Cereal Phosphatases

1. THE ASSAY OF FREE WHEAT PHOSPHOMONOESTERASE AND CHARACTERIZATION OF THE FREE PHOSPHATASES OF WHEAT

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Relatively little attention has been paid to plant phosphatases in comparison with those found in animal tissues, despite the fact that the first dephosphorylating enzyme discovered was phytase in cereal grains (Suzuki, Yoshimura & Takaishi, 1907). Phosphatases of various types have since been found to have a wide distribution, and it is increasingly clear that, together with the phosphorylases, they have an important role in carbohydrate and mineral metabolism in both plants and animals. The present study was undertaken following the observation that wheaten flour contains a phosphatase which, under appropriate conditions, will dephosphorylate co-carboxylase (aneurin pyrophosphate) more rapidly and efficiently than the phosphatase contained in taka-diastase.

Collatz & Bailey (1921) and Herd (1931) studied phosphatase activity of flours by measuring the electrical conductivities of aqueous flour extracts before and after incubation, but results were equivocal and at best this method is very non-specific. Berliner & Rüter (1928) provided evidence of a phosphatase of special type in wheat-flour extracts, which would dephosphorylate soya-bean phosphatide.

Uzawa (1932) reported that rice bran contained a phosphomonoesterase with pH activity optimum of c. 5.0. This enzyme was similar to several acid phosphatases of mammalian viscera in respect of optimal pH, relative rate of hydrolysis of α - and β -glycerophosphate ($\beta > \alpha$), and also in the absence of any activation by Mg^{++} . He also reported a phosphodiesterase associated with the monoesterase of rice bran, but, while he was able satisfactorily to free the monoesterase from diesterase, he was unable to free the diesterase from monoesterase.

Ignatieff & Wasteneys (1936) studied the distribution of phosphomonoesterase in wheat prior to and during germination, with β -glycerophosphate as substrate, while similar studies were made by Hilbe & Marron (1940) with the histological technique of Gomori (1939), in which sections of the material containing the enzyme were caused, to produce calcium phosphate (and subsequently a grey-black stain from reduced silver phosphate).

Nemec (1928) and Lüers & Malsch (1929) carried out similar studies, particularly on barley, also assessing phosphomonoesterase activity.

Sarma & Giri (1942) have demonstrated that the phosphomonoesterase of rice and ragi can exist in two forms, one 'bound' and not extractable from the finely powdered material by water, while the other portion is freely water-soluble. The marked increase in phosphomonoesterase activity noted on germination is only due in part to the concomitant freeing of 'bound' enzyme, and, therefore, a real increase occurs in the dephosphorylating activity of the grain as a whole as its metabolic activity becomes accelerated.

Giri (1941) has also shown that rice, barley, maize and other cereals (but not wheat) contain a pyrophosphatase. It has been established from the work of Takahashi (1932), Munemura (1933), Bauer (1935, 1936) and Giri (1936), by means of differential heat and pH inactivation and by adsorption techniques, that this enzyme is different from the phosphomonoesterase with which it is associated. This finding is supported by theoretical considerations of enzyme specificity.

In the enzymic hydrolysis of co-carboxylase by wheat extracts (which, as will be shown later, also contain pyrophosphatase), phosphomonoesterase is particularly concerned owing to the fact that acid solutions of co-carboxylase are unstable and spontaneously become converted to aneurin monophosphate + phosphoric acid.

The excellent review of phosphatases by Folley & Kay (1936) includes a systematic classification in which wheat phosphatases do not appear. It is desirable to know whether such cereal phosphatases are at all typical in respect of any of the characteristics selected in order to effect this classification.

The study of cereal phosphatases is beset by some primary problems which have so far been only partially surmounted. As already mentioned, Sarma & Giri (1942) have shown that the enzymes occur in 'free' and 'bound' states, and that aqueous extraction of grain removes only the 'free' phosphatase. A point of major importance with cereals is that in the normal grain or flour there is too little moisture

present for much enzyme activity to occur, e.g. respiration rate in normal grain is very slow. Immediately the grain is moistened or the flour is taken up in water, enzyme-substrate reactions occur more readily and orthophosphate is liberated from compounds of phosphorus in the grain, a certain portion of the phosphatase being concomitantly destroyed. This effect is lowered by reducing the temperature at which extraction is carried out, but this brings about a reduced rate of extraction of the enzymes.

