To the three standards and the test solution add 1 ml. of 1 % $HgCl_2$ solution and mix completely with the KCl extract; then add 4 ml. of 0·06 N-NaOH solution with constant mixing. The temperature of the solution when the alkali is added may vary from 0 to 25° without affecting the result, provided that it is the same for test and standards. Heat in a water-bath at 40° for 15 min., cool and add 10 ml. acetone by blowing from a pipette. Transfer to the glass cell of a Spekker fluorimeter and match against the quinine sulphate standard, with a purple filter (no. 7) in front of the right photocell and two strong neutral filters in front of the left photocell. Plot the drum readings given by the three standards against the corresponding amounts of aneurin added and determine the aneurin content of the test solution by interpolation. The glass cells should always be placed with the same side in front of the photocell. The covers are not required.

The method is lengthy, but if samples are incubated overnight at 37° it is possible to complete six determinations each day.

DISCUSSION

With the method described above the recovery of aneurin added to oats was found to be $100 \pm 5 \%$. Duplicates usually agree to within 2 %. A higher degree of accuracy cannot be expected in view of the number of manipulations involved. The recovery values show that the adsorption technique and the oxidation to thiochrome are free from appreciable error, and although no proof has been obtained that the aneurin liberated from oats by 0·1 g. papain represents the total aneurin content, no more

aneurin is extracted when the proteolytic activity is increased by means of HCN.

Existing evidence indicates that the thiochrome reaction is specific for aneurin. The possibility that the result obtained by the procedure described above includes substances other than aneurin is extremely remote, being restricted to substances capable of being destroyed by sulphite as well as of being oxidized to a fluorescent derivative.

All results have been calculated in terms of air-dry aneurin. The moisture content of the sample of aneurin used to prepare standards was 4·15 %. If desired, the results may be converted to the oven-dry basis.

The method has been tested only with oats but may, with suitable modification, be applicable to other cereals and foodstuffs.

SUMMARY

1. A method is described for the oxidation of aneurin to thiochrome by means of HgO dissolved in KCl solution. The method enables pure solutions of aneurin to be analyzed rapidly and accurately, but is not directly applicable to extracts of oats.

2. An adsorption technique is described which employs powdered Decalso to remove all the aneurin from oat extracts and is sufficiently selective for the HgO method to be subsequently applied.

3. The effect of digestion with proteolytic enzymes on the extraction of combined aneurin from oats has been studied and a procedure which gives the maximum yield of aneurin is described.

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Purification and Properties of Yeast Pyrophosphatase

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An enzyme which hydrolyzes inorganic pyrophosphate to orthophosphate appears to have a wide distribution. It has been demonstrated in muscle (Lohmann, 1928); in intestinal mucosa, kidney, bone, lung and liver (Kay, 1928); in blood cells

(Roche, 1931); in placenta and foetal liver (Minniti, 1939); and in various seeds (Fleury & Courtois, 1937). Bauer (1936) showed it to be present with phosphomonoesterase in autolysates of bottom yeast and obtained it free from the latter enzyme. Apart

from separations of this type, no rigorous purification has been attempted.

The pyrophosphatase from baker's yeast has now been prepared free from other phosphatases, and its specificity and kinetics studied. The best preparation, having a Q_P value of 10^5 at 38°, was essentially homogeneous electrophoretically, but attempts to crystallize it have been unsuccessful.

METHODS

Materials. Pyrophosphate: Kahlbaum, $Na_4P_2O_7.10H_2O$, free from orthophosphate; adenosine triphosphate (ATP) and K diphenylpyrophosphate preparations have been described (Bailey, 1942). Triphosphate, $Na_5P_3O_{10}.6H_2O$, was supplied by Dr A. Kleinzeller, and prepared by the method of Huber (1937); flavinadenine dinucleotide was a preparation from yeast containing 3 mg./g. and supplied by Dr A. Pirie; carnosine was prepared from horse flesh and twice recrystallized (N, found 24·6%; calc. 24·8%).

