2. Trichloroacetic acid used for the removal of protein may interfere with colour development when Crandall & Anderson's method is used.

3. By increasing the concentrations of ferric nitrate and nitric acid in the reagent to optimum conditions, quantitative results were obtained.

4. A photoelectric method for the determination

of thiocyanate in blood serum which gives accurate results is described.

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A New Method for the Oxidation of Aneurin to Thiochrome and a Procedure for the Determination of Aneurin in Oats

By W. I. M. HOLMAN, *The Rowett Institute, Aberdeen*

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It was desired as part of an investigation on the composition of oats to determine the aneurin content of a large number of samples with sufficient accuracy to distinguish relatively small differences. The methods of Hennessy & Cerecedo (1939) and Harris & Wang (1941) did not give in our hands the required degree of precision. The method of Nicholls, Booth, Kent-Jones, Amos & Ward (1942) for flour is not applicable to oats since the sample is not digested with a proteolytic enzyme before extraction and only free aneurin is determined. It was considered advisable to study in some detail the oxidation of aneurin to thiochrome, the extraction of aneurin from oats and the purification of extracts by the use of a selective adsorbent.

Existing methods for the determination of aneurin by oxidation to thiochrome are modifications of the method of Jansen (1936). Various workers have drawn attention to factors likely to lead to error in the application of this method, viz. ferricyanide concentration (Nicholls *et al.* 1942); partition between aqueous and *isobutanol* layers (Herd, Mundy & Ridyard, 1943); instability of *isobutanol* solutions of thiochrome in ultra-violet (u.v.) light (Nicholls *et al.* 1942; Wokes, Organ, Still & Jacoby, 1944).

EXPERIMENTAL

Oxidation of aneurin to thiochrome

In a search for an oxidizing agent weaker than ferricyanide, which might render the oxidation less difficult to control and the extraction with *isobutanol* unnecessary, a number of metal oxides used as

oxygen electrodes was examined. Such oxides provide a small but constant partial pressure of oxygen. Heating an alkaline solution of aneurin with HgO for 10 min. at 100° was found to be the most promising, but the presence of solid HgO caused erratic results, apparently because of adsorption of thiochrome on the HgO. A saturated solution of HgO in 25% (w/v) KCl solution which is alkaline in reaction gave not only reproducible results but also a much greater yield of thiochrome, as indicated by the intensity of its fluorescence after heating for 10 min. at 100°.

A more satisfactory method of preparing such a solution is by mixing solutions of HgCl₂, KCl (or NaCl) and KOH (or NaOH):

Precipitation of mercuric oxychloride does not occur if the proportion of HgCl₂ is sufficiently small. When 5 ml. of 25% (w/v) KCl solution were used the maximum amount of 1% HgCl₂ solution which could be added without risk of precipitation of mercuric oxychloride when the solution was subsequently made alkaline was found to be 1 ml. To 5 ml. of a freshly prepared solution of aneurin in 25% KCl was added 1 ml. of 1% HgCl₂ solution followed by 4 ml. of NaOH solution. A fluorescence developed which, when the solution was heated, soon reached its maximum intensity. The rate of development increased as the temperature was raised, but destruction of thiochrome occurred at high temperatures. At 40°, the most suitable temperature, the fluorescence reached its maximum intensity in 15 min., and destruction did not occur on further heating. The concentration of alkali used affected the development of the fluorescence slightly, but the temperature of the solution when the alkali was added had a greater effect. The maxi-

imum intensity of fluorescence obtainable by suitable heat treatment after the addition of the alkali varied according to the temperature of the solution when the alkali was added. The highest result was obtained by adding the alkali at 0° and then heating the solution for 15 min. at 40°.

It was found necessary always to mix the HgCl_2 completely with the KCl solution before adding the alkali, otherwise mercuric oxychloride was precipitated and did not subsequently dissolve, leading to low and erratic results. It was also found essential for the solution of aneurin in 25% (w/v) KCl to be neutral or acid in reaction and for the alkali to be added after the HgCl_2 . Otherwise the development of the fluorescence was almost entirely suppressed. A faint opalescence appeared when the alkali was added and the solution heated, but it could be dispersed by addition of an equal volume of acetone, which also increased the intensity of fluorescence. A higher proportion of acetone caused precipitation of KCl.

Fig. 1. Calibration curves for thiochrome and quinine sulphate using the Spekker fluorimeter.