Preliminary experiments, however, indicated that so far as phosphomonoesterase activity is concerned, the gain in rapidity of extraction at 20° as compared with 4° much more than offsets the small loss of activity due to increased enzyme action at the higher temperature. All extractions were therefore carried out at room temperature (approx. 20°). The following work is concerned almost exclusively with 'free' cereal phosphatase as found in raw non-germinated grain.

METHODS

RAPID METHODS OF ASSAY OF CEREAL PHOSPHOMONOESTERASE ACTIVITY IN TERMS OF ALREADY ESTABLISHED UNITS

Probably the most widely used method for the estimation of phosphatase activity (in blood serum, plasma, etc.) is that of King & Armstrong (1934), which employs disodium monophenylphosphate as substrate. The method is designed to measure mammalian alkaline phosphomonoesterase and achieves considerably greater sensitivity and speed than methods using β -glycerophosphate as substrate, such as that of Bodansky (1932, 1933). It appeared to be very convenient for use with wheat extracts because such extracts contain no phenol, and the amount of inorganic phosphorus in them is too small to affect appreciably the velocity of the reaction employed.

King & Armstrong's method has been modified by Gutman & Gutman (1938*a, b*, 1940) for the assay of mammalian acid phosphatase by changing the buffer from veronal to citrate of pH 4.9. For assay of cereal phosphatase, many of the considerations which led to the choice of citrate buffer and a pH of 4.9 do not obtain, e.g. there is no alkaline phosphatase present in wheat extracts (see later), and therefore there is no reason why buffering should not take place at the enzyme optimum; there is further no reason why citrate (with its slightly inhibitory effect upon final molybdate colour development) should be used in preference to acetate buffer.

The pH optimum of wheat phosphomonoesterase is 5.15 (Fig. 2) and, in the preparation of buffer substrate for this pH, cognizance was taken of the fact that the addition of disodium monophenylphosphate causes a slight alkaline shift in acetate buffer. The only fundamental difference in the following methods A and B lies in the phenol reagents used.

Phosphatase assay—method A: modification of the procedure of King & Armstrong (1934)

Reagents

Buffer substrate consisting of M/200 (1.09 g./l.) of ether-washed, phenol-free disodium phenylphosphate, dissolved

in M/5 sodium acetate-acetic acid buffer of pH 5.1. This reagent is prepared weekly from a stock solution of buffer, and, after saturation with chloroform, is preserved in the refrigerator. The solid disodium phenylphosphate is also kept at 4°.

Folin & Ciocalteu (1929) reagent diluted 1 in 3 with distilled water.

Na₂CO₃ solution, 20% (w/v) of anhydrous Analar reagent in distilled water.

Procedure

Two 4 ml. samples of buffer substrate are pipetted into two tubes in a water-bath at 38°, and to one tube (the control) is added 1.8 ml. of diluted Folin & Ciocalteu reagent. After temperature equilibrium has been established, 0.2 ml. of a previously centrifuged and filtered flour extract is added to both tubes. The contents of the tubes are well mixed, and precisely 30 min. after the addition of the flour extract to the second tube the Folin & Ciocalteu reagent is added to this tube also. Unlike the original King & Armstrong method, no precipitate forms at this stage, owing to the exceedingly small amount of protein present in the flour extract. Filtering is therefore unnecessary, and to each of the tubes is added 1.5 ml. of 20% (w/v) Na₂CO₃ solution; they are mixed with a footed glass rod and allowed to remain in the bath for 10 min. for full colour development. Comparison is made in the Spekker photoelectric absorptiometer using 1 cm. cells and Ilford Spectrum Red (no. 608) filters, the instrument having been previously calibrated against standard phenol solutions over a suitable range.