Activity estimations. The inorganic orthophosphate formed after incubating the enzyme with Na pyrophosphate in presence of Mg^{++} was estimated by the Fiske-Subbarow method. The enzyme (0·1–1 ml.) was incubated at 38° with 0·2 ml. M/10-$MgCl_2$, 0·2 ml. M/100 sodium pyrophosphate, and M/35 veronal-acetate buffer pH 7·2 (Michaelis, 1931) to a total volume of 5 ml. A series of tubes containing varying dilutions of the enzyme was always employed, and after 30 min. the samples were rinsed out into 25 ml. flasks containing the molybdate reagent, which stopped the reaction. The pyrophosphatase unit was defined as the amount of enzyme producing 1 mg. of orthophosphate P in 30 min. at 38° under the above conditions. The curve obtained by plotting orthophosphate P formed in 30 min. against enzyme concentration was linear up to 80% hydrolysis, and the activity value was calculated from this linear portion. An amount of enzyme equivalent to about 0·1 unit was required for each estimation; even in the crudest preparations the total amount of protein taken was so small that it did not interfere with the phosphate estimation. Since 1 mol. of pyrophosphate gives rise to 2 mol. of orthophosphate, the above unit corresponds in respect of turnover number to a unit based on the hydrolysis or transfer of 1 mg. P/hr. in the case of other enzymes. Activities when expressed as units/mg. protein N can be converted to Q_P values, which denote the volume in μl. of a hypothetical gas/mg. protein/hr., assuming 1 mol. of orthophosphate produced to be equivalent to 1 mol. of gas, by multiplying by the factor $22,400/31 \times 6$.

PURIFICATION

The chief procedures employed were fractional precipitation with $(NH_4)_2SO_4$, and fractional adsorption on calcium phosphate gel followed by elution with strong salt solution. The following summary of the main preparation describes the procedure.

1st stage. 70 lb. 'Encore' baker's yeast were minced and warmed to 38°, and mixed with 1·9 l. of toluene. After 30 min., 25 l. of tap water at 38° were added, and 18 hr. later the digest was centrifuged. The residue was mixed with 6·5 l. of water and spun down, and the semi-fluid

portion, after the clear supernatant was decanted, was mixed with an equal volume of water and again centrifuged. The combined supernatants (53·3 l.) contained 1,197,000 units with an activity of 6·3 units/mg. total N.

2nd stage. The autolysate was dialyzed by trickling at a rate of 0·5 l./hr. through 15 ft. of collapsed cellophan tubing contained in a sink of running tap water. Dialysate: 58·2 l., 1,800,000 units and activity 12·2 units/mg. total N. The increase in activity is partly due to removal of dialyzable N and partly to the removal of inhibitors.

3rd stage. 5·82 l. of calcium phosphate gel (4·4% dry weight) were mixed with the dialysate, spun off and discarded, and a further 18 l. of gel were added to adsorb nearly the whole of the enzyme. The gel was centrifuged down and mixed with 700 g. $(NH_4)_2SO_4$ and 240 g. $NaHCO_3$, the final pH being 7·5. Elution was then effected by extracting four times with $(NH_4)_2SO_4$ solution (10 g./100 ml. water). Eluate: 18·7 l.; 1,084,000 units.

4th stage. 5·3 kg. $(NH_4)_2SO_4$ were added to the eluate, and after standing for 2 days at 0° the precipitate was spun off and discarded. A further precipitate was obtained by addition of 1 kg. $(NH_4)_2SO_4$ to the supernatant, and this was filtered off on a Buchner funnel, dissolved in water and dialyzed. Dialysate: 585 ml.; 515,000 units; 192 units/mg. N.

