid pyrophosphatase (acid PP-ase). The incubation mixture consisted of 4 ml. of the buffer, pH 5.3 (sometimes the buffer of pH 4.9 was used), 0.5 ml. of the neutralized pyrophosphate and 0.5 ml. of the enzyme solution. The incubation period was 10–15 min. at 38–40°. Enzyme action was stopped with 2.5 ml. of 10% (w/v) trichloroacetic acid (TCA). After filtration, the orthophosphate in a sample of the filtrate was estimated by the method of Fiske & Subbarow (1925) using freshly prepared 1-amino-2-naphthol-4-sulphonic acid reagent.

Alkaline pyrophosphatase (alkaline PP-ase). Here the incubation mixture consisted of 3.9 ml. of the buffer,

pH 8.7, 0.1 ml. of *M*- $MgCl_2$ (sometimes 3 ml. of buffer and 1 ml. of 0.1*M*- $MgCl_2$ were used) and 0.5 ml. of the enzyme solution. Other details are the same as those given under acid PP-ase.

Acid phosphatase. The incubation mixture consisted of 3.6 ml. of buffer, pH 5.3, 0.5 ml. of 0.1*M* glycerophosphate, 0.4 ml. of 0.1*N*-HCl and 0.5 ml. of the enzyme solution. Addition of HCl was necessary to restore the pH, which is elevated by the glycerophosphate. The incubation period varied from 15 to 60 min. Other details are the same as those given under acid PP-ase.

RESULTS

Enzyme activities at different pH values, with and without Mg^{2+}

The activities of both the PP-ases with the crude and purified enzyme and of the acid phosphatase with the crude enzyme were studied at different

Fig. 1. Effect of pH on the phosphatase activity of crude potato extract, with and without added Mg^{2+} . Sodium β -glycerophosphate, 0.01*M*; Mg^{2+} , 0.02*M*; incubation period, 1 hr., ■, Without added Mg^{2+} ; O, with added Mg^{2+} .

pH values and in the presence and absence of 0.02*M*- Mg^{2+} . The addition of 0.5 ml. of neutralized pyrophosphate to tubes containing Mg^{2+} and buffer at and above pH 7.9 caused a fall in pH. The buffer was unable to control the pH in these cases. Addition of different quantities of dilute alkali or alternatively addition of unneutralized pyro-

phosphate instead of neutralized pyrophosphate, was found necessary in those cases. Also there was a pH alteration in tubes containing buffer at and below pH 6.1 on the addition of glycerophosphate. In these cases the pH was restored by adding the requisite quantities of dilute HCl.

The acid phosphatase activity of the crude enzyme is shown in Fig. 1. Figs. 2 and 3 illustrate the PP-ase activities of the crude and purified enzymes, respectively. It is evident from Fig. 1 that there is only one phosphomonoesterase in

with or without added Mg^{2+} , have been unsuccessful.

From Fig. 2, it may be seen that in the absence of Mg^{2+} the PP-ase activity is high between pH 4 and 6, showing a peak at pH 5.0, while in the alkaline range the activity is negligible. Mg^{2+} inhibits this acid enzyme to a variable extent. Thus at pH 4.9 the inhibition is about 20 %, whereas at pH 6.1 it is 80 %. At pH 6.7 the inhibition is small, perhaps because the activity due to the magnesium-sensitive alkaline PP-ase is superimposed.

Fig. 2

Fig. 3

Figs. 2 and 3. Effect of pH on the pyrophosphatase activity of crude (Fig. 2) and purified (Fig. 3) potato extracts, with and without added Mg^{2+} . $Na_4P_2O_7$, 0.001 M; Mg^{2+} , 0.02 M; incubation period, 15 min. ■, Without added Mg^{2+} ; ○, with added Mg^{2+} .

vegetable tissues, highly active over a rather broad pH range, viz. pH 4.3–6.7, the activity being maximum between pH 5 and 6. Mg^{2+} produces slight and almost uniform inhibition of the enzyme activity throughout the pH range 4.9–6.7. In the alkaline pH range there is no phosphomonoesterase activity either with or without Mg^{2+} . Attempts to demonstrate the presence of an alkaline phosphatase using sodium α -glycerophosphate or sodium phenyl phosphate as substrate, either

While there is low PP-ase activity in the alkaline zone in the absence of Mg^{2+} , there is marked activity in the presence of Mg^{2+} , with an optimum at pH 8.6–9.6. This optimum is slightly higher than that reported for a similar enzyme in animal tissues (Naganna & Menon, 1948; Gordon, 1950; Swanson, 1952) and in yeast (Bailey & Webb, 1944; Kunitz, 1952).