Phosphatase assay—method B: modification of the Scharer (1938) method

Reagents

Buffer substrate as already described for method A.

Alkaline buffer solution made by mixing equal volumes of M/5 boric acid in M/5 KCl, and $\frac{1}{2} \times 1.07$ NaOH solution.

Solution of 2:6-dibromoquinone-chloroimide, 0.04 g. in 10 ml. of 95% ethanol, prepared weekly and kept in an amber glass bottle. A suitable dropper giving 0.18–0.22 mg. of chloroimide/3 drops should be chosen for use with the reagent.

Procedure

Two 5 ml. samples of buffer substrate are pipetted into two tubes in the water-bath at 38°. To the control tube is added 2 ml. of alkaline buffer solution (borate-NaOH) followed by 0.2 ml. of flour extract. After temperature equilibrium has been established, 0.2 ml. of flour extract is added to the second tube. After precisely 30 min., 2 ml. of alkaline buffer solution are added to this tube and the contents are mixed. Both tubes are removed from the bath and allowed to cool and 3 drops of chloroimide reagent are added to each. The indophenol blue colour is allowed to develop for 15 min., after which the tubes are matched in the same way and with the same filters as in method A.

When using method B, it is necessary to ensure (1) that the pH of the resulting solution after the alkaline buffer is added is 9.5 ± 0.2 , as otherwise colour development is interfered with, and (2) that no saliva (containing alkaline salivary phosphatase) finds its way into the tubes. NaF may be added to the alkaline buffer solution at the rate of 5 g./l. to inhibit the action of any such alkaline phosphatase.

In the use of either of methods A or B, extreme cleanliness of tubes, flasks, etc., is essential to success and reproducibility of results, and chromic acid cleaning has been found to be the only safe way of avoiding trouble from this source.

Probable error of methods A and B

Method A has given a probable error on triplicate determinations of $\pm 2.7\%$ ($P=0.99$), while the comparable figure for method B is $\pm 2.95\%$. There is, therefore, little to choose between them on this score.

Expression of results

The unit of phosphatase as defined by King & Armstrong (1934) is the amount of enzyme which will set free 1 mg. of phenol under the conditions of the test. Instead of quoting units/100 ml. of serum, the content/g. is a convenient form of expressing the activity of cereals. Thus the free phosphatase activity of a cereal, in units, is the number of mg. of phenol freed by the aqueous extract from 1 g. of the cereal under the conditions of the test. The advantage of this means of expression lies principally in the fact that it avoids the setting up of new units and draws a close parallel between plant and animal assays.

Preparation of cereal extracts

Distilled water saturated with chloroform was used for extractions, and the relative amounts of flour and water used were kept constant at 1 g. + 25 ml. After being centrifuged and filtered, this extract was diluted 1 in 6 when normal whole-wheat flour extracts were being assayed, a greater dilution being necessary or germinated (or malted) cereals and a lesser dilution for low extraction flours or samples in which the enzyme has been inactivated by heat or other means.

EXPERIMENTS

The rate of extraction of phosphatase from finely ground whole-cereal grains was studied, using method A for assay. In Table 1 are given the phosphatase contents indicated after various periods of extraction (with frequent shaking). The flour residues after 24 hr. extraction were again extracted with a fresh lot of 25 ml. of water, and this second extraction was allowed to proceed for 12 hr. A third aqueous extraction failed to remove any measurable amount of phosphatase, but assays on the solid residue showed high 'bound' activity.

Table 1. *Free phosphatase content of whole-grain flour as indicated after various lengths of aqueous extraction*

Cereal	Time of extraction (King-Armstrong units)						2nd extraction (12 hr.)
	$\frac{1}{2}$ hr.	1 hr.	2 hr.	4 hr.	8 hr.	24 hr.	
Wheat	11.4	12.3	12.45	12.9	13.7	14.8	1.4
Oats	5.25	5.55	6.00	7.5	8.1	8.85	0.00
Barley	2.1	2.25	3.2	3.4	3.65	4.00	0.9

The rate of extraction of the enzyme is clearly so slow between 8 and 24 hr. that little inaccuracy is introduced by adopting a convenient time of

16-20 hr. as standard for the extractions; no significant differences occurred between assays carried out on extracts made within this time range.