5th stage. 200 ml. of phosphate gel were added, spun off and discarded, involving a loss of 59,000 units. The supernatant was fractionated by adding solid $(NH_4)_2SO_4$ and the precipitates filtered off, dissolved and dialyzed free of salt:

Fraction		
1	172,000 units at 405 units/mg. N; 60%	
2	152,000 units at 675 units/mg. N; 62%	Saturation
3	151,000 units at 340 units/mg. N; 64%	with
4	43,000 units at 230 units/mg. N; 66%	$(NH_4)_2SO_4$
5	24,000 units at 112 units/mg. N; 70%	

6th stage. Fractions 1, 2 and 3, containing 455,000 units, were combined, dialyzed, adsorbed on 300 ml. of phosphate gel, and eluted as before. Eluate: 690 ml.; 362,000 units.

7th stage. The eluate was again fractionated with $(NH_4)_2SO_4$, the fourth fraction, obtained between 62 and 64% saturation, having the highest activity: 109,000 units at 790 units/mg. non-dialyzable N; total protein N, 0·14 g.

8th stage. This purified fraction after dialysis was brought to pH 5 with N/70-HCl, dialyzed for 4 hr., re-acidified to pH 5, and the precipitate thus formed spun off and discarded. The supernatant was neutralized to pH 6·5 and centrifuged clear. Supernatant: 82 ml.; 91,000 units; 835 units/mg. N.

This preparation represents a 4·6% recovery and a 133-fold purification from the yeast autolysate stage. It was stored under concentrated $(NH_4)_2SO_4$ solution and was quite stable at 0°. Although crystallization has not been effected, the electrophoresis of a 1·2% solution in 0·05M-phosphate buffer pH 6 showed a single boundary over a period of 3 hr.; thereafter a trace of a slow-moving component could just be detected. At pH 6, the protein was negatively charged, and the mobility of the main ascending boundary was $3·1 \times 10^{-5}$ cm.^{2} V.$^{-1}$ sec.$^{-1}$.

PROPERTIES

Activation by metallic cations. The enzyme is quite inactive except in the presence of the Mg ion, which cannot be replaced by the ions Ca, Mn, Zn or Be. Under the conditions of the standard test, the activation is maximal in 0·002M-MgCl$_2$ and does not change if the Mg^{++} concentration is increased 20-fold. Fig. 1, illustrating these results, shows that 50 % activation is obtained with a Mg^{++} concentration of 0·0007M.
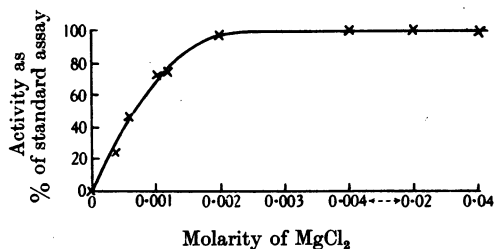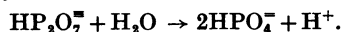

Fig. 1. Activation of yeast pyrophosphatase by Mg^{++} in M/35 veronal-acetate buffer pH 7·2 and M/2500-Na$_4$P$_2$O$_7$.

Specificity. The enzyme appears to catalyze specifically the reaction

$$HP_2O_7^- + H_2O \rightarrow 2HPO_4^- + H^+.$$

α-Glycerophosphate, hexosediphosphate, Na metaphosphate, Na triphosphate and ATP when tested under the conditions described for Na pyrophosphate were not attacked. Potassium diphenylpyrophosphate was tested manometrically (using sodium pyrophosphate as control) and flavinadenine dinucleotide by its ability to act as prosthetic group to d-amino-acid oxidase before and after treatment with pyrophosphatase. These substrates likewise remained intact. It thus appears that the pyrophosphate radical cannot in any way be substituted, either by a further esterification with phosphate (as in Na triphosphate and ATP) or by organic radicals (as in diphenylpyrophosphate and flavinadenine dinucleotide). It may be noted that various authors (Neuberg & Wagner, 1926; Kurata, 1931; Takahashi, 1932), using crude enzyme preparations from animal sources, have reported the breakdown of diphenylpyrophosphate; the inability of the purified yeast enzyme to do so was not unexpected in view of its inactivity towards ATP and Na triphosphate.

