PROPERTIES OF WOOL ROOT PHOSPHATASES

By J. M. GILLESPIE*

[*Manuscript received April* 20, 1956]

Summary

Wool roots prepared by the wax sheet technique contain pyrophosphatase, phosphatase, and metaphosphatase. Of these the pyrophosphatase is the most active. It appears to carry an essential –SH group, is activated by Mg^{++} ions, and shows optimum activity at about pH 6·5. The phosphatase is not activated by metals nor inactivated by chelating agents or –SH inhibitors. Its optimum pH is not sharp but appears to be in the range 5–7. The metaphosphatase is activated by Mg^{++} and has an optimum pH of about 7.

I. INTRODUCTION

Pyrophosphatase, which is known to play a part in nucleotide metabolism (Kornberg 1950) is widely spread in nature. A comprehensive review of its distribution has been made by Roche (1950).

During the course of investigations concerning the enzymes in the wool root follicle, it was observed that pyrophosphatase and also certain other phosphatases were present. The activity of this group of enzymes appeared to be considerably greater than that of other enzyme systems in the wool root such as esterase, protease, and enzymes concerned with oxidation-reduction reactions. Ellis, Gillespie, and Lindley (1950) reported preliminary findings on the phosphatases. The present paper is concerned with the more detailed properties of the pyrophosphatase, phosphatase, and metaphosphatase of the wool root.

II. MATERIALS AND METHODS

Sodium pyrophosphate was recrystallized twice from distilled water to free it completely from orthophosphate. Chemically pure sodium β-glycerophosphate was also used.

Pyrophosphatase activity was estimated by incubating 1·0 ml of enzyme solution or wool root suspension for 30 min at 37°C with 3·0 ml of a substrate buffered at pH 7·0. The final solution usually contained 0·5 mM sodium pyrophosphate, 75 mM sodium veronal, and 2 mM $MgCl_2$. The reaction was stopped by adding 2·0 ml of 20 per cent. trichloroacetic acid, the mixture filtered, and an aliquot taken for an inorganic phosphorus determination by the method of King (1932). Pyrophosphatase activity is recorded as the amount of orthophosphate liberated by 10 mg of wool roots in 30 min. The other phosphatases were estimated in a similar fashion using the appropriate substrate.

A mixed buffer containing 50 mM acetate and 50 mM borate, adjusted to the appropriate pH with acid or alkali, was used in experiments to assess the effect of pH on the activity of the enzymes.

* Biochemistry Unit, Wool Textile Research Laboratory, C.S.I.R.O., Melbourne.

Wool roots were obtained from Merino cross type sheep, the method of preparation of roots being substantially that of Ellis (1948). Briefly, sheep skins were obtained within 30 min of removal from the animal, clipped to a wool length of about 3 mm, the wool embedded with a hot beeswax-rosin mixture, and the whole cooled to 10°C. The skin could then be stripped off, leaving the wool embedded in the wax with the root-ends protruding. The roots were removed with fine hair clippers and stored at −20°C or freeze-dried. In either case the phosphatases retained their activity for long periods.

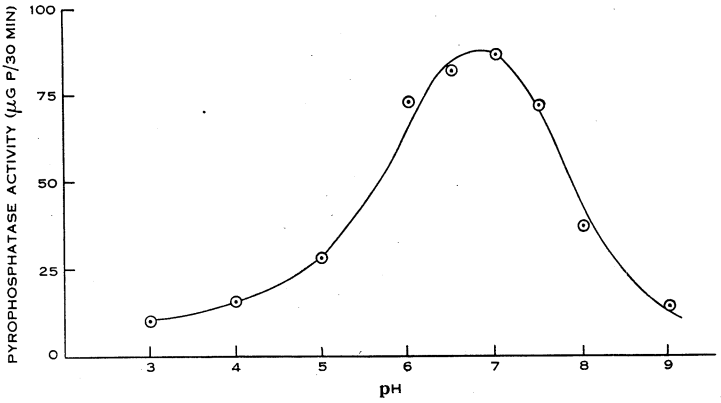

Fig. 1.—pH-Activity curve of wool root pyrophosphatase. System contained 50 mM acetate, 50 mM borate, 0·5 mM pyrophosphate, 2 mM Mg^{++}, and 10 mg wool roots; incubated for 30 min at 37°C.

Although the wax-sheet technique for preparing wool roots is very simple, it should be pointed out that it is very difficult to obtain reproducible yields of wool roots. For example one skin may yield 1 g of roots whilst several apparently similar ones may yield only 20 mg. This may be related to variations in the length of shaft in the skin, for it is suspected that a minimum length is needed for satisfactory cutting by the clippers.