It is worthy of note that the amount of enzyme extracted by water under standard conditions of time and temperature is not independent of the relative amounts of flour and water used. Thus, 1 g. of wholemeal flour extracted with 150 ml. of water gives an extract of consistently and markedly lower activity than that of the same flour extracted by the standard method described above (1 g. + 25 ml. extract diluted 1 in 6 after centrifuging).

The determination of various characteristics of wheat phosphomonoesterase

Determination of optimal pH of wheat phosphomonoesterase

This determination was carried out on four different substrates: aneurin monophosphate (acidified solution of co-carboxylase), disodium phenylphosphate and α - and β -glycerophosphates, with acetate buffer (and in one instance phthalate also). As will appear later, there are probably at least two phosphomonoesterases in wheat, and the optimal pH found here refers therefore to the presumptive mixture of them as naturally occurring. As no success has yet attended efforts to separate entirely the two (or more) enzymes, their individual pH optima, if different, cannot yet be determined, but measurements on wheat enzyme fractions which appeared to contain markedly different proportions of the two enzymes have shown no significant departure from the values reported here on the naturally occurring mixture.

Preliminary experiments indicated an optimal pH of approximately 5.0. The following are the protocols of a typical experiment on the more accurate determination of this pH.

Acetate buffer of strength $M/10$ was made up at nominal pH's from 4.75 to 5.25 at steps of 0.05 pH. 5 g. of whole-ground wheat were suspended in 95 ml. of buffer (using a Waring blender), a few drops of toluene were added, and extraction was allowed to proceed at room temperature for 16 hr., with frequent agitation. The flour was centrifuged off, the extract was made up to 95 ml. and its pH was determined (to 0.01 pH) electrometrically with a glass electrode. It was then placed in a flask and immersed in a water-bath at 38° until equilibrium was established. 5 ml. of a previously acidified solution of Merck co-carboxylase (54.6 mg./l.) in the appropriate buffer at 38° were added rapidly, the solutions were mixed, the time was noted, and samples were withdrawn at 5, 10, 30 min., 1 and 2 hr. intervals. Immediately they were withdrawn, these samples were run into an equal volume of ice-cold $M/20$ NaF solution which, as had been previously shown, effectively inhibited further enzyme activity. Aneurin was measured in these samples immediately by the photoelectric fluorimetric technique evolved by the present author and described in a report of the Accessory Food Factors Committee of the

Medical Research Council (1943). The velocity of the enzymic dephosphorylation of the aneurin monophosphate is almost linear for the first 15 min. under these conditions, despite the low substrate concentration, as can be seen from Fig. 1.

Fig. 1. The dephosphorylation of aneurin monophosphate by aqueous flour extract in $M/10$ acetate buffer at pH 5.0; initial substrate concentration $2.73 \mu\text{g.}$ of co-carboxylase/ml.

Fig. 2. pH activity curve of wheat phosphomonoesterase ($M/10$ acetate buffer; $2.73 \mu\text{g.}$ co-carboxylase/ml., 40°).

In Fig. 2 is shown the amount of aneurin freed at various pH's after a constant time of incubation ($\frac{1}{2}$ hr.), from which it is clear that the optimum pH of the enzyme lies between pH 5.1 and 5.2. Experiments similar to the above, using $M/200$ α - and β -glycerophosphate as substrates and using Allen's (1940) method for estimation of freed inorganic phosphorus, and experiments using the method outlined for assay of phosphatase activity in cereal extracts with phenylphosphate and buffers of varying pH, have confirmed the optimal pH at 5.15. The activity is still very considerable at pH 4.0 but disappears between 4.0 and 3.0; on the alkaline side

activity disappears between pH 6.0 and 7.0. By reason of these facts and that co-carboxylase and aneurin do not lose vitamin activity in solution at pH 4.0 and 38° overnight, a wheat extract (dialyzed free of aneurin) is a useful source of phosphatase in the assay of co-carboxylase by chemical means and has been found to be superior to taka-diastase in many respects. This subject will be dealt with more fully elsewhere.

