Noc, F. (1904). *Ann. Inst. Pasteur*, **18**, 387.
Quastel, J. H., Tennenbaum, M. & Wheatley, A H. M. (1936). *Biochem. J.* **30**, 1668.
Quastel, J. H. & Wheatley, A. H. M. (1938). *Biochem. J.* **32**, 936.
Slotta, K. H. & Fraenkel-Conrat, H. (1938). *Ber. dtsch. chem. Ges.* **71**, 1076.
Stumpf, P. K., Zarudnaya, K. & Green, D. E. (1947). *J. biol. Chem.* **167**, 817.
Swanson, M. A. & Artom, C. (1950). *J. biol. Chem.* **187**, 281.
Taborda, A. R., Taborda, L. C., Williams, J. N. & Elvehjem, C. A. (1952). *J. biol. Chem.* **194**, 227.
Weil-Malherbe, H. (1937). *Biochem. J.* **31**, 2202.
Zamecnik, P. G., Brewster, L. E. & Lipmann, F. (1947). *J. exp. Med.* **85**, 381.
Zeller, E. A. (1948). *Advanc. Enzymol.* **8**, 459.
Zeller, E. A. (1950). *Helv. chim. acta*, **33**, 821.
Zeller, E. A. (1951). In *The Enzymes*, **1**, part 2, p. 987. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press.

The Phytase of Wheat

By F. G. PEERS
Research Association of British Flour Millers, Cereals Research Station, St Albans

(*Received* 15 *January* 1952)

Phytase catalyses the hydrolysis of phytic acid (inositol hexaphosphoric acid) to inositol and free orthophosphate. It was one of the first enzymes which liberate inorganic phosphate from organic phosphorus compounds to be described (Suzuki, Yoshimura & Takaishi, 1907), and it has a wide distribution in plant and animal tissues, in many species of fungi and in certain bacteria. It has not, however, been so intensively studied as many of the other phosphatases, although in recent years Courtois and his collaborators have carried out extensive work on its occurrence, specificity and mechanism of action (cf. Fleury & Courtois, 1937, 1947; Courtois, 1945, 1947; Courtois & Biget, 1943; Courtois & Joseph, 1947; Courtois & Plumel, 1947; Courtois & Perez, 1948).

In cereal grains a considerable proportion of the total phosphorus is present as phytate; in wheat, for example, the phytin P constitutes 70–75 % of the total P of the grain (Booth, Carter, Jones & Moran, 1941). This is probably the source of the suggestion, frequently made, that a principal function of phytase in grains is to provide inorganic phosphate from phytate in the initial stages of germination. In this connexion Bernardini & Morelli (1912), Albaum & Umbreit (1943), de Turk, Holbert & Howk (1933) and Giri & Sreenivasan (1938) have shown that the proportion of inorganic phosphate to phytate increases progressively during germination with wheat, oats, maize and rice respectively. It has also been observed that the phytase activity of grains increases on germination, in barley by Luers & Malsch (1929), in wheat by Bernardini & Morelli (1912) and in oats by Albaum & Umbreit (1943). However, Essery (1951), although observing an increased phytase activity when barley grains germinate, found that the concomitant decrease of phytate P is relatively small and he doubts the validity of the simplified conception of phytase function mentioned above.

The relatively high content of phytate in cereals is a factor of importance in human and animal nutrition, particularly where whole grain or high-extraction products are concerned (the ratio of phytate P to total P increases markedly in the outer layers of the grain). This is because phytate can interfere with the assimilation of calcium and iron, and possibly other metal ions, due to these metals forming phytates which are highly insoluble over a wide pH range. McCance & Widdowson (1935) observed that human subjects on a high-phytate, low-calcium diet develop a negative calcium balance, and this observation provided the basis for the present practice of adding calcium carbonate to high-extraction flours. If phytate is hydrolysed by phytase then the immobilization of calcium will be decreased, and the interesting observation of Walker, Irving & Fox (1948), that in some subjects on high-phytate, low-calcium diets there is a gradual adaptation so that calcium balance is eventually restored, may perhaps be explained by an increased phytase activity of the intestinal flora.

It is useful, for both nutritional and practical reasons, to know how phytase is distributed among the different anatomical parts of the wheat grain and on its thermal stability in wheat and wheat products. No detailed work on these questions appears to have been published, and they have therefore been examined as part of a general study of wheat phytase which is described in this paper.

EXPERIMENTAL

Materials

Sodium phytate. Two products have been obtained from Ciba Ltd., 'phytin' and 'sodium phytinate'. The phytin (mixed calcium and magnesium salt of phytic acid), gives, on analysis, the correct phosphorus content and the inorganic phosphate content is about 1 % of the total P. If phytin is used as the enzyme substrate difficulties arise from the

separation of calcium phosphate from solution as the reaction proceeds, and the incomplete solubility of phytin at pH values greater than 5·0.

The 'sodium phytinate' supplied by Ciba Ltd. contained about 25% of its total P as free orthophosphate and was thus far from pure. Posternak (1919), Harrison & Mellanby (1939) and Courtois (1948) have indicated that sodium phytate ($C_6H_6O_{24}P_6Na_{12}.35H_2O$) may readily be crystallized. Many attempts to crystallize this salt in these laboratories have been unsuccessful and this has also been the experience of Essery (private communication).