When suitable conditions were adopted, highly reproducible results were obtained for the oxidation of 0.2 μg. aneurin. The fluorescence of the solution in u.v. light was matched in a Spekker fluorimeter against an equal volume of a standard solution of quinine sulphate in 0.1 N- H_2SO_4 containing 1 μg./ml., with a violet filter (no. 7) in front of the right photocell. The aneurin content could be obtained from the drum reading by reference to a calibration curve prepared with known amounts of pure aneurin. Such a curve is shown in Fig. 1. It is apparent that the drum reading does not decrease proportionately as the amount of aneurin increases. That this lack of proportionality does not arise from a variation in the proportion of thiochrome produced from different amounts of aneurin is indicated by

the close similarity between the curve for aneurin and that for quinine sulphate, which is also included in the figure. It should be noted that the drum reading of the fluorimeter decreases as the intensity of fluorescence increases.

The results tabulated in Table 1 illustrate the effects of varying the experimental conditions on the values obtained as indicated by the drum readings. Except where otherwise stated, 4 ml. of 0.06 N-NaOH were added at room temperature, the fluorescence was developed by heating for 15 min. at 40° and, on cooling, 10 ml. of acetone were added. The tabulated results furnished a guide for establishing the procedure outlined on p. 394. It may be noted that with the present procedure the intensity of fluorescence decreases very gradually with prolonged exposure to u.v. light, the loss during 5 min. being equivalent to only about 3% of the thiochrome. The band of u.v. light passes through only a small part of the solution and deterioration is confined to that region. It has been found that if the solution is mixed before each reading, at least four successive readings, each involving a 15 sec. exposure, may be taken without any detectable diminution of the fluorescence.

The use of Decalso for the adsorption of aneurin

The method described above was applicable only to pure solutions of aneurin. Extracts of oats contain extraneous materials which fluoresce in u.v. light, as well as substances which interfere with the oxidation to thiochrome. The bulk of these impurities must be removed before the oxidation to thiochrome is performed. Several adsorbents were examined for this purpose, viz. Decalso, Superfiltrol and Lloyd's reagent. Decalso, although a much weaker adsorbent than Lloyd's reagent or Superfiltrol, was found to be much more selective, and has the further advantage that adsorbed aneurin may be readily eluted with 25% KCl solution. Lloyd's reagent and Superfiltrol adsorbed a greater proportion of impurities along with the aneurin, and aneurin could only be eluted by an alkaline solution. In such cases it would therefore be necessary to oxidize to thiochrome in the presence of the adsorbent. Jowett's (1940) technique was tested in preference to that of Hennessy & Cerecedo (1939). It requires the use of much smaller amounts of adsorbent and in consequence the adsorption of impurities is greatly reduced. The procedure is more rapid and is capable of being more closely standardized.

The effect of various factors on the adsorption of aneurin by powdered Decalso was therefore studied using either Walpole's acetate buffers diluted from 0.2 to 0.05 M or Clark & Lubs's 0.05 M-phthalate buffers. The technique employed was as follows:

The solution containing aneurin was mixed with powdered Decalso for 1 min. and, after centrifuging, the supernatant liquid was poured off. When two successive adsorptions were necessary this liquid was transferred to a second tube containing a further lot of Decalso. After mixing and centrifuging, the supernatant liquid was rejected. The residual Decalso in the second tube was transferred with 10 ml. of water to the first tube and the total contents of the tube well mixed. After centrifuging, the supernatant liquid was siphoned off to avoid risk of stirring up the Decalso, which disperses when the salt concentration is reduced. The residue was extracted for several minutes in a boiling water-bath with 10 and then 5 ml. of 25% (w/v) KCl solution and the combined extracts were made up to 15 ml. with KCl solution and mixed; 5 ml. were taken for oxidation to