Experiments on precisely similar lines to those described above, but using various dilutions of enzyme solution with a constant amount of buffer substrate of fixed concentration, have shown that the velocity of the dephosphorylation reaction is directly proportional to enzyme concentration over a wide range.

Wheat contains no alkaline phosphomonoesterase, this having been demonstrated on a disodium phenylphosphate substrate at various pH's ranging from 8 to 10, with borate, veronal and glyoxaline buffers. This observation accords well with the fact that the pH of wheat, either resting or during germination, lies below 6.0.

The thermal destruction of free wheat phosphomonoesterase in solution and its critical inactivation temperature

Preliminary experiments carried out on dialyzed wheat extracts buffered with acetate at pH 5.15 and in the absence of added substrate indicated that destruction was at an appreciable rate at 45° and became very rapid at over 60° . More detailed experiments were carried out on 24 hr., room temperature, 20% extracts of finely ground whole-wheat flour in chloroform water, which were centrifuged and filtered and then dialyzed in cellophane (sausage skin) bags for 12 hr. against flowing tap water, then for a further 36 hr. against frequently changed distilled water.

Ninety ml. of 0.25 M -sodium acetate-acetic acid buffer, pH 5.15, in a 150 ml. Erlenmeyer flask, were immersed in a water-bath maintained at the desired temperature $\pm 0.25^\circ$ and were allowed to reach temperature equilibrium. 10 ml. of the dialyzed enzyme solution were then run into the flask, which was agitated until temperature equilibrium was reached again ($1\frac{1}{2}$ –2 min.), the time of addition having been noted on a stop-watch. At precise time intervals (noted in Fig. 3) small samples were withdrawn and run into thin-walled $\frac{1}{8}$ in. test-tubes immersed in an ice-bath. Phosphatase assays were carried out on these samples immediately, and after 24 and 48 hr. in the refrigerator, by the modified King-Armstrong method (A) previously described. The destruction observed under these conditions appears to be irreversible and is constant for any given temperature/time over wide ranges of enzyme and buffer concentrations. It therefore appears to be true thermal destruction. Fig. 3 indicates some of these rates of destruction graphically. The critical inactivation temperature (i.e. that temperature at which 50% of the enzyme activity is

destroyed in 1 hr.) is 53°. It is of interest to note that the critical inactivation temperature of wheat protease as determined by Mounfield (1936) is 54°.

Fig. 3. The thermal destruction of free wheat phosphomonoesterase in M/20 pH 5.15 acetate buffer.

Prior to extraction from the wheat grain, the phosphomonoesterase is very much more resistant to heat treatment. For example, it has been shown that no loss of enzyme occurs in wheat of 14% moisture content after heating at 67° for 36 min., and if wheat of very low moisture content be used this temperature may be raised to 90–100° without appreciable loss. As might be expected from theoretical considerations, this destruction of free phosphatase *in situ* is closely bound up with protein denaturation. This subject, with its many practical implications, will be dealt with elsewhere.

Activation and inhibition of free wheat phosphomonoesterase

Wheat-enzyme preparations were made by ethanolic precipitation of centrifuged and filtered 20% aqueous extracts of wheat. The precipitates were taken up in water, filtered and dialyzed against frequent changes of distilled water for 48 hr. They were then found to be free of Mg^{++} when tested by the method of Weisselberg (1931) with *p*-nitrobenzene-azo- α -naphthol. The volumes of the solutions were adjusted to give suitable activities for use on disodium phenylphosphate and β -glycerophosphate (M/200) substrates at pH 5.15.