The purified enzyme (Fig. 3) shows a broad PP-ase activity optimum ranging from pH 4.6 to 6.7. The

high activity observed at pH 6.7 with the purified enzyme could not be observed with the crude enzyme. This suggests that the crude enzyme extracts contain some inhibitors which are removed during purification. Crude extracts prepared from several lots of stored potatoes gave pH activity curves similar to those shown in Fig. 2, while those from newly harvested potatoes gave results similar to those given in Fig. 3. It is therefore clear that some unknown inhibitors must have been elaborated in the potatoes on storage. Thoai (1941) also reported the occurrence in *Basidiomycetes* of two PP-ases with pH optima 3.8–4.0 and 5.6–6.4, respectively, and that the second enzyme was accompanied by a natural inhibitor which could be removed by adsorption on alumina. Furthermore, he found that both the enzymes were unaffected by Mg^{2+} . The effect of Mg^{2+} is quite different in the present studies. It inhibits activity throughout the acid range, being very marked at pH 6.1–6.8. This is the pH range at which any natural inhibitor, if present, would exert its inhibitory effect. It is therefore possible that a separate enzyme, active at pH 6.1–6.8, exists in plants. However, it should be remembered that the activities observed at each pH need not necessarily be due to a single enzyme. Though it is not possible to give the exact optimum pH of each enzyme, it may be tentatively considered that there are two acid enzymes one of which acts best at a pH around 5 and the other at pH 6–7, the former being insensitive to Mg^{2+} and any natural inhibitors while the latter is greatly inhibited by them. In addition, it is clear that an alkaline PP-ase, active only in the presence of added Mg^{2+} , exists in potatoes.

Enzyme fractionation with ethanol, acetone and ammonium sulphate

The precipitates obtained by treating 100 ml. portions of crude potato extract separately with 50 ml. of ethanol, 33 ml. of acetone or 22.4 g. of ammonium sulphate had negligible enzyme activities. The supernatants from the above were further fractionated at final concentrations of 66 % (v/v) ethanol, 50 % (v/v) acetone and 0.7 saturation with ammonium sulphate. The precipitates in every case were dissolved in 100 ml. of water and the activities determined. The fractionations were made below 10°. From the results given in Table 1 it will be seen that while the acid PP-ase is unaffected by the three fractionating agents the alkaline PP-ase is completely inactivated when treated with ethanol or acetone. It may be noted that earlier workers (Pfankuch, 1936; Giri, 1937) used ethanol and acetone for the fractional purification of the enzymes, and these fractionating agents must have incidentally destroyed the alkaline PP-ase. With ammonium sulphate nearly two-thirds

of the original alkaline PP-ase activity was present in the 0.3–0.7 saturation fraction. So far as the acid phosphatase activity is concerned the acetone fraction proved to be the most active.

Table 1. *Effect of fractionating agents on the potato pyrophosphatases and acid phosphatase*

Enzyme fraction	Activity of fraction (% of crude activity)		
	Acid PP-ase	Alkaline PP-ase	Acid phosphatase
Acetone (25–50 %, v/v)	84	0	76
Ethanol (33–66 %, v/v)	90	0	70
Ammonium sulphate (0.3–0.7 saturation)	91	65	54

Table 2. *Stability of potato pyrophosphatases and acid phosphatase at different pH values at 38°*

Purified potato extracts were incubated with buffer (or water), with and without added Mg^{2+} , at different pH values for 1 hr. at 38°. The activities were determined and the percentage of enzyme destruction calculated.