1. 3 μ g. aneurin adsorbed from 10 ml. 0.05 M-acetate by 0.2 g. Decalso	pH of solution Recovery of aneurin (%)	3.6 87	4.0 93	4.4 95	4.8 96	5.2 95				
2. 3 μ g. aneurin adsorbed from 0.05 M-acetate (pH 4.8) by 0.2 g. Decalso	Volume of solution (ml.) Recovery of aneurin (%)	5 96	10 96	15 96	20 98	25 98				
3. 3 μ g. aneurin adsorbed from 0.05 M-acetate (pH 4.8) by two successive 0.1 g. lots of Decalso	Volume of solution (ml.) Recovery of aneurin (%)	5 99	10 97	15 97	20 99	25 98				
4. 3 μ g. aneurin adsorbed from 10 ml. 0.05 M-phthalate by 0.2 g. Decalso	pH of solution Recovery of aneurin (%)	2.5 84	3.0 90	3.5 92	4.0 93	4.5 87	5.0 81.5	5.5 74	6.0 57	
5. 3 μ g. aneurin adsorbed from 0.05 M-phthalate (pH 4.0) by 0.2 g. Decalso	Volume of solution (ml.) Recovery of aneurin (%)	5 91	10 93	15 91	20 90	25 88				
6. 3 μ g. aneurin adsorbed from 10 ml. 0.05 M-phthalate (pH 4.0) by different amounts of Decalso	Amount of Decalso (g.) Recovery of aneurin (%)	0.02 73	0.04 85	0.06 91	0.08 93	0.10 93	0.15 93	0.20 93		
7. Different amounts of aneurin adsorbed from 10 ml. 0.05 M-phthalate (pH 4.0) by 0.2 g. Decalso	Amount of aneurin (μ g.) Recovery (%)	0.5 100	1.0 96	1.5 94	2.0 95	2.5 95	3.0 93	4.5 93	6.0 92	
8. 3 μ g. aneurin adsorbed from 0.05 M-phthalate (pH 4.0) by two successive 0.1 g. lots of Decalso	Volume of solution (ml.) Recovery of aneurin (%)	5 97	10 100	15 98	20 95	25 92				
Different amounts of aneurin adsorbed from 10 ml. 0.05 M-phthalate (pH 4.0) by two successive 0.1 g. lots of Decalso	Amount of aneurin (μ g.) Recovery (%)	0.5 100	1.0 100	1.5 100	2.0 100	2.5 100	3.0 100	4.5 100	6.0 100	

thiochrome. Under these conditions aneurin was extracted completely from Decalso. No destruction of aneurin occurred at 100° since the extracts were always acid in reaction as a result of base exchange with the Decalso. The adsorption was not improved by raising the temperature or by increasing the time of mixing the Decalso with the solution.

When acetate buffers were used the optimum pH of adsorption was 4.8. The degree of adsorption was not affected by the volume of solution and only very slightly improved by performing two successive adsorptions with Decalso. The values obtained for the recovery of aneurin varied slightly when the experiments were repeated even though the same sample of Decalso was used. A precipitate was formed when acetone was added after the oxidation to thiochrome. This precipitate, which consisted of substances dissolved from the Decalso, was centrifuged off before the intensity of fluorescence was measured.

When phthalate buffers were used the optimum pH was 4.0. The degree of adsorption varied according to the volume of solution and the amounts of aneurin and Decalso present. No precipitate was formed when the acetone was added after the oxidation to thiochrome. Constant results were obtained when the experiments were repeated with the same sample of Decalso. The recovery of aneurin was improved considerably by performing two successive adsorptions and under certain conditions was complete.

Different samples of Decalso were found to vary in activity. Two samples purchased at different times gave 93% recovery when 0.1 g. powdered Decalso was used to adsorb 3 µg. aneurin from 10 ml. of 0.05M-potassium acid phthalate solution, but two further samples gave recoveries of only 56.5 and 85.5%. The activity of these two samples could not be improved by preliminary purification.

The results of a series of experiments are shown in Table 2. The sample of Decalso giving 93% recovery under the conditions specified above was used. It is apparent that the conditions employed in Exp. 9 are satisfactory, but it must be noted that these results apply only when a sample of Decalso of equal or greater activity is used.

Determination of aneurin in oat extracts

Samples of oats after digestion with papain and/or takadiastase (see next section) were extracted with 0.05M-potassium acid phthalate solution and analyzed according to the technique used in Exp. 9, Table 2. Unsatisfactory results were obtained owing to the presence of impurities. It was found that two successive adsorptions were inadequate but that after five adsorptions no further aneurin was recovered. Recovery values for added aneurin were still low, and since similar low recoveries for aneurin added to the final KCl extracts were obtained it is obvious that

Table 3. *Effect of different enzyme treatments on the extraction of aneurin from oats*