Sodium phytate solutions, prepared as follows from commercial phytin, have therefore been used as the substrate: About 10 g. of phytin are dissolved in 50 ml. of water with the aid of the minimum amount of concentrated HCl. 20% (w/v) $FeCl_3$ in 2N-HCl is added until the supernatant obtained on centrifuging gives no further precipitate with the reagent. The white precipitate of ferric phytate is washed by centrifugation with three 50 ml. portions of N-HCl and five of water. The ferric phytate is then evenly suspended in water and 10N-NaOH added with vigorous stirring until, on centrifuging, a water-clear, colourless supernatant is obtained. This supernatant is decanted, filtered, the pH adjusted to 5·15 with HCl and the volume made up to 200 ml. before determination of inorganic and total P. Usually the orthophosphate content is less than 0·01% of the total P and the yield is about 70%, although the latter varies somewhat, probably due to the degree of fineness of suspension of the ferric phytate prior to double decomposition.

The work of Hanes & Isherwood (1949) on the separation of phosphate esters on paper chromatograms, suggested a possible means of testing the purity of sodium phytate solutions prepared in this way. Using *tert.*-butanol-picric acid with 24 hr. downward runs on Whatman no. 1 filter paper, the preparations showed only one phosphorus-containing spot, but a solution of Ciba 'sodium phytinate' showed three such spots; one due to phytate, one to orthophosphate and a third, having an intermediate R_F value, due to unidentified material.

Using the procedure of Hanes & Isherwood (1949) attempts were also made to separate the possible intermediates of phytase action, i.e. the various inositol phosphates. Using a wide range of solvent systems, it was found possible to demonstrate the accumulation of free organic phosphate by paper chromatography, but not the existence of intermediate inositol phosphates.

Preparation of partially purified phytase. 30 g. of a wholemeal from Manitoba wheat are extracted for 6 hr. at 0–4° with 175 ml. distilled water (pH of the suspension is about 6·0). The suspension is centrifuged to remove the bulk of the solid matter and the supernatant filtered to give 150 ml. of a clear solution, pH 5·9. This is poured with vigorous stirring into 600 ml. ice-cold acetone, allowed to stand 10 min. and the bulk of the supernatant liquor decanted. The precipitate is collected on a Büchner funnel and washed with acetone, acetone-ether (1:1) and finally ether: about 1·4 g. of a white powder are obtained. This is extracted for 2 hr. at 0–4° with 75 ml. distilled water and then filtered. The solution is saturated with $(NH_4)_2SO_4$, kept for 2 hr. at 0–4° and the precipitate which forms is collected on a Buchner funnel and dried *in vacuo* over anhydrous $CaCl_2$.

The dry material (0·4 g.) is dissolved in 20 ml. distilled water and dialysed 48 hr. at 0–4° against three changes of 2 l. distilled water. A slight precipitate which forms during dialysis is filtered off. The phytase activity of the final solution recovered on a dry-weight basis is usually about 20 times that of the original wholemeal with a recovery of about 25%.

Methods

Phosphate estimation. The methods used for the determination of total P and orthophosphate P were those described by Allen (1940): the colour densities were read on a Spekker instrument against a reagent blank using spectrum red (H. 508) and heat-resistant filters. The amidol bisulphite reagent used was made up fresh daily and filtered before using.

It was noticed, in the estimation of orthophosphate P in the presence of phytate P, that the $HClO_4$ appeared to hydrolyse phytate relatively rapidly at room temperature. Experiments showed that phytate and pyrophosphate were hydrolysed by the $HClO_4$ at room temperature liberating free orthophosphate, but that α-glycerophosphate and phenyl phosphate were resistant to hydrolysis in this way. The rate of hydrolysis of phytate by $HClO_4$ increases rapidly with temperature but is zero at 4°. Following these observations, since most of the estimations were of orthophosphate in the presence of phytate, solutions in which orthophosphate was to be determined were read on the Spekker between 5 and 10 min. after mixing with the reagents and making to volume.

In the course of inhibitor studies on phytase it was noted that the hydrolysis of phytate by $HClO_4$ appears to be catalysed by tellurite and selenite ions.

Method of following enzyme digestions. Material containing the enzyme, either a wholemeal or a purified preparation, equivalent to 200–1000 'phytase units' (in this paper one phytase unit is the amount of enzyme releasing 1 μg. of orthophosphate P from $1·6 \times 10^{-3}$M-phytate at pH 5·15 and 55° in 1 hr.) is weighed or pipetted into a 50 ml. Erlenmeyer flask. To this is added 5 ml. of 0·2M-acetate buffer, pH 5·15, containing 0·004M-$MgSO_4$, prewarmed to 55°, followed by the appropriate amount of phytate solution at 55° to bring the total volume to 10 ml. and the final substrate concentration to approximately 300 μg./ml. phytate P (i.e. about $1·6 \times 10^{-3}$M). A 2 ml. sample of the suspension or solution is removed immediately and the remainder incubated at 55°, further 2 ml. samples being removed after appropriate time intervals.

The 2 ml. samples are added to 1 ml. 10% (w/v) trichloroacetic acid, filtered and portions of the clear filtrates are used for the determination of orthophosphate P.