pH *activity curve.* The buffers employed were the Michaelis veronal-acetate mixture (M/35) and carnosine (M/20). pH values were measured in the glass electrode at 20° and corrected where necessary for the pH-temperature effect, since incubations were carried out at 38°. The reaction mixture (4·5 ml. of buffer, 0·2 ml. M/10-MgCl$_2$, 0·1 ml. enzyme and 0·2 ml. M/100 pyrophosphate) was incubated for 15 min., and the orthophosphate liberated at various

pH values compared with that obtained in the standard veronal buffer at pH 7·2. In the pH range in which veronal and carnosine buffers overlap, the activity of the enzyme was similar in the two buffers, showing an optimum at pH 6·9–7·0 (Fig. 2), agreeing with that found by Bauer (1936) for the pyrophosphatase of bottom yeast, and also similar to that found by Lohmann (1928) for the pyrophosphatase of muscle extract. The curve indicates that the activity of the enzyme extends even beyond the explored pH range of 5·7–8·8.
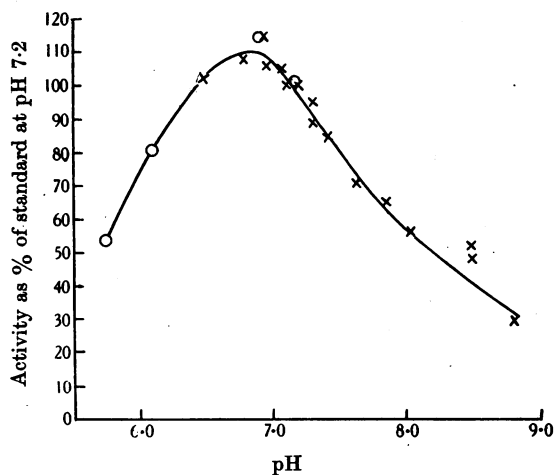

Fig. 2. pH-activity curve of yeast pyrophosphatase. Buffers: M/35 veronal-acetate (crosses) and M/20 carnosine (circles) containing M/250-MgCl$_2$ and M/2500-Na$_2$P$_4$O$_7$.

In animal tissues, several pyrophosphatases with differing pH optima have been reported (Takahashi, 1932; Minniti, 1939; Bamann & Gall, 1937). The last authors obtained from liver three such enzymes which were separated one from another, showing optima at pH 4, 5·7 and 8. Even in the crudest yeast extract, only the pyrophosphatase with optimum pH 7 has been reported (cf. Malkov & Kal, 1939).

Progress curve and effect of substrate concentration. The affinity of the enzyme for its substrate is too great to permit the direct measurement of initial hydrolysis rates at the very low pyrophosphate concentrations necessary to reduce the enzyme activity below the optimal. Using, for example, a pyrophosphate concentration of 0·0004M, hydrolysis is linear with time until the reaction is 80 % complete, and falls to about 50 % of the maximum velocity when hydrolysis is about 93 % complete (Fig. 3). These results suggest a Michaelis constant of not more than 3×10^{-5}: such a high affinity is, we believe, unique for a hydrolyzing enzyme.

The activity of the enzyme shows a surprising sensitivity to increase of substrate concentration,

and begins to decrease when the pyrophosphate concentration exceeds 0·003 M (Fig. 4). The velocity of hydrolysis falls to 50 % of the maximal in 0·01 M-substrate, and to zero in 0·03 M. This effect, which
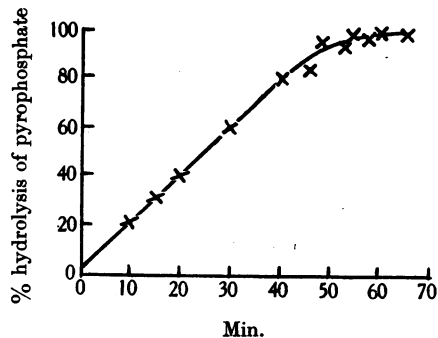