Method of extraction of aneurin	Weight* of dry matter (g.)	Aneurin added (µg.)	Aneurin found (µg.)	Aneurin content of dry matter (µg./g.)	Recovery of added aneurin (%)	Degree of proteo- lysis (%)
Oatmeal						
No digestion with enzymes: two extractions at 70° with 0.05M-phthalate	1.3693	Nil	5.36	3.91	—	—
	1.3693	1.5	6.87	—	100.7	—
	1.3693	3.0	8.19	—	94.3	—
Digestion with 0.1 g. takadiastase: two cold extractions with phthalate	1.3700	Nil	6.02	4.39	—	3.1
	1.3718	1.5	7.47	—	96.0	—
	1.3688	3.0	9.00	—	99.7	—
Digestion with 0.05 g. papain and 0.1 g. takadiastase	1.3708	Nil	6.87	5.01	—	7.9
	1.3692	3.0	9.84	—	99.3	—
Digestion with 0.1 g. papain and 0.1 g. takadiastase	1.3701	Nil	7.50	5.47	—	10.4
	1.3667	3.0	10.50	—	101.0	—
Digestion with 0.15 g. papain and 0.1 g. takadiastase	1.3690	Nil	6.87	5.02	—	9.8
	1.3690	3.0	9.96	—	103.0	—
Digestion with 0.2 g. papain and 0.1 g. takadiastase	1.3637	Nil	6.57	4.82	—	9.8
Oats (with husks); variety 'Star'						
No digestion with enzymes: two extractions at 70° with 0.05M-phthalate	1.3135	Nil	4.44	3.38	—	—
	1.3148	1.5	5.97	—	101.3	—
	1.3139	3.0	7.47	—	101.0	—
Digestion with 0.1 g. takadiastase: two cold extractions with phthalate	1.3168	Nil	3.99	3.03	—	—
	1.3162	1.5	5.55	—	104.0	—
	1.3168	3.0	7.01	—	100.7	—
Digestion with 0.5 g. papain and 0.1 g. takadiastase	1.3161	Nil	5.58	4.24	—	—
	1.3168	3.0	8.64	—	102.0	—
Digestion with 0.1 g. papain and 0.1 g. takadiastase	1.3255	Nil	5.70	4.30	—	—
	1.3251	3.0	8.66	—	98.7	—
Digestion with 0.15 g. papain and 0.1 g. takadiastase	1.3213	Nil	5.40	4.09	—	—
	1.3223	3.0	8.27	—	95.3	—
Digestion with 0.2 g. papain and 0.1 g. takadiastase	1.3273	Nil	5.40	4.07	—	—

* Approximately 1.5 g. of fresh material were weighed out in every case and the exact corresponding weight of dry matter is given.

impurities adsorbed along with the aneurin and extracted by the KCl solution interfered with the oxidation to thiochrome. The interfering substances could not be removed from the oat extracts by ultrafiltration, solvent extraction or precipitation methods. The error arising from their presence could be eliminated by destroying the aneurin in part of the KCl extract with sulphite, removing SO_2 and then adding definite amounts of aneurin to samples of the solution and oxidizing to thiochrome.

4 μg . aneurin dissolved in 12 ml. of 25% (w/v) KCl solution were completely destroyed by heating for 1 hr. at 100° with 0.2 ml. of 12.5% (w/v) $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ solution. If the solution was acidified with HCl and SO_2 removed by aspiration there was no interference with the oxidation of aneurin added after the destruction. Oat extracts were analyzed by adsorbing the aneurin from 10 ml. by five successive 0.1 g. lots of Decalco, extracting the combined lots of Decalco with 15 ml. of 25% KCl solution and taking 3 ml. of the extract for oxidation to thiochrome in the usual manner, 2 ml. of 25% KCl solution being added before the HgCl_2 and NaOH solutions. The aneurin in the remaining 12 ml. of the KCl extract was destroyed by neutralizing with NaOH and heating for 1 hr. at 100° with Na_2SO_3 ; after removal of SO_2 , the solution was made up to 12 ml., and 3 ml. portions included in a series of standards before oxidation to thiochrome. The aneurin content of the test solution was obtained by plotting the drum readings given by the three standards against the corresponding amounts of aneurin added and interpolating on the curve.

This technique is capable of giving accurate results only if there is no appreciable alteration of substances other than aneurin during the heat treatment with Na_2SO_3 . The 'blank' fluorescence was unaltered when the heat treatment was extended to 2 or 3 hr., and it is therefore unlikely that it was affected by heating for 1 hr. The recovery of different amounts of aneurin added to oat extracts was complete, thus indicating that no serious change occurred in the substances which interfere with the oxidation to thiochrome.