RESULTS

Properties of phytase in wholemeal and in a partially purified state

Progress curves. Using 2 g. samples of Manitoba wholemeal in one case and 1·25 ml. samples of a purified preparation (1925 units/ml.) in the other, progress curves of the hydrolysis of phytate have been plotted (Fig. 1). In both experiments the final volume was 25 ml. in standard buffer; the phytate concentration was $1·35 \times 10^{-3}$M in the wholemeal experiment and $1·6 \times 10^{-3}$M in the purified enzyme

experiment. The liberation of orthophosphate P was followed as described under Methods.

pH *Curves.* Samples of Manitoba wholemeal (500 mg.) and 1 ml. samples of a purified preparation diluted to contain 1000 units/ml. have been used to establish the pH-activity relationship at 55°. Buffers of 0·1 M-acetate over the range pH 3·8–5·8 were used to maintain the pH. The results, as shown in Fig. 2, agree very closely for both experimental series and show an optimum pH of 5·15 with a rapid diminution in activity on either side of this optimum. Various figures ranging from 5·0 to 5·5 have been reported in the literature as the optimum pH of phytase: for example, Luers & Silbereisen (1927) quote 5·2 for the optimum pH of malt phytase measured at 48°.

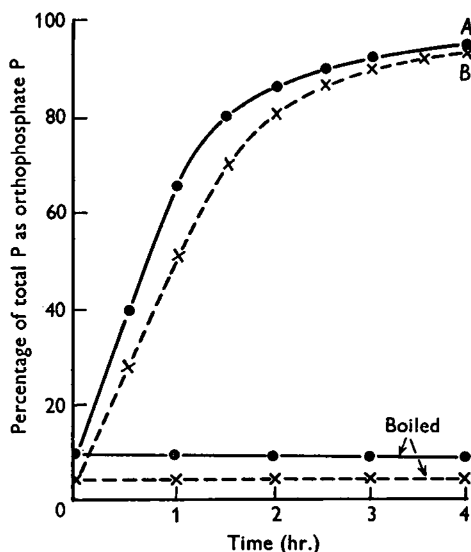

Fig. 1. Progress curves for phytase at 55°. Curve *A*, wholemeal (2 g. in 25 ml. buffered substrate, pH 5·15); curve *B* purified phytase preparation (1925 units in 25 ml. buffered substrate, pH 5·15). The boiled controls are also shown, the wholemeal being suspended in 10 ml. of water and boiled for 5 min.

Effect of enzyme concentration. Varying amounts, up to 750 mg. of the Manitoba wholemeal, and up to 3 ml. of a purified preparation (1925 units/ml.) were assayed for activity under the usual conditions. The results, which are shown in Fig. 3, show linearity of response up to 50 mg. wholemeal/ml. digest and 400 units purified enzyme/ml. digest: the latter corresponds to rather less than twice the enzyme concentration of the former. Luers & Silbereisen (1927) obtained a corresponding value of 200 units/ml. digest for the limit of linearity of response with malt phytase.

Effect of substrate concentration. Using 250 mg. samples of wholemeal and 1 ml. samples of a purified

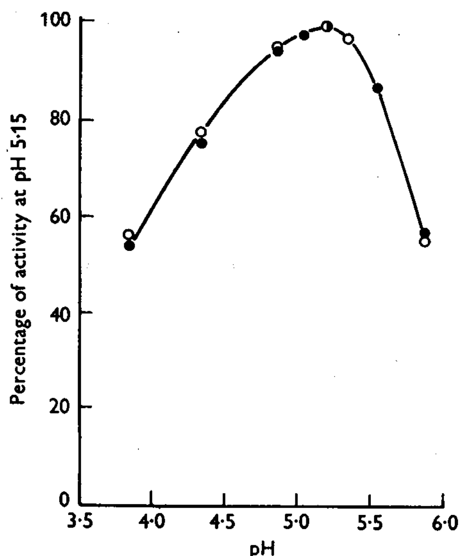

Fig. 2. Influence of pH on phytase activity. Initial reaction velocities (expressed as percentages of the maximum activity) measured at 55° in 0·1 M-acetate buffers in the presence of 0·002 M-$MgSO_4$ and $1 \cdot 61 \times 10^{-3}$ M-phytate; using wholemeal (●), and a purified preparation (○).

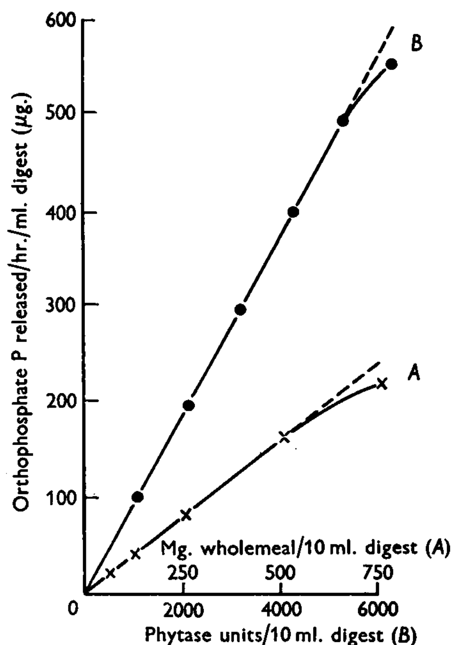

Fig. 3. Effect of enzyme concentration at 55°. Initial reaction velocities measured in buffered substrate, pH 5·15; wholemeal (*A*), purified phytase preparation (*B*).

phytase preparation (1000 units/ml.), initial reaction velocities have been measured at initial phytase concentrations varying from $0{\cdot}27\times10^{-3}$ to $1{\cdot}74\times10^{-3}$M with the wholemeal, and from $0{\cdot}79\times10^{-3}$ to $3{\cdot}58\times10^{-3}$M with the purified phytase preparation. Reciprocal plots made as described by Lineweaver & Burk (1934) give straight lines and from the intercepts on the arcs the Michaelis constant (K_m) and the maximum reaction velocity (V) have been calculated and are given in Table 1.