The results obtained with various substances which commonly act as activators and inhibitors of phosphatases are shown in Table 2. The absence of

Table 2. Activation and inhibition of free wheat phosphomonoesterase (M/200 substrate in M/20 buffer, pH 5.15)

Substance	Concentrations used	Effect
Mg^{++} (as $MgSO_4$)	$M \times 10^{-6}$ to 10^{-4}	None
HCN	$M \times 10^{-2.7}$ to 10^{-5}	None
Iodoacetic acid	$M \times 10^{-2}$ to 10^{-6}	None
NaF	$< M \times 10^{-4.7}$	None
NaF	$> M \times 10^{-4.7}$	Some inhibition
NaF	$> M \times 10^{-2}$	Total inhibition

Mg^{++} activation is in accord with the findings noted in the review of Folley & Kay (1936) in respect of rice bran phosphomonoesterase and the mammalian acid phosphoesterases with which the rice-bran enzyme is classed.

The nature of fluoride inhibition of wheat phosphomonoesterase

Disodium phenylphosphate buffer substrate of pH 5.0 (M/20 acetate buffer) was prepared at M/200, M/100 and M/50 strengths, each solution being also made $M \times 10^{-3}$ in respect of NaF. This concentration of NaF had been previously shown to bring about approximately 60% inhibition of activity in M/200 substrate. These solutions were treated in precisely the same manner as in determining the unit activity of cereal extracts, using a purified enzyme solution in suitable quantity, the same amount of enzyme solution being added to each tube. Similar experiments were carried out in the absence of fluoride.

The percentage inhibition of the constant quantity of enzyme used in each case was also roughly constant, indicating therefore that NaF is non-competitive but exerts its effect directly as a 'poison' to the enzyme.

The various free phosphatases of wheat

(a) *Phosphomonoesterases*. In the experiments on determination of optimal pH it was noticed that the activity of a given wheat-enzyme solution appeared to be greater in respect of α - than β -glycerophosphate despite the pH optima for the two substrates being identical. This observation was not in accord with the findings of Uzawa (1932) with rice-bran phosphomonoesterase, which dephosphorylated β -very much more rapidly than α -glycerophosphate.

In Fig. 4 is illustrated a typical experiment on a wheat extract. The freed orthophosphate was estimated by the method of Allen (1940) modified by the replacement of the 60% perchloric acid by 5N- H_2SO_4 . (Pringle (1943) has shown that the replacement of 60% perchloric acid by 5N- H_2SO_4 (after the work of Berenblum & Chain, 1938) is quite satisfactory.) This 5N- H_2SO_4 was used to stop the enzymic reaction after the appropriate time intervals.

It is clear that α - is hydrolyzed much more rapidly than β -glycerophosphate by aqueous extracts of wheat. It was therefore thought possible that wheat might contain two phosphomonoesterases, one similar to that described by Uzawa (hydrolyzing β - more rapidly than α -glycerophosphate) and another

substrates, namely, diphenyl- and triphenylphosphates, sodium pyrophosphate and hexosediphosphate, all at M/200 in chloroform-saturated M/20 acetate buffer, pH 5.0. 4 ml.

Fig. 4. The dephosphorylation of α - and β -glycerophosphate (0.004 M in M/20 acetate buffer pH 5.1) at 37° by centrifuged and filtered aqueous extracts of wheat. 4 ml. of substrate + 4 ml. of wheat extract used in each case.

present to a greater extent and showing an opposite relative affinity for the two glycerophosphate substrates. To test this theory, aqueous extracts of wheat were fractionated as follows:

- (i) by precipitation with $(\text{NH}_4)_2\text{SO}_4$ followed by dialysis of the re-dissolved precipitate,
- (ii) by precipitation with ethanol followed by dialysis of the re-dissolved precipitate.

In the case of (i) precipitation was carried out in three stages: (a) $\frac{1}{3}$ saturation, (b) $\frac{2}{3}$ to $\frac{3}{4}$ saturation, and (c) $\frac{3}{4}$ to full saturation of the initial filtered 20% aqueous wheat extract. Fraction (b) was most active, (c) almost inactive, and (a) possessed an activity about one-sixth that of (b), but all fractions behaved in the same way in respect of more rapid hydrolysis of α - than β -glycerophosphate. The behaviour of 50 and 50–75% ethanol precipitation fractions was similar to that obtained with the ammonium sulphate. Pairs of enzyme solutions were prepared from fractions (a) and (b) and also from the ethanol precipitations which were of equal activity in respect of disodium phenylphosphate, and these were used in suitable equal amounts on α - and β -glycerophosphate substrates. The results obtained in respect of ethanol fractions are shown in Figs. 5 a, b.