Fig. 3. Progress curve of $Na_4P_2O_7$ hydrolysis by pyrophosphatase. M/250-$MgCl_2$, M/35 veronal-acetate pH 7·2, initial $Na_4P_2O_7$ concentration M/2500.

is not abolished by a 10-fold increase in the Mg^{++} concentration, and is therefore not due to substrate-activator combination, is in fact a true inhibition, reversed simply by diluting. When, for example, the enzyme is incubated with M/250 substrate, no hydrolysis occurs, but after diluting 10–20 times (maintaining the Mg^{++} concentration at its previous value) enzyme action proceeds at the maximal rate. This complete inhibition of an enzyme by its sub-
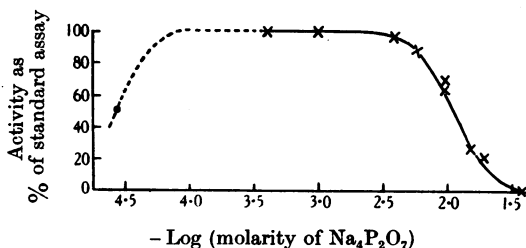

Fig. 4. Effect of substrate concentration on pyrophosphatase activity. M/250-$MgCl_2$, M/35 veronal-acetate buffer pH 7·2. Dotted portion inferred from progress curve, Fig. 3.

strate in comparatively low concentration appears to be unique. In other cases, e.g. liver esterase (Bamann, 1929) and urease (Howell & Sumner, 1934), the activity decreases at fairly high substrate concentrations, but the inhibition is never complete. (The activity-substrate concentration curves for xanthine oxidase, reported by Dixon & Thurlow (1924), are similar to that for pyrophosphatase; the analogy is, however, only superficial, since the effect of substrate on the oxidase is due to competition of xanthine or of hypoxanthine with the methylene blue used as hydrogen acceptor.)

It must be noted here that this substrate effect necessitated the use of low concentrations (M/250–M/1000) of potential substrates in the foregoing specificity experiments.

The effect of other cations on Mg-activated pyrophosphatase. The activity of Mg-activated pyrophosphatase was found by Bauer (1937) to decrease in presence of Ca ions, an effect partially overcome by raising the Mg^{++} concentration. This type of competitive inhibition appears from the work of Greville & Lehmann (1943, 1944) to be applicable to other enzyme systems requiring a divalent ion activator. Thus the Ca-activated adenosine triphosphatase activity of myosin and the Ca-activated process of plasma clotting are both inhibited by Mg ions; conversely, the Mg-activated adenosine triphosphatase activity of electrical tissue is inhibited by Ca ions.

The effect of a number of ions on the purified yeast pyrophosphatase in presence of Mg^{++} has been examined. The experimental conditions were similar to those of the standard test; where necessary, reagents added were first neutralized to the pH of the buffer. Table 1 shows that, at low pyrophosphate

Table 1. *Inhibition of* Mg-*activated pyrophosphatase by cations*

(Standard test conditions, 0·004 M-$MgCl_2$)

$Na_4P_2O_7$ (M)	Ionic ratio [cation]/[Mg]	Percentage inhibition				
		Ca	Zn	Mn	Al	Be
0·0004	1·0	100	100	100	—	100
0·0004	0·1	68	84	86	—	78
0·002	0·5	90	84	77	70	76
0·004	0·1	69	40	20	20	10

concentrations, all ions tested act as effective inhibitors; by increasing the substrate concentration, the effect of Mn^{++}, Al^{+++}, Be^{++} is largely abolished, with that of Ca^{++} remaining unchanged and Zn^{++} occupying an intermediate position. These data are explained by postulating two effects: the one, manifest at low substrate concentrations and due to combination of ion and substrate, such that activity is reduced by substrate depletion; the other, a true inhibition, due to a competition with Mg^{++} for the active centre of the enzyme, as demonstrated by Ca^{++} and to some extent by Zn^{++}. These latter metals are closely related to Mg in the periodic table. The unequivocal resolution of the two effects would probably be achieved at still higher substrate concentrations, but, as already shown, the enzyme is then inhibited by its substrate.