Preliminary incubation	Percentage of enzyme destroyed		
	Alkaline PP-ase	Acid PP-ase	Acid phosphatase
Buffer at pH 8.7 and Mg^{2+}	8	—	—
Buffer at pH 8.7	31	74	98
Water at pH 7.0 and Mg^{2+}	11	—	—
Water at pH 7.0	25	10	4
Buffer at pH 7.0 and Mg^{2+}	22	—	—
Buffer at pH 7.0	40	18	14
Buffer at pH 4.9 and Mg^{2+}	100	—	—
Buffer at pH 4.9	100	2	2

Stability of the enzymes at different pH values at 38°

Several workers have recorded that PP-ases are inactivated at reactions other than their optimum pH (Jacobson, 1933; Bamann & Gall, 1937; Fleury & Courtois, 1938). To study the effects of pH and any inhibition by the buffer constituents on the potato enzymes, two sets of tubes were taken, each containing the purified enzyme and (a) buffer of pH 8.7, (b) buffer of pH 7.0, (c) water at pH 7.0 or (d) buffer of pH 4.9. To one set Mg^{2+} was added. The tubes were incubated at 38° for 1 hr., cooled in ice, and enzyme activities determined after adding the requisite amounts of acid or alkali to bring the extracts to the desired pH, the respective buffer and substrate, and Mg^{2+} where necessary. The results are given in Table 2.

At the alkaline pH both the acid enzymes are largely inactivated while more than two-thirds of alkaline enzyme remained unaffected. The presence of Mg^{2+} protected the alkaline enzyme to a great

extent. At the acid pH the acid enzymes are practically unaffected while the alkaline PP-ase is completely destroyed. Mg^{2+} could not protect the alkaline enzyme against this inactivation. At neutrality the alkaline enzyme is more inactivated than the acid enzymes. Mg^{2+} was able to protect the alkaline enzyme to a certain extent. The values determined for the alkaline enzyme treated with water and buffer indicated that the buffer constituents exert a certain amount of inhibition. However, the extent of buffer inactivation was not so pronounced as was observed with crude erythrocyte and animal tissue PP-ases (Naganna, 1950, 1951b). It is possible that the effects of buffer constituents depend on the purity of the enzyme.

Fig. 4. Effect of Mg^{2+} on potato pyrophosphatases. $Na_4P_2O_7$, 0.001M; incubation period, 15 min. ■, Pyrophosphatase activity of the crude extract at pH 8.7; ○, pyrophosphatase activity of the purified extract at pH 8.7; △, pyrophosphatase activity of the crude extract at pH 4.9.

Effect of Mg^{2+} on acid and alkaline PP-ases

The inhibition of the acid PP-ase and the activation of the alkaline PP-ase by Mg^{2+} are shown in Fig. 4. Mg^{2+} in concentrations up to 0.01M exerts no inhibitory effect on the acid PP-ase. There is about 10% inhibition at 0.02M- Mg^{2+} and 30% at 0.05M- Mg^{2+} , while higher concentrations of Mg^{2+} produced greater inhibition. At such higher concentrations the magnesium-activated alkaline enzyme also was inhibited.

The alkaline enzyme is activated to the maximum extent with 0.02M- Mg^{2+} . With 0.001M- Mg^{2+} the activation is only 10% of the maximum, whereas with 0.002M- Mg^{2+} it is 50% and with 0.005M- Mg^{2+} it is about 75%. Similar observations were made with animal tissues (Naganna, 1951b; Swanson, 1952). Vegetable alkaline PP-ase therefore resembles the animal tissue alkaline PP-ases with regard to Mg^{2+} optimum concentration, but differs from the PP-ases of yeast and firefly (Kunitz, 1952; McElroy *et al.* 1951), which require only 0.001M- Mg^{2+} for full activation. It remains to be investigated whether the vegetable and the animal tissue alkaline PP-ases in the purest form could be activated completely with 0.001M- Mg^{2+} .

Fig. 5. Effect of calcium on magnesium activation of alkaline pyrophosphatase from purified potato extract. $Na_4P_2O_7$, 0.001M; pH 8.7; incubation period, 15 min. ■, Mg^{2+} , 0.02M, Ca^{2+} varied; ○, Ca^{2+} , 0.002M, Mg^{2+} varied; △, Ca^{2+} , 0.004M, Mg^{2+} varied.

Antagonistic action of Ca^{2+} on the Mg^{2+} activation of the alkaline PP-ase

Calcium in relatively small concentrations was found to inhibit the Mg^{2+} -activated PP-ases of yeast (Bauer, 1937; Bailey & Webb, 1944; Kunitz, 1952), erythrocytes (Naganna & Menon, 1948),

animal tissues (Lohmann, 1933; Naganna, 1949, 1951*b*; Gordon, 1950; Swanson, 1952) and firefly (McElroy *et al.* 1951). This inhibition is said to be due to the antagonistic action of the two bivalent cations on the enzyme. The results illustrated in Fig. 5 show that the Mg^{2+} -activated alkaline PP-ase also is inhibited by Ca^{2+} , the extent of inhibition being about 80 % when the $Ca^{2+}:Mg^{2+}$ ratio is 0.1. The nature of the curve is more or less identical with that obtained for the yeast (Bailey & Webb, 1944) and erythrocyte (Naganna & Menon, 1948) enzymes.