Table 1. *Effect of substrate concentration on phytase activity*

(The following figures have been calculated from graphs of $1/v$ against $1/s$: the initial reaction velocities (I.R.V.) at various substrate concentrations being measured under the standard conditions.)

	Michaelis constant (K_m)	Maximum I.R.V. (V) (μg. P/hr./ml. digest)
Wholemeal exp.	$0{\cdot}29\times10^{-3}$M	127
Purified enzyme exp.	$0{\cdot}33\times10^{-3}$M	127

Effect of temperature of incubation. Samples of Manitoba wholemeal (500 mg.) were used, in 20 ml. of buffer-substrate-$MgSO_4$, over a range of incubation temperatures. The progress curves at the different temperatures are plotted in Fig. 4 and these show the optimum temperature of activity to

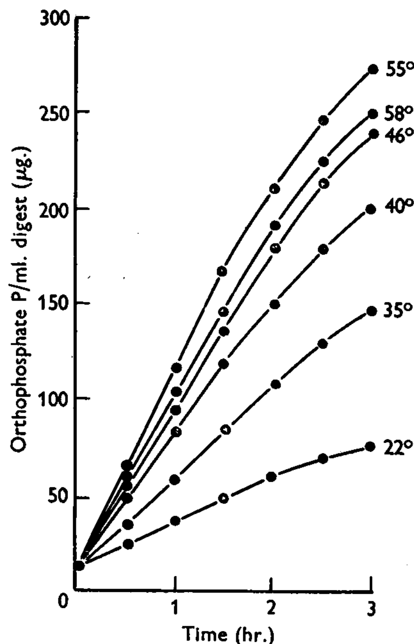

Fig. 4. Effect of temperature on the progress curve of phytase in wholemeal. Measured in buffered substrate, pH 5·15.

be about 55°. Luers & Silbereisen (1927) obtained a value of 48° for the optimum temperature of malt phytase, while Collatz & Bailey (1921) and Kolobkowa (1936) found 55° as optimum for wheat phytase.

Later experiments on the thermal inactivation of phytase showed that some inactivation of the enzyme occurs at 55° in the absence of the substrate and hydrolysis products. It is not known to what extent these may protect the enzyme from heat inactivation. The temperature coefficients $\left(\frac{q_{t+10^\circ}}{q_t}\right)$ have been calculated from the data given in Fig. 4 and are given in Table 2.

Table 2. *Temperature coefficients of activation for wholemeal phytase*

(Derived from the data constituting Fig. 4.)

Temperature interval	$\frac{q_{t+10^\circ}}{q_t}$
25–30°	1·64
35–45°	1·74
45–55°	1·20

Table 3. *Activation of a dialysed phytase preparation by* Mg^{++} *ions*

(Initial reaction velocities measured in 0·1M-acetate, pH 5·15 and $1{\cdot}58\times10^{-3}$M-phytate at 55°.)

Molarity of Mg^{++} ions	I.R.V. (μg. P/hr./10 ml. digest)
0·0	108
0·0002	188
0·0005	228
0·001	246
0·002	254
0·005	253
0·01	254

Activation by magnesium ions. A phytase preparation consisting of an exhaustively dialysed, filtered, aqueous extract of the Manitoba wholemeal was assayed at different concentrations of $MgSO_4$, other conditions being as described in the Methods section. Before dialysis this preparation was assayed at 253 units/ml. The results, given in Table 3, show that some fall in activity occurs on prolonged dialysis, which may be restored by the addition of Mg^{++} ions at concentrations of 0·002M and greater. The reduction in activity, however, is not complete and phytase differs in this respect from certain other phosphatases which are completely inactive in the absence of Mg^{++} ions.

Effect of various salts on phytase activity. Using a filtered 3 hr. aqueous extract of wholemeal as the enzyme preparation (275 units/ml.) a number of inorganic and organic salts were tested for inhibitory or stimulatory properties at a final concentration of 0·01M. The enzyme digestions were

carried out as usual except that no $MgSO_4$ was added. The results are recorded in Table 4, and it is seen that sodium azide, alkaline oxalates and calcium chloride apparently activate the enzyme: the $CaCl_2$ probably does so by disturbing the equilibrium through removal of calcium phosphate from solution. Sodium citrate also appears to activate the enzyme although the response is somewhat irregular.

Sodium fluoride, potassium cyanide and yeast extract, final concentration 0·5 % (w/v), partially inhibit the enzyme whereas zinc and manganese sulphates completely inhibit. Zinc and manganese ions probably act by removing the phytate ions from solution since they both cause precipitation in the mixtures.

Table 4. *The action of various salts on the phytase activity of a filtered, aqueous extract of wholemeal*

(Activities are measured at 55° in 0·1M-acetate, pH 5·15, and 1.58×10^{-3}M-phytate. The salts, are added to a final concentration of 0·01M; the yeast extract to 0·5% (w/v).)