When the Ca effect was examined more systematically, using 0·0004 M-pyrophosphate and (a) constant [Mg^{++}] with varying [Ca^{++}], or (b) variable [Mg^{++}] with constant [Ca^{++}], the activities were found to depend entirely on the [Ca^{++}]/[Mg^{++}]

ratio. All points from all series of experiments thus fall on the one curve of Fig. 5, showing convincingly the competition which exists between Ca^{++} and Mg^{++} for some active group. From the curve, the remarkable effect of Ca^{++} is clearly shown; when, for example, the Ca^{++} concentration is only half that of Mg^{++}, the enzyme is completely inactive, and even a Ca^{++}/Mg^{++} ratio of $1:10$ produces a 65% inhibition.
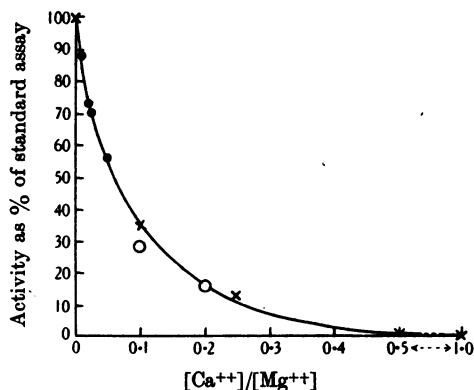

Fig. 5. Pyrophosphatase activity as a function of $[Ca^{++}]/[Mg^{++}]$ ratio. $M/2500$-$Na_4P_2O_7$, $M/35$ veronal-acetate buffer pH 7·2. Cation concentrations: 0·004M-Mg^{++} and varying Ca^{++} (crosses); 0·004M-Ca^{++} and varying Mg^{++} (circles); 0·0004M-Ca^{++} and varying Mg^{++} (dots). In all cases sufficient Mg^{++} was present to activate the enzyme completely in absence of Ca^{++}.

Inhibitors. The inhibitor after dissolving in water was added to the enzyme diluted under the conditions of the standard activity assay. After 15 min. at 38°, the pyrophosphate was added to a concentration of 0·0004M. The most powerful inhibitors, NaF, Cu^{++} (as sulphate), and $CH_2ICOONa$ gave 50% inhibition at molarities of 2×10^{-5}, 4×10^{-5}, and 5×10^{-3} respectively. Metaphosphate (0·01M) and cyanide (0·002M) were without effect.

The inhibition produced by alloxan (50% with 0·01M-reagent) is readily reversed by addition of 0·04M-cysteine, but not by KCN—results which suggest that SH groups are essential for the enzyme activity (cf. Hopkins, Morgan & Lutwak-Mann, 1938). In this connexion it was found that crude pyrophosphatase preparations were more active by some 20% in 0·02M-cysteine. The purified enzyme, freshly diluted, does not respond to activation in this way, but when the activity is allowed to fall by about 40% after the enzyme is stored in high dilution at pH 7 and 0° for 2 weeks, a partial reactivation occurs both in presence of cysteine and of KCN. The inhibition of the enzyme by iodoacetate gives further evidence of its SH character.

SUMMARY

1. The pyrophosphatase of baker's yeast has been purified. The preparation, having at 38° a Q_P value of 10^5, showed only a trace of impurity in the Tiselius electrophoretic apparatus.

2. Inhibition and reversal experiments suggest that pyrophosphatase is an SH enzyme, specifically activated by the Mg ion.

3. Specificity studies indicate that only inorganic pyrophosphate is attacked, the optimal pH being 6·9–7·0.

4. The enzyme has a very high affinity for its substrate (Michaelis constant $c.\ 3 \times 10^{-5}$), but is completely inactive in substrate concentrations greater than 0·03M, an effect reversible by dilution.

5. The activation by Mg^{++} is antagonized by Ca^{++} to an extent depending only on the $[Ca^{++}]/[Mg^{++}]$ ratio.

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