In most enzyme tests a suitable amount of roots was added directly to the substrate. Simple extraction with water or salt solutions removed only small amounts of the pyrophosphatase, but better extraction was obtained after treatment with high intensity sound waves. The metaphosphatase and phosphatase were readily extracted with pH 4·5 buffer or with salt solution after autolysis.

III. Results

(*a*) *Pyrophosphatase*

(i) *Influence of pH on Activity.*—The pH-activity curve is shown in Figure 1. Maximum activity occurred at about pH 6·5.

(ii) *Activation by* Mg^{++}.—The influence of Mg^{++} on enzyme activity at a substrate concentration of 0·5 mM is shown in Figure 2. Maximum activity was attained at a Mg^{++} concentration over 0·001M. The enzyme was almost inactive in the absence of added Mg^{++}.

(iii) *Influence of Substrate Concentration on Activity.*—The relation between substrate concentration and reaction velocity at constant Mg^{++} concentration is shown in Figure 3. Increasing the pyrophosphate concentration increased the rate of hydrolysis up to a concentration of about 1 mM under standard conditions.

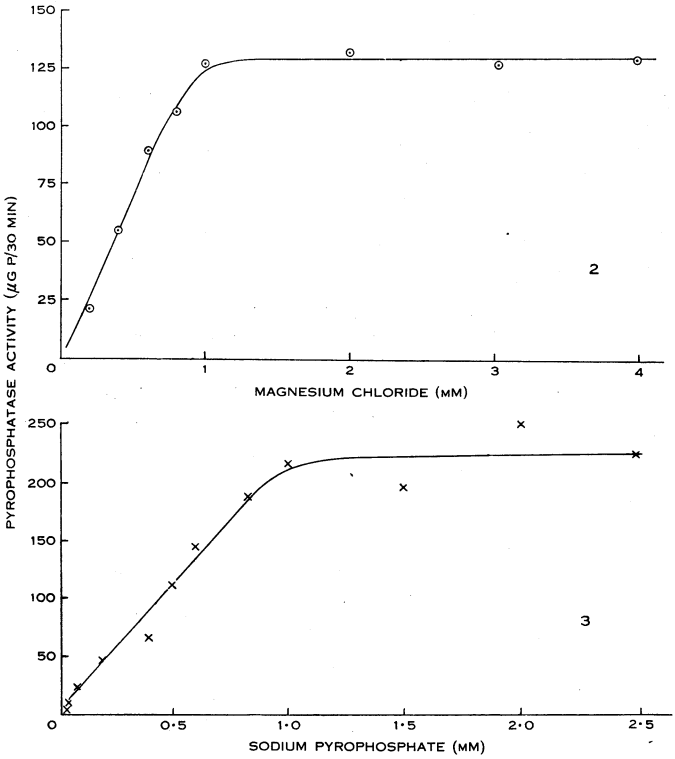

Fig. 2.—Activation of wool root pyrophosphatase by Mg^{++}. System also contained 75 mM veronal, 0·5 mM pyrophosphate, 10 mg wool roots; pH 7·0; incubated for 30 min at 37°C.

Fig. 3.—Influence of substrate concentration on the reaction rate of wool root pyrophosphatase. System also contained 75 mM veronal, 2 mM Mg^{++}, 10 mg wool roots; pH 7·0; incubated for 30 min at 37°C.

(iv) *Progress of Reaction.*—The relation between time and per cent. hydrolysis was almost linear up to 80 per cent. hydrolysis as shown in Figure 4.

(v) *Inhibitors.*—Table 1 lists the effect of some inhibitors on the wool root enzyme. The inactivation by oxygen could be completely reversed by the addition of cysteine, and a similar loss of activity on long storage at room temperature could also be reversed by reduction.

(vi) *Effect of Dialysis.*—After dialysis against distilled water, wool roots and their extracts showed no pyrophosphatase activity, but activity could be completely restored by the addition of cysteine and Mg^{++} (see Table 2).

(vii) *Other Sources of Pyrophosphatase.*—Qualitative tests showed that bovine horn pre-keratin and the roots of rabbit and human hair also contained pyrophosphatase.

(*b*) *Metaphosphatase*

(i) *Optimum pH.*—It can be seen from Figure 5 that the highest activity is obtained near pH 7, but that the enzyme retains considerable activity over a wide pH range.

(ii) *Activation.*—The enzyme required Mg^{++} ions for its activity and was not activated by Ca^{++}, Zn^{++}, Mn^{++}, or Co^{++} ions.