Substance added	Activity (μg. P/hr./ml. enzyme)	Percentage of control
None	273, 275, 274	—
Sodium lactate	273	100
Sodium sulphate	274	100
Magnesium sulphate	273	100
Sodium fluoride	138	50
Potassium cyanide	192	71
Yeast extract (Difco)	190	70
Sodium citrate	430, 303, 357	156, 111, 130
Sodium oxalate	340	124
Ammonium oxalate	342	125
Sodium azide	310, 312	113
Calcium chloride*	364, 360	132
Zinc sulphate*	0	0
Manganese sulphate*	0	0

* These three salts all caused precipitation in the digestion mixtures.

Sodium lactate, sodium sulphate and magnesium sulphate have no effect on the activity of the enzyme under these conditions.

Reproducibility of the assay of phytase in wholemeal. Manitoba wholemeal (250 mg., 88·2 % dry matter) was used for the determination of phytase activity, under the standard conditions, on seven different occasions over a period of 3 weeks. The values obtained for the initial reaction velocity expressed as μg. inorganic P released/hr./mg. dry weight were: 5·26, 5·13, 5·21, 5·29, 5·19, 5·24, 5·25; giving a mean value of 5·23 with a standard deviation (σ) of 0·053.

Thermal inactivation of phytase

Inactivation in steam-treated whole wheat grains. Samples of Manitoba wheat (500 g.) were heated to various temperatures by live steam, the wheat being thoroughly shaken in the stream of steam. The temperature was then maintained by feathering the steam supply. After a total time in the steamer of 6 min. the wheat was rapidly cooled and dried back to 500 g. weight at 30° before milling to 100 % extraction. Samples of the resulting wholemeals (500 mg.) were assayed for phytase activity and the results are shown in Table 5. The moisture content of the wheat increased from 12 % to about 18 % during the time in the steamer.

Inactivation in wholemeal. As the rate of thermal inactivation of an enzyme may vary with the moisture content of the medium and since, in the preceding experiment, the moisture content was not controlled, the following experiment was carried out with wholemeal (88·2 % dry matter) from Manitoba wheat.

Samples (1 g.) of wholemeal were sealed into thin-walled glass ampoules which were immersed in a water bath maintained at various temperatures up to 100° for exactly 10 min. The ampoules were then

Table 5. *Inactivation of phytase in whole wheat by steaming*

(The activities of wheat treated with steam to maintain various temperatures for 6 min. prior to milling to a wholemeal, determined at 55° in 0·1M-acetate, pH 5·15, 0·002M-$MgSO_4$ and 1.61×10^{-3}M-phytate.)

Temperature of treatment (°)	Activity (% of untreated control)
62·8	98·5
73·8	77·0
82·2	17·0
100	12·6

rapidly cooled, opened and the phytase activity of the wholemeal determined.

The results (Fig. 5) indicate that inactivation becomes measurable, for a period of 10 min. heating, at temperatures greater than 80°. A similar experiment was set up in which the ampoules were heated at 81° for varying periods of time up to 1·5 hr. The results of this experiment are shown in Fig. 6 where the logarithm of percentage activity remaining is plotted against time of heating. The curve is not linear, which indicates that the heat inactivation of phytase, under these conditions, does not follow the unimolecular rate law which is frequently observed with other enzymes.

Inactivation in solution. The inactivation of phytase in solution (705 units/ml.) has been similarly investigated at temperatures from 40 to 70°. From the results, which are shown in Fig. 7, it was decided that 55 and 65° were suitable temperatures for observing the rate of inactivation. The results of these experiments are shown in Fig. 8 where deviation from the unimolecular expression for heat inactivation is again apparent.

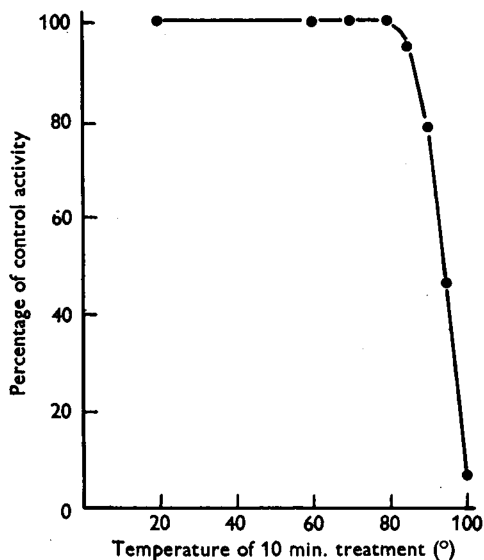

Fig. 5. Thermal inactivation of phytase in wholemeal. Activities of samples of wholemeal heated to various temperatures in sealed ampoules for 10 min., determined in buffered substrate, pH 5·15, at 55°.

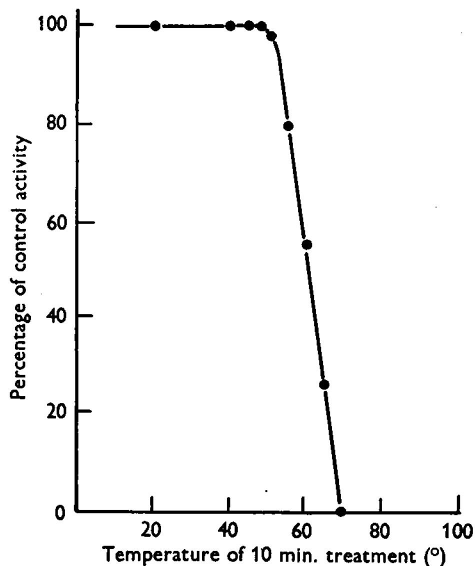

Fig. 7. Thermal inactivation of phytase in solution (705 units/ml.). Activities of samples of a partially purified solution of phytase heated to various temperatures in sealed ampoules for 10 min., determined in buffered substrate, pH 5·15, at 55°.