Fig. 6. Progress curves of purified potato pyrophosphatases and acid phosphatase. ■, $Na_4P_2O_7$, 0.001M; pH 8.7; Mg^{2+} , 0.02M; △, $Na_4P_2O_7$, 0.005M; pH 4.9; ○, glycerophosphate, 0.01M; pH 5.3.

Progress curves

The rates of hydrolysis of pyrophosphate at pH 4.9 and 8.7 and of glycerophosphate at pH 5.3 by the purified enzymes are shown in Fig. 6. The rate of hydrolysis of pyrophosphate by the alkaline enzyme may be regarded as of zero order, since the progress curve is linear up to 65 % hydrolysis of the substrate. The rate of hydrolysis by the acid PP-ase corresponds with a first-order reaction. The nature of the glycerophosphate hydrolysis also is similar to

that of pyrophosphate hydrolysis at acid pH. The results obtained for alkaline PP-ase here are analogous to those obtained with yeast and animal tissue PP-ases.

Substrate effect

It has already been pointed out that pyrophosphate in high concentrations greatly interferes with the phosphomolybdate colour development (Schmidt & Thannhauser, 1943; Naganna & Menon, 1948). Glycerophosphate also was found to interfere with the colour development above 0.03M concentration. Further, the blanks with different concentrations of either of the substrates gave different values. It was also observed (Naganna, 1952) that the slow rate of hydrolysis of pyrophosphate by the acid molybdate was very much increased after adding the 1-amino-2-naphthol-4-sulphonic acid reagent, as judged by the intensity of the blue colour.

Duplicate experiments were therefore set up with both substrates at different concentrations, together with blanks in duplicate at each concentration. Both the experimental and blank tubes were incubated simultaneously. Enzyme was added to the blanks only after the incubation, immediately followed by TCA. The filtrates were diluted to lower the pyrophosphate concentration to less than 0.001M and the glycerophosphate concentration to less than 0.01M. The dilution factor was the same for each experiment and its blank. The blue colours were measured in a photoelectric colorimeter exactly 1 min. after adding the 1-amino-2-naphthol-4-sulphonic acid reagent. The results are illustrated in Fig. 7.

From curve A (Fig. 7) the Michaelis constant (K_m) for the potato acid phosphatase is 0.01M. This value agrees very well with that reported by Pfankuch (1936). The K_m for the acid PP-ase is relatively very low; it is about 0.0008M (curve B). Such high affinities of the acid PP-ase for the substrate have not been reported earlier. Fleury & Courtois (1937) reported K_m values of 0.01 and 0.005M for the acid PP-ases of takadiastase and emulsin, respectively.

For the potato alkaline PP-ase the data obtained is such that it is not possible to evaluate the Michaelis constant. The maximum velocity is reached at as low a concentration as 0.001M pyrophosphate. With lower concentrations the velocity of hydrolysis decreased only by about 20 %, and with still lower concentrations the entire substrate was hydrolysed. The use of a very dilute enzyme solution also showed no improvement. It can therefore be concluded that the affinity of the potato enzyme for pyrophosphate in alkaline medium in the presence of optimum amounts of Mg^{2+} is immeasurably large. Bailey & Webb (1944) also made a similar observation with yeast PP-ase. Unlike other hydrolytic enzymes which have relatively low affinities for their substrates, the

alkaline PP-ase resembles the oxidation-reduction enzymes in having a very high affinity for its substrate. This property constitutes the most important characteristic of the alkaline PP-ase.

Energies of activation

The three enzyme activities were determined in triplicate at 29, 35 and 40°. The incubation periods were 7.5 min. for the PP-ases and 15 min. for the acid phosphatase. The logarithms of the velocities of hydrolysis ($\log_{10} K$) are plotted against the

10000 cal. for enzymic hydrolysis and 29000 cal. (Weil-Malherbe & Green, 1951) for the uncatalysed hydrolysis of pyrophosphate. It is also of interest that similar values have been obtained for the activation energy for the enzymic hydrolysis of pyrophosphate irrespective of the source of the enzyme (potato, erythrocyte or yeast) and the state of purity of the enzyme (crystalline or otherwise).