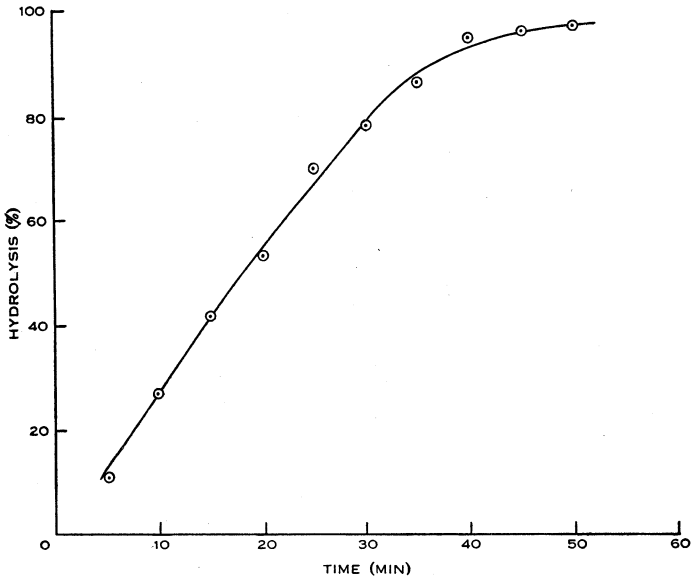

Fig. 4.—Influence of time on the hydrolysis of pyrophosphate by wool root pyrophosphatase. System contained 20 mM Mg^{++}, 75 mM veronal, 0·5 mM pyrophosphate, 10 mg wool roots; pH 7·0; incubated for 30 min at 37°C.

(*c*) *Phosphatase*

(i) *pH-Activity Curve.*—The relation between pH and enzyme activity is shown in Figure 6. The curve is rather spread out, but the maximum activity seems to be in the pH region 5–7.

(ii) *Activation.*—This enzyme appeared to be fully active in the isolated wool roots, and no increase in activity was observed after adding Ca^{++}, Mg^{++}, Zn^{++}, Mn^{++}, or Co^{++} ions. It was not inactivated by 100 mM concentrations of fluoride, 8-hydroxyquinoline, or iodoacetic acid.

(iii) *Tests Against Other Substrates.*—Table 3 shows the results obtained using a number of organic phosphorus compounds as substrates.

IV. Discussion

The present work suggests that the pyrophosphatase of the wool root resembles those of yeast and erythrocytes, especially in respect of the action of inhibitors and

Table 1

Effect of some inhibitors on the activity of wool root pyrophosphatase

Wool root suspension incubated with inhibitors in the presence of Mg^{++} for 30 min at 37°C before addition of buffer substrate. Final conditions: 2 mM Mg^{++}, 0·5 mM pyrophosphate, pH 7. Complete system incubated at 37°C for further 30 min

Inhibitor	Concn. (mM)	Inhibition (%)
p-Chloromercuribenzoate	10	100
Iodoacetate	50	95
o-Iodosobenzoate	10	100
Fluoride	0·1	100
8-Hydroxyquinoline	0·1	100
Citrate	0·1	85
Ca^{++}	10	100
Cu^{++}	1	100
Formalin	10	100
Oxygen (bubbled)	—	100

activators. The optimum pH near 6·5 is close to that reported for the pyrophosphatase of yeast (pH 7) by Bailey and Webb (1944) and for those of many animal

Table 2

Effect of dialysis on the activity of wool root pyrophosphatase

Wool root extract dialysed against running tap water for 24 hr and then against frequently-changed distilled water for 24 hr at 2°C. Final conditions: 0·5 mM pyrophosphate, pH 7. System incubated at 37°C for 30 min

Preparation	Activity (μg P/30 min)
Wool root extract (control)	10
Wool root extract + 20 mM cysteine	24
Wool root extract + 10 mM Mg^{++}	60
Wool root extract + 20 mM cysteine + 10 mM Mg^{++}	66
Dialysed wool root extract	0
Dialysed wool root extract + 20 mM cysteine	10
Dialysed wool root extract + 10 mM Mg^{++}	0
Dialysed wool root extract + 20 mM cysteine + 10 mM Mg^{++}	70

tissues, but it is lower than the optimum value of pH 8·3 for enzymes from insect muscle (Gilmour and Calaby 1953) and from the fire-fly (McElroy, Coulombre, and Hays 1951).