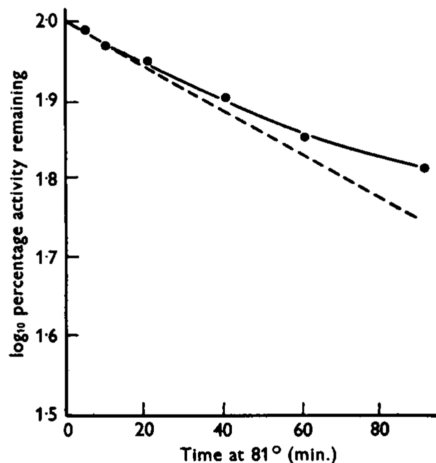

Fig. 6. Thermal inactivation of phytase in wholemeal. Activities of samples of wholemeal heated to 81° in sealed ampoules for various times, determined in buffered substrate, pH 5·15, at 55°.

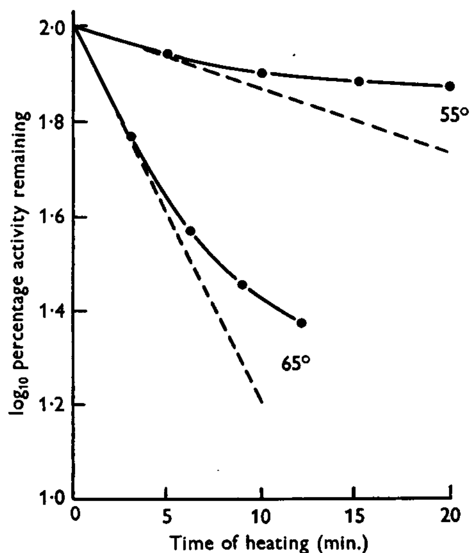

Fig. 8. Thermal inactivation of phytase in solution (705 units/ml.). Activities of samples of a phytase preparation heated to 55 and 65° for varying periods of time determined in buffered substrate, pH 5·15, at 55°.

By constructing the tangents to the two curves obtained in Fig. 8 we can derive the 'pseudomonomolecular' rate constants: $k_{55^\circ}=0{\cdot}028$, $k_{65^\circ}=0{\cdot}180$. An indication of the order of the energy of inactivation can then be obtained by substituting these values in the Arrhenius equation:

$$E=\frac{RT_1T_2}{(T_2-T_1)}\log_e\frac{k_2}{k_1},$$

whence $E=41\,600$ cal.

The value of the temperature coefficient of inactivation $\frac{k_{t+10}}{k_t}$ for the interval 55–65° is 6·5.

Distribution of phytase in the wheat grain

The various anatomical fractions of the wheat grain have been dissected out and assayed for phytase activity. The results for the soft wheat, Cappelle Deprez, are shown in Table 6, and they provide direct evidence for the suggestion of Widdowson (1941), made on the basis of baking tests, that phytase is more dispersed throughout the wheat grain than its substrate, phytin. It has also been found that an increase of 6·5-fold in the phytase activity occurs on germination of this wheat.

Typical values are non-germinated activity 3·17, and 5-day-germinated 20·6 phytase units/mg. dry weight.

Table 6. *Distribution of phytase in the wheat grain* (*Cappelle Deprez*)

Fraction	% by wt. of whole grain	Phytase activity (μg. P/hr./mg. dry wt.)	Percentage of total phytase
Wholemeal	100	3·41	100
Endosperm	82·5	1·29	34·1
Germ	1·0	9·07	2·9
Scutellum	1·5	31·8	15·3
Epidermal layers	4·5	1·32	1·9
Testa + cross-layer	3·5	4·34*	4·8
Aleurone	7·0	17·7	39·5

* This figure is obtained by difference from the figures obtained for the epidermal layers and the total pericarp.

Phytase content of various wheats

A number of different wheats, all harvested in 1951, have been examined for phytase activity. Small samples were ground to wholemeals in a mechanical mill and the phytase activities measured by the standard procedure. The results, shown in Table 7, confirm the suggestion of Courtois & Perez (1948) that hard wheats have a higher phytase activity than soft wheats, but the variation in activity between species and between different varieties of one species (i.e. *Triticum vulgare*) is not very great.

Table 7. *Phytase activity of various wheats* (1951 *harvests*)

(250 mg. samples of wholemeals assayed in buffered substrate, pH 5·15, at 55°.)

Wheat	Phytase activity (μg. P/hr./mg. dry wt.)	Hard or soft
Manitoba grade 1	5·22	Hard
Manitoba grade 2	5·44	Hard
Manitoba grade 3	4·93	Hard
Manitoba grade 4	5·41	Hard
Manitoba grade 5	5·18	Hard
American, Soft White	3·76	Soft
American, No. 2 Red Winter	4·08	Soft
American, Dark Spring	4·88	Hard
English, Pilot	5·22	Hard
English, Holdfast	5·52	Hard
English, Bersee	4·21	Soft
English, Jubiligem	4·76	Soft
Plate	4·54	Soft
Australian	4·06	Soft
Triticum vulgare	4·80*	—
T. polanicus	4·97	—
T. durum	4·28	—
T. spelta	3·82	—
T. turgidum	4·44	—
T. dicoccum	4·47	—

* This figure is the mean of the fourteen figures obtained for the bread wheats.