Fig. 7. Effect of substrate concentration on purified potato pyrophosphatases and acid phosphatase. Curve A, acid phosphatase at pH 5.3; curve B, acid pyrophosphatase at pH 4.9; curve C, alkaline pyrophosphatase at pH 8.7 in the presence of Mg^{2+} (0.02M).

reciprocals of the absolute temperatures ($1/T$) as shown in Fig. 8. From the slopes of the lines the energies of activation were calculated. The values obtained for all three enzymes are found to lie in the range 8300–9800 cal. For glycerophosphate hydrolysis by bone phosphatase a value of 9940 cal./mole was reported by Bodansky (1939), and for pyrophosphate hydrolysis values of about 9500 cal. were found by Kunitz (1952) and Naganna (1952) with the yeast and erythrocyte enzymes, respectively. It is evident that the values reported here are in very close agreement with the values reported by Bodansky and Kunitz. The biological importance of enzymes in bringing about reactions with a comparatively low energy of activation is obvious when one compares the activation energies of about

Fig. 8. Effect of temperature on the rate of hydrolysis by the acid phosphatase and pyrophosphatases of potato. Experiments were done in triplicate at 29, 35 and 40°. Curve A, hydrolysis of $Na_4P_2O_7$ at pH 5.3 for 7.5 min.; curve B, hydrolysis of glycerophosphate at pH 5.3 for 15 min.; curve C, hydrolysis of $Na_4P_2O_7$ at pH 8.7 for 7.5 min. in the presence of Mg^{2+} (0.02M).

Effect of inhibitors

The effects of various substances, known to inhibit the several PP-ases and acid phosphatase, were studied with a view to noting the differences in behaviour of the three enzymes with respect to each inhibitor. The inhibitors were added to the buffered enzyme mixture before adding the substrate, which was added after 5 min. The results are given in Table 3.

There is a close resemblance between the acid PP-ase and the acid phosphatase in their behaviour towards many inhibitors. Molybdate and tungstate, which are known to inhibit several vegetable and

animal tissue phosphatases (Bossard, 1947; Courtois & Anagnostopoulos, 1949), were found to inhibit both the acid PP-ase and acid phosphatase in very low concentrations, whereas the alkaline PP-ase was unaffected by them. Thiocyanate, which was earlier reported to be an inhibitor of potato acid phosphatase (Helferich & Stetter, 1947), inhibited both the acid PP-ase and phosphatase, but in relatively high concentrations. The alkaline PP-ase was inhibited only to a slight extent by SCN^- . Metal ions produced greater inhibition of the alkaline PP-ase than the acid enzymes. Ca^{2+} and Ba^{2+}

to substrate depletion by precipitation as metal pyrophosphates. Fluoride was toxic to all three enzymes. Inhibition produced by oxalate was very low in all. Cyanide was inhibitory, more to the acid enzymes, only in 0.02M concentration, while 0.001M produced no inhibition. Formaldehyde behaved as a specific inhibitor towards alkaline PP-ase. The acid enzymes were unaffected by even 0.02M formaldehyde; higher concentrations, however, produced a slight inhibition of the acid enzymes. The integrity of the amino group is therefore essential for the alkaline PP-ase activity.

Table 3. *Inhibition of potato acid phosphatase and PP-ases*

Various inhibitors (0.1–0.5 ml.), adjusted to the respective pH, were added to the buffered enzyme mixtures. After standing for 5 min. at room temperature, the substrates were added and the activities determined.