It has been shown that the wool root pyrophosphatase requires Mg^{++} for activity and the optimum concentration corresponds with a molar ratio of Mg^{++} to

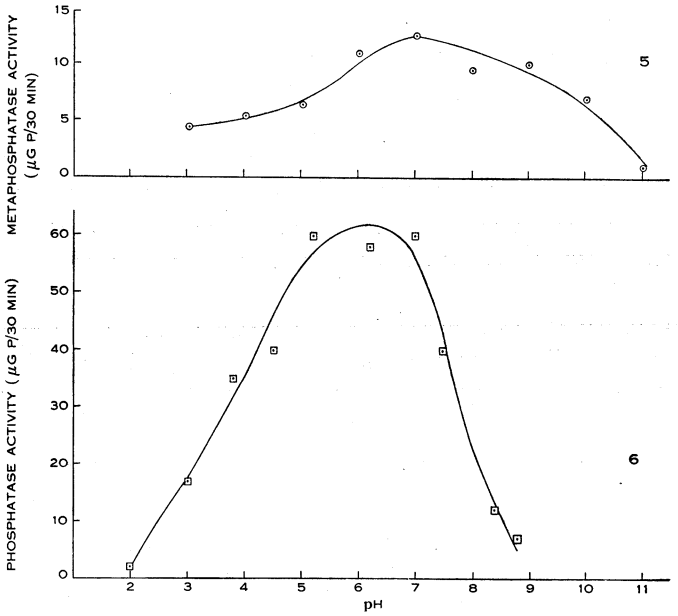

Fig. 5.—pH-Activity curve of wool root metaphosphatase. System contained 50 mM acetate, 50 mM borate, 1·0 mM metaphosphate, 50 mg wool roots; incubated for 30 min at 37°C.

Fig. 6.—pH-Activity curve of wool root phosphatase. System contained 50 mM acetate, 50 mM borate, 1·0 mM β-glycerophosphate, 50 mg wool roots; incubated for 30 min at 37°C.

substrate of about 2:1 which is the same as that reported for the pyrophosphatases of muscle and liver (Lohman 1933), erythrocytes (Bloch-Frankenthal 1954), and

TABLE 3

PHOSPHATASE HYDROLYSIS OF SOME ORGANIC PHOSPHORUS SUBSTRATES

Final solution contained 20 mg wool roots, 1·0 mM substrate buffered to pH 6, 20 mM acetate. System incubated at 37°C for 30 min

Substrate	Activity (μg P/30 min)
α-Glycerophosphate	5
β-Glycerophosphate	20
Fructose-1, 6-diphosphate	6
Adenylic acid	7
Disodium phenylphosphate	22·5
Phytin	0

insect muscle (Gilmour and Calaby 1953). The significance of this has been recently discussed by Bloch-Frankenthal (1954).

Wool roots do not appear to contain large amounts of the enzyme in comparison with the crude enzyme preparations reported on by other workers. It is difficult to make an exact comparison, but, using the figures of Heppel and Hilmoe (1951) for their best method of extraction, dried yeast yielded something over three times the activity given by an equal weight of dried wool roots.

Since all the reagents reacting with –SH groups reduce the activity it seems that this enzyme has an essential –SH group. The concentration required for inactivation was often higher than that reported by other workers and this may have been due to reaction of the reagents with –SH associated with the wool root protein.

Inhibition by formaldehyde may indicate that intact amino groups in the enzyme are also essential but this is not certain since this reagent also reacts with –SH groups.

Good reproducibility was very difficult to obtain because of the varying amounts of shaft and root in different preparations. In a typical preparation, less than 30 per cent. of the protein was easily water soluble and very little enzyme was extracted with it. A variation of up to 50 per cent. in pyrophosphatase activity was observed between different preparations, and it was not possible therefore to calculate the reaction rates.

V. References

Bailey, K., and Webb, E. C. (1944).—*Biochem. J.* **38**: 394.
Bloch-Frankenthal, L. (1954).—*Biochem. J.* **57**: 87.
Ellis, W. J. (1948).—*Nature* **162**: 957.
Ellis, W. J., Gillespie, J. M., and Lindley, H. (1950).—*Nature* **165**: 545.
Gilmour, D., and Calaby, J. H. (1953).—*Enzymologia* **16**: 34.
Heppel, L. A., and Hilmoe, R. J. (1951).—*J. Biol. Chem.* **192**: 87.
King, E. J. (1932).—*Biochem. J.* **26**: 292.
Kornberg, A. (1950).—*J. Biol. Chem.* **182**: 779.
Lohmann, K. (1933).—*Biochem. Z.* **262**: 137.
McElroy, W. D., Coulombre, J., and Hays, R. (1951).—*Arch. Biochem.* **32**: 207.
Roche, J. (1950).—"The Enzymes." Vol. 1. Pt. 1. p. 493. (Ed. J. B. Sumner and K. Myrbäck.) (Academic Press Inc.: New York.)