DISCUSSION

The experiments described in this paper show that the optimum temperature for the hydrolytic activity of phytase is 55°. This figure is comparatively high although, according to Sumner & Somers (1943), it is not unusual for some plant enzymes to have optimum temperatures of activity between 50 and 60°. The experiments on the rate of thermal inactivation of the enzyme show that it is relatively stable and the high temperature for optimum activity is, presumably, partly a reflexion of this thermostability. These temperature relations of wheat phytase have some practical interest since, due to its comparative thermostability, the enzyme will largely retain its activity through the processes of grain drying and of heat treatment where this is practised as a method of improving the milling and baking qualities of wheats. The high optimum

temperature means that, in the making of bread, an appreciable degree of phytase activity occurs during both panary fermentation and the early oven stages. This in turn results in up to 60 % of the phytic acid present in flour being hydrolysed to inositol and free orthophosphate during the baking process (Pringle & Moran, 1942).

Information on the distribution of enzymes between the various anatomical fractions of the wheat grain is of both fundamental and practical interest. Engel (1945, 1947), Engel & Bretschneider (1947) and Engel & Heins (1947), by histological methods using serial sections of the wheat berry, have shown that esterase, proteinase and peptidase activities are found mainly in the embryo, scutellum and aleurone fractions. The data on phytase, recorded in the present paper, appears to be the first to give an approximate quantitative picture of the distributions of an enzyme of the wheat grains. Phytase activity was found to be higher in the scutellum and aleurone fractions and its distribution is thus similar to that of the enzymes studied by Engel. It is also of interest to note that Hinton (1947) and Heathcote, Hinton & Shaw (1952) have shown that these two areas are also the sites of highest concentration of thiamine and nicotinic acid.

With the phytase preparations used in this paper there is, consistently, an initial linear relationship between time and the amount of orthophosphate released. If the course of the hydrolysis of phytate is considered as six consecutive bimolecular reactions with equal velocity constants (k) and if it is assumed that the first step is a 'pseudomonomolecular' reaction, it can be shown that,

$$x = 1 - e^{-kt}\left(1 + \frac{5kt}{6} + \frac{k^2t^2}{3} + \frac{k^3t^3}{12} + \frac{k^4t^4}{72} + \frac{k^5t^5}{720}\right),$$

where x represents the fractions of phytate P released as orthophosphate P in time t. This equation gives a curve which is of the same form as the experimentally observed progress curves. The fit of this theoretical curve is good up to the point of about 65 % phosphate released. Beyond this point the experimental points fall below the theoretical curve. This would be expected if there were inhibition of the enzyme by orthophosphate such as was observed by Luers & Silbereisen (1927) with malt phytase.

SUMMARY

1. Some properties of wheat phytase have been studied using both wholemeal and a partially purified preparation. The optimum pH of the enzyme is 5·15, optimum temperature 55°, Michaelis constant (K_m) $0{\cdot}3 \times 10^{-3}$ M: the enzyme is activated by magnesium ions at an optimum concentration of 0·002 M.

2. A procedure has been worked out for the partial purification (about 20-fold on a dry matter basis) of the enzyme from wholemeal.

3. The distribution of phytase among the anatomical fractions of the wheat grain has been determined.

4. An increase of 6·5-fold in the phytase activity has been observed when a soft wheat (Cappelle Deprez) germinates.

5. The phytase activity of a number of different wheats has been determined, and it has been found that hard wheats have a higher activity than soft wheats.

6. The influence of a number of inorganic and organic salts on phytase action has been determined. Heavy metal salts completely inhibit the enzyme probably by removing the phytate from solution.

7. With the enzyme in solution the rate of thermal inactivation does not follow the unimolecular expression but an approximate value for the heat of inactivation is 41 000 cal.

The author is indebted to Dr J. J. C. Hinton for dissecting certain of the fractions of the wheat grain described in Table 6.

REFERENCES

Albaum, H. & Umbreit, W. (1943). *Amer. J. Bot.* **30**, 553.
Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Bernardini, L. & Morelli, G. (1912). *R.C. Acad. Lincei Sed. solen.* **21**, 357.
Booth, R. G., Carter, R. H., Jones, C. R. & Moran, T. (1941). *J. Soc. chem. Ind., Lond.*, **60**, 903.
Collatz, F. A. & Bailey, C. H. (1921). *J. industr. Engng Chem.* **13**, 317.
Courtois, J. (1945). *Bull. Soc. Chim. biol., Paris*, **27**, 411.
Courtois, J. (1947). *Biochim. biophys. Acta*, **1**, 256.
Courtois, J. (1948). *Bull. Soc. Chim. biol., Paris*, **30**, 37.
Courtois, J. & Biget, P. (1943). *Bull. Soc. Chim. biol., Paris*, **25**, 103.
Courtois, J. & Joseph, G. (1947). *Bull. Soc. Chim. biol., Paris*, **29**, 951.
Courtois, J. & Perez, C. (1948). *Bull. Soc. Chim. biol., Paris*, **30**, 195, 631.
Courtois, J. & Plumel, M. (1947). *Actualités Biochim.* **10**, 81.
Engel, C. (1945). *Rec. Trav. chim. Pays-Bas*, **64**, 318.
Engel, C. (1947). *Biochim. biophys. Acta*, **3**, 278.
Engel, C. & Bretschneider, L. H. (1947). *Biochim. biophys. Acta*, **1**, 357.
Engel, C. & Heins, J. (1947). *Biochim. biophys. Acta*, **2**, 190.
Essery, R. E. (1951). *J. Inst. Brew.* **48**, 125.
Fleury, P. & Courtois, J. (1937). *Enzymologia*, **1**, 377.
Fleury, P. & Courtois, J. (1947). *Biochim. biophys. Acta*, **1**, 270.
Giri, K. V. & Sreenivasan, A. (1938). *Biochem. Z.* **296**, 428.

Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Harrison, D. C. & Mellanby, E. (1939). *Biochem. J.* **33**, 1660.
Heathcote, J. G., Hinton, J. J. C. & Shaw, B. (1952). *Proc. Roy. Soc.* B, **139**, 276.
Hinton, J. J. C. (1947). *Proc. Roy. Soc.* B, **134**, 418.
Kolobkowa, E. W. (1936). *Biochemistry, Leningr.*, **1**, 512.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Luers, H. & Malsch, L. (1929). *Wschr. Brau.* **46**, 143.
Luers, H. & Silbereisen, K. (1927). *Wschr. Brau.* **44**, 263, 273.
McCance, R. A. & Widdowson, E. M. (1935). *Biochem. J.* **29**, 2694.
Posternak, S. (1919). *C.R. Acad. sci., Paris*, **168**, 1216.
Pringle, W. J. S. & Moran, T. (1942). *J. Soc. chem. Ind., Lond.*, **61**, 108.
Sumner, J. B. & Somers, G. F. (1943). *Chemistry and Methods of Enzymes*, New York: Academic Press.
Suzuki, V., Yoshimura, K. & Takaishi, M. (1907). *Bull. Coll. Agric. Tokyo*, **7**, 503.
Turk, E. E. de, Holbert, J. R. & Howk, B. W. (1933). *J. Agric. Res.* **46**, 121.
Walker, A. R. P., Irving, J. T. & Fox, F. W. (1948). *Biochem. J.* **42**, 452.
Widdowson, E. M. (1941). *Nature, Lond.*, **148**, 219.

Serum Esterases

1. TWO TYPES OF ESTERASE (A AND B) HYDROLYSING *p*-NITROPHENYL ACETATE, PROPIONATE AND BUTYRATE, AND A METHOD FOR THEIR DETERMINATION

BY W. N. ALDRIDGE

Medical Research Council Unit for Research in Toxicology, Serum Research Institute, Carshalton, Surrey

(*Received* 25 *January* 1952)

Huggins & Lapides (1947) have developed a sensitive method for the determination of esterases using as substrates *p*-nitrophenyl esters of the lower fatty acids. Although these esters are esters of phenols it was assumed that they were hydrolysed by what is usually termed 'ali-esterase'; no evidence was given on this point. It has been shown that other enzymes will hydrolyse *p*-nitrophenyl esters: Zeller, Fleischer, McNaughton & Schweppe (1949) have demonstrated that a purified erythrocyte cholinesterase will hydrolyse *p*-nitrophenyl acetate, and Whittaker (1951) has produced some evidence that both the true and pseudocholinesterases of human blood will hydrolyse a variety of substituted phenyl acetates. It is, therefore, not at all clear what enzymes were being determined in the methods developed by Huggins & Lapides (1947).

During the course of work on the enzyme which will hydrolyse diethyl *p*-nitrophenyl phosphate (E 600) it was found that there are in most sera two quite distinct types of esterase which will hydrolyse *p*-nitrophenyl acetate, propionate and butyrate. One type is inhibited, like cholinesterase, by 10^{-8} M-E 600, while the other is not inhibited at all. This paper describes the work leading to the development of quantitative methods for the estimation of these two types of esterase and which is part of a thesis which has been accepted by the University of London for the degree (external) of Doctor of Philosophy in the Faculty of Science.

METHODS

p-Nitrophenyl acetate, propionate and butyrate were prepared by a procedure essentially the same as described by Huggins & Lapides (1947). *p*-Nitrophenol was esterified by the corresponding acyl chloride in benzene in the presence of magnesium turnings and it was found that far better yields of ester were obtained in benzene saturated with water than in dry benzene. The acetate and propionate were crystallized from methanol. The solubilities of these esters in water at 25° are approximately 10 times less than those given by Huggins & Lapides (1947).

Estimation of esterase activity. Since these esters are unstable, a colorimetric method based on the determination of *p*-nitrophenol is not suitable. A manometric method has been developed, based on the CO_2 liberated from bicarbonate buffer by the 2 moles of acid produced during the hydrolysis. The substrate suspension was prepared by dissolving 100 mg. of the esters in 0·5 ml. of methanol with warming and then blowing the solution rapidly from a Pasteur pipette into 25 ml. of bicarbonate buffer ($NaHCO_3$, 0·031 M; NaCl, 0·162 M; gelatin, 0·1% w/v) and shaking vigorously to obtain a fine suspension of the ester. 1·0 ml. of the enzyme preparation (suitably diluted with the bicarbonate buffer) in the side arm of a Warburg flask was tipped into 3·0 ml. of substrate suspension after the flask had been gassed with 5% (v/v) CO_2 + 95% N_2 and equilibrated. The output of CO_2/min. is calculated from readings taken every 5 min.