Inhibitor	Final concn. (M)	Inhibition (%)		
		Alkaline PP-ase	Acid PP-ase	Acid phosphatase
Na_2MoO_4	2×10^{-6}	0	72	78
	2×10^{-5}	0	100	100
Na_2WO_4	2×10^{-6}	0	74	80
	2×10^{-5}	0	100	100
NH_4SCN	2×10^{-3}	0	0	0
	1×10^{-1}	9	44	42
$\text{Co}(\text{NO}_3)_3$	2×10^{-4}	26	6	0
	2×10^{-3}	86	15	6
MnCl_2	2×10^{-4}	19	0	0
	2×10^{-3}	77	86	0
CuSO_4	2×10^{-4}	100	9	76
	1×10^{-3}	100	72	100
ZnSO_4	2×10^{-4}	91	0	55
	1×10^{-3}	100	12	100
CaCl_2	2×10^{-3}	73	0	0
BaCl_2	2×10^{-3}	22	0	0
NaF	1×10^{-3}	91	78	86
$\text{K}_2\text{C}_2\text{O}_4$	2×10^{-2}	12	8	8
HCHO	2×10^{-2}	80	0	0
	5×10^{-2}	90	12	18
KCN	1×10^{-3}	0	0	0
	2×10^{-2}	48	90	90

have practically no effect on the acid enzymes while the alkaline PP-ase was inhibited by both, more by Ca^{2+} . The alkaline PP-ase was greatly inhibited by Co^{3+} , Mn^{2+} , Cu^{2+} , and Zn^{2+} , the latter two metals being most toxic. Attempts to produce any activation with minute amounts of Co^{3+} or Mn^{2+} , as was obtained with yeast PP-ase by Kunitz (1952), have been unsuccessful. Very low concentrations of Zn^{2+} also were tried but no activation was observed. With respect to these metal ions the acid enzymes showed some differences. While the acid phosphatase is unaffected by Co^{3+} and Mn^{2+} , the acid PP-ase is inhibited by both, the extent of inhibition being great with Mn^{2+} . These inhibitions may be due

*Effect of mapharside and cysteine
on the alkaline PP-ase*

To ascertain whether the potato alkaline PP-ase is a thiol enzyme the effects of mapharside and cysteine were studied. Mapharside was reported to be a powerful inhibitor of thiol enzymes by Gordon & Quastel (1948). At 10^{-5}M , mapharside was found to inhibit nearly completely the alkaline PP-ase of brain (Gordon, 1950), erythrocytes and other animal tissues (Naganna, 1952). With potato alkaline PP-ase, however, practically no inhibition could be observed even with $5 \times 10^{-4}\text{M}$ mapharside.

Cysteine, which is an activator of thiol enzymes, was ineffective towards potato alkaline PP-ase. Incubation of fresh or old purified enzyme with cysteine for periods of 15 min. or 18 hr. at 3–5° produced practically no activation. Also cysteine could not prevent the spontaneous inactivation of the enzyme kept at 30°. The crude enzyme extracts, however, showed slight activation with cysteine.

DISCUSSION

The potato alkaline PP-ase can easily be characterized by its absolute dependence on Mg^{2+} , reaction kinetics and behaviour towards inhibitors. Perhaps its most important property is its very high affinity for the substrate, a property which may be considered as of great significance in the regulation of tissue levels of diphosphopyridine nucleotide and flavin-adenine dinucleotide (Kornberg, 1950; Schrecker & Kornberg, 1950). It seems to have a wide distribution in nature for it is present not only in yeast and animal tissues but also in all vegetable tissues, green leaves being particularly rich sources (Naganna, Venugopal & Sripathi, 1955).

Even though this alkaline PP-ase resembles the magnesium-activated PP-ases of yeast and animal tissues in many of the properties, there seem to be some significant differences. While the PP-ases of yeast (Bailey & Webb, 1944), brain (Gordon, 1950), erythrocytes (Naganna, 1950) and other animal tissues (Naganna, 1952) could be activated by cysteine, no such activation could be observed with potato alkaline PP-ase. McElroy *et al.* (1951) also found that cysteine had no effect on firefly PP-ase. Swanson (1952) reported that liver PP-ase gave equivocal results with thiol reagents. Furthermore, mapharside, which is an efficient inhibitor of thiol enzymes, had no effect on potato alkaline PP-ase. The apparent thiol nature or otherwise of the several PP-ases seems to depend on the source of the enzyme and perhaps more on the state of purity of the enzyme studied. It appears that potato alkaline PP-ase does not require the integrity of SH groups for its activity.

SUMMARY

1. Potatoes contain a labile alkaline pyrophosphatase besides the already known acid phosphatase and acid pyrophosphatase. No alkaline phosphatase could be demonstrated. The alkaline PP-ase is active only in the presence of 0.01–0.02 M- Mg^{2+} .