It is clear that the ratios of rate of hydrolysis of α -: β -glycerophosphate vary markedly from fraction to fraction. This circumstance is difficult to explain except on the assumption that wheat extracts contain two or more phosphomonoesterases which differ in their relative affinities for the two glycerophosphates.

(b) *Free phosphatases other than phosphomonoesterases in wheat.* The fractionated enzyme solutions used in the immediately preceding experiments were applied to other

Fig. 5a.

Fig. 5b.

Fig. 5. The dephosphorylation of α - and β -glycerophosphate (0.004 M in M/20 acetate buffer) by (a) '50% ethanol' precipitate from aqueous wheat extract; (b) '50–75% ethanol' precipitate from aqueous wheat extract.

samples of both buffer substrate and enzyme solutions were used as in the previous experiments, the reactions being stopped in the case of the phenylphosphates by the Folin & Ciocalteu reagent (prior to the estimation of free phenol)

and with 2 ml. of 5*N*-H₂SO₄ (prior to the estimation of orthophosphate by Allen's method) in the case of the pyrophosphate and hexosediphosphate. Control tubes were incubated (with the enzyme reaction stopped by the appropriate reagent) for the same length of time and under precisely the same conditions as the experimental tubes.

In contrast with monophenyl-, triphenylphosphate was completely unaffected by the wheat-enzyme preparations and diphenylphosphate was very much less readily attacked. In the latter case, the $\frac{1}{2}$ saturation fraction had about $\frac{1}{25}$ of the activity on diphenyl- that it exhibited on monophenylphosphate, whereas the $\frac{1}{2}$ to $\frac{3}{4}$ saturation fraction has a comparable figure of only about $\frac{1}{250}$. This finding takes no account of the fact that the phosphodiesterase involved may have a different pH optimum from the phosphomonoesterases previously dealt with, but raises the possibility that the phosphodiesterase is either (1) heterogeneous or (2) has a solubility in ammonium sulphate solutions which differs markedly from the solubilities of the phosphomonoesterases.

The test as carried out here is undoubtedly a measure of diesterase activity at pH 5.0, as, owing to the very much greater speed of the monoester reaction, the overall speed of the reaction is entirely governed by the activity of the diesterase.

Sodium pyrophosphate and hexosediphosphate were hydrolyzed by both wheat-enzyme fractions, solutions of equal activity in respect of monophenylphosphate substrate showing in both instances almost double the activity in the $\frac{1}{2}$ to $\frac{3}{4}$ saturation fraction of that in the $\frac{1}{2}$ saturation fraction. This $\frac{1}{2}$ to $\frac{3}{4}$ ratio of about 1:2 here is in marked contrast to the comparable ratio of approximately 5:1 in respect of diesterase activity.

It does, however, strengthen the probability that both the pyrophosphatase and hexosediphosphatase of wheat are not heterogeneous.

DISCUSSION

The concept of 'bound' and 'free' phosphatase in cereals put forward by Sarma & Giri (1942) has been confirmed, but it is clear that the proportions of 'bound' and 'free' enzyme are not absolute in the way that, for example, solubilities of salts are absolute. The way in which the amount of 'free' phosphatase varies with differing relative amounts of solid and liquid phase during extraction suggests that the enzyme is behaving in a manner similar to that found with proteins—within limits, the higher the concentration of salts (extracted from the flour), the higher the solubility. Preliminary experiments on the extraction of phosphatase with protein-dispersing solvents (2% aqueous KCl, 3% aqueous KBr, etc.) tend to confirm the view that the 'free' phosphatase is probably bound to, or closely associated with, the soluble proteins of wheat in the

same way that adenosinetriphosphatase is associated with myosin. The finding of Sarma & Giri that some of the 'bound' phosphatase of rice and ragi becomes freed on germination also supports this view, as the proteolysis which takes place on germination would tend to free the phosphatase previously bound to the insoluble proteins of the grain.