2. The alkaline PP-ase is destroyed at acid pH while the acid enzymes are inactivated at alkaline pH. Fractionation with ethanol or acetone resulted in total destruction of the alkaline PP-ase, whereas the acid enzymes were unaffected. The alkaline PP-ase could be purified only by fractionation with ammonium sulphate.

3. Molybdate and tungstate strongly inhibited the acid enzymes while the alkaline PP-ase was unaffected. Ca^{2+} had no effect on the acid enzymes but inhibited the alkaline enzyme. Fluoride was toxic to all the enzymes. Formaldehyde inhibited the alkaline PP-ase, indicating that intact amino groups are essential for its action. The alkaline PP-ase is probably not a SH-enzyme, as there was neither activation by cysteine nor inhibition by mapharside.

4. Data on the reaction rates, Michaelis constants and energies of activation for the three enzymes are presented.

REFERENCES

- Bailey, K. & Webb, E. C. (1944). *Biochem. J.* **38**, 394.
 Bamann, E. & Gall, H. (1937). *Biochem. Z.* **293**, 1.
 Bauer, E. (1937). *Hoppe-Seyl. Z.* **248**, 213.
 Bodansky, O. (1939). *J. biol. Chem.* **129**, 197.
 Bossard, M. (1947). *Bull. Soc. Chim. biol., Paris*, **29**, 218.
 Courtois, J. & Anagnostopoulos, C. (1949). *Enzymologia*, **13**, 183.
 Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Fleury, P. & Courtois, J. (1937). *Enzymologia*, **1**, 377.
 Fleury, P. & Courtois, J. (1938). *Enzymologia*, **5**, 254.
 Gilmour, D. & Calaby, J. H. (1953). *Enzymologia*, **16**, 34.
 Giri, K. V. (1937). *Hoppe-Seyl. Z.* **245**, 185.
 Gordon, J. J. (1950). *Biochem. J.* **46**, 96.
 Gordon, J. J. (1952). *Biochem. J.* **51**, 97.
 Gordon, J. J. & Quastel, J. H. (1948). *Biochem. J.* **42**, 337.
 Helferich, B. & Stetter, H. (1947). *Liebig's Ann.* **558**, 234.
 Jacobson, E. (1933). *Biochem. Z.* **263**, 302.
 Kornberg, A. (1950). *J. biol. Chem.* **182**, 779.
 Kunitz, M. (1952). *J. gen. Physiol.* **35**, 423.
 Kurata, K. (1931). *J. Biochem., Tokyo*, **14**, 25.
 Lohmann, K. (1933). *Biochem. Z.* **262**, 137.
 Lüers, H., Zychlinski, B. & Bengtsson, K. (1931). *Wschr. Brau.* **48**, 519.
 McElroy, D., Coulombre, J. & Hays, R. (1951). *Arch. Biochem. Biophys.* **32**, 207.
 Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
 Munemura, S. (1933). *J. Biochem., Tokyo*, **17**, 343.
 Naganna, B. (1949). *Curr. Sci.* **18**, 207.
 Naganna, B. (1950). *J. biol. Chem.* **183**, 693.
 Naganna, B. (1951a). *Curr. Sci.* **20**, 101.
 Naganna, B. (1951b). *Curr. Sci.* **20**, 231.
 Naganna, B. (1952). D.Sc. Thesis, Andhra University, India.
 Naganna, B. & Menon, V. K. N. (1947). *Curr. Sci.* **16**, 226.
 Naganna, B. & Menon, V. K. N. (1948). *J. biol. Chem.* **174**, 501.
 Naganna, B., Venugopal, B. & Sripathi, C. E. (1955). *Biochem. J.* **60**, 224.
 Norberg, B. (1950). *Acta chem. scand.* **4**, 601.
 Pfankuch, E. (1936). *Hoppe-Seyl. Z.* **241**, 34.
 Schmidt, G. & Thannhauser, S. J. (1943). *J. biol. Chem.* **149**, 369.
 Schrecker, A. W. & Kornberg, A. (1950). *J. biol. Chem.* **182**, 795.
 Swanson, M. A. (1952). *J. biol. Chem.* **194**, 685.
 Thoai, N. (1941). *Trav. Soc. Chim. biol.* **23**, 1183.
 Uzawa, S. (1932). *J. Biochem., Tokyo*, **15**, 19.
 Weil-Malherbe, H. & Green, R. H. (1951). *Biochem. J.* **49**, 286.