The high concentration and variety of phosphatases contained in wheat clearly signify that their role in germination and the utilization of the food reserves in the endosperm of the grain must be a major one. Ignatieff & Wasteneys (1936) observed that the concentration of phosphatase in the leaves is higher than that in any other part of the plant during its entire life period, which would indicate that the enzyme plays a part in the intense metabolic activity which takes place in such tissue—perhaps a part in photosynthesis. It would seem, therefore, that plant phosphatases must play quite as vital a part in plant metabolism as they do in animals.

SUMMARY

1. Two rapid methods are described for the assay of free phosphomonoesterase activity in wheat and other cereals in terms of already established units.
2. The optimal pH of free wheat phosphomonoesterase was found to be 5.15. The activity is still considerable at pH 4.0, but disappears between pH 4.0 and 3.0 on the acid side and between 6.0 and 7.0 on the alkaline.
3. The rate of thermal destruction of this phosphatase in solution has been determined; it is appreciable at 45° and very rapid at 60° and over. The critical inactivation temperature is 53° and this thermal destruction is irreversible.
4. Neither MgSO₄ nor HCN activated wheat phosphomonoesterase, the former at 10⁻⁶ to 10⁻⁴M and the latter at 10⁻²⁷ to 10⁻⁵M concentrations. Iodoacetic acid did not inhibit the enzyme at 10⁻² to 10⁻⁶M, but NaF was strongly inhibitory.
5. Evidence is presented to show that there are probably at least two phosphomonoesterases in wheat which differ in their relative solubilities in ethanol and (NH₄)₂SO₄ solutions, and of which one at least dephosphorylates α -glycerophosphate more rapidly than β -glycerophosphate. The total free phosphomonoesterase activity of wheat is greater in respect of α - than β -glycerophosphate.
6. Wheat also contains free hexosediphosphatase and pyrophosphatase (probably single enzymes in each case) and a phosphodiesterase which may possibly be heterogeneous.

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Studies on Diffusing Factors

4. THE ACTION OF REDUCING AGENTS ON HYALURONIC ACID AND OTHER POLYSACCHARIDES

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Robertson, Ropes & Bauer (1940) suggested that the spontaneous loss of viscosity of vitreous humour might be due to its high ascorbic acid content. McClean & Hale (1940) showed that ascorbic acid, with or without hydrogen peroxide, increased dermal permeability and lowered the viscosity of hyaluronic acid. Thiolacetic acid, quinol, pyrogallol, 'metol', sodium sulphite and hydrogen sulphide caused a fall in the viscosity of hyaluronic acid; this fall was not immediate but progressed over a period (McClean & Hale, 1941). Activation of an enzyme present in the hyaluronic acid solution was excluded by pre-heating the solution. No reducing sugar could be detected in the substrate after its viscosity had been lowered by reducing agents, as it can after hydrolysis by hyaluronidase.

Robertson, Ropes & Bauer (1941) showed that the viscosity of other polysaccharides, such as starch and pectin, also could be decreased by reducing agents; not all organic reducing agents were equally effective, and the presence of hydrogen peroxide appeared to be necessary, since the effect was in-

hibited by catalase. When their viscosity was decreased in this way the polysaccharides were no longer precipitable as asbestos-like clots. It was suggested that the change of viscosity might be due to a dephosphorylation of the viscous polysaccharide. Pirie (1942) showed that hyaluronic acid obtained from tumours contained copper, that the action of ascorbic acid on hyaluronic acid was catalyzed by copper, and that this could be inhibited by sodium diethyldithiocarbamate.

The present work was undertaken to investigate the action of reducing agents on hyaluronate and other polysaccharides and the part played by copper.

RESULTS

Sodium sulphate and oxidized thiolacetic acid or the oxidation products of ascorbic acid (all 0.01M-solutions) do not lower the viscosity of the hyaluronate (Table 1). Thus it may be presumed that the progressive lowering of viscosity referred to above depends upon the presence of an active reducing agent and not upon alterations in the salt content.