The results of a series of recovery tests are shown in Table 3. Aneurin was added initially to weighed samples of oats before extraction. Different methods of extraction were used. The recoveries are in all cases satisfactory.

Extraction of combined aneurin from oats

The method described above was used to study the effect of different enzyme treatments on the extraction of aneurin from oats. Weighed samples of oats were heated for 5 min. at 100° with 20 ml. of 0.05 M-potassium acid phthalate solution to destroy natural enzymes. Destruction of aneurin

does not occur under these conditions if sulphites are absent. Different amounts of papain and takadiastase were added to the cooled samples, which were then digested for 16 hr. at 37° . A little chloroform was added to inhibit growth of micro-organisms. After the extracts had been centrifuged their aneurin content was determined. The results, which are given in Table 3, show that considerably more aneurin is extracted after digestion with papain than when no enzyme or takadiastase alone is used and that 0.1 g. papain liberates more aneurin than 0.05 or 0.15 g. To estimate the extent to which the proteins of oats were hydrolyzed by these different treatments, the amino N content of the extracts was determined by the copper method of Pope & Stevens (1939). 'Blanks' on the enzymes were subtracted and the results, expressed as percentages of the total N in the sample, are included in Table 3. It is apparent that in all cases the proteins are hydrolyzed to only a small extent.

It is possible that further hydrolysis of the proteins may liberate more combined aneurin. An experiment was therefore conducted using 0.1 g. papain and 0.1 g. takadiastase with the addition of 1 ml. of 4% HCN to increase the proteolytic activity, the samples being digested for either 16 or 48 hr. at 37° . The effect of digesting for 16 hr. at 37° with 0.1 g. pepsin in 0.2% HCl, followed by digestion for 16 hr. at 37° with 0.1 g. takadiastase at pH 4.0, was also tested. The extracts were analyzed for aneurin and amino N. It was found necessary to dilute the extracts with 0.05 M-potassium acid phthalate solution before making the aneurin determinations, otherwise the greater amounts of impurities present caused low results to be obtained. The results, which are given in Table 4, show that although the degree of proteolysis is increased considerably, no more aneurin is extracted than when 0.1 g. papain was used without HCN. It is therefore probable that this maximum value represents the total aneurin content.

Procedure for determination of aneurin in oats

Reagents. The reagents should be prepared from 'Analar' grade chemicals and distilled water showing no fluorescence. Glassware must be scrupulously clean.

(1) 0.05 M-potassium acid phthalate solution. Dissolve 10.21 g. $\text{COOH} \cdot \text{C}_6\text{H}_4 \cdot \text{COOK}$ in distilled water and dilute to 1 l.

(2) Papain and takadiastase. The enzyme preparations used should be tested for freedom from aneurin. Takadiastase containing either talc or lactose as a diluent may be used.

Table 4. *Effect of digestion with papain and HCN or pepsin on the extraction of aneurin from oatmeal*

Method of extraction of aneurin	Weight* of dry matter (g.)	Aneurin added (μg .)	Aneurin found (μg .)	Aneurin content of dry matter ($\mu\text{g}/\text{g}$.)	Recovery of added aneurin (%)	Degree of proteo- lysis (%)
Digestion with 0.1 g. papain, 0.1 g. takadiastase and 1 ml. of 4% HCN for 16 hr. at 37°	1.3697	Nil	7.56	5.52	—	21.6
	1.3688	3.0	10.50	—	98.3	—
Digestion with 0.1 g. papain, 0.1 g. takadiastase and 1 ml. of 4% HCN for 48 hr. at 37°	1.3697	Nil	7.56	5.52	—	29.8
	1.3682	3.0	10.44	—	96.0	—
Digestion with 0.1 g. pepsin followed by 0.1 g. takadiastase	1.3687	Nil	7.44	5.43	—	27.3
	1.3681	3.0	10.26	—	94.3	—

* See footnote to Table 3.

(3) Powdered Decalso. Grind the 60-90-mesh Decalso supplied by the Permutit Co. Ltd. in a mortar and pass through a 200-mesh sieve. Stir with distilled water and centrifuge for 30 sec. at 3000 r.p.m. Pour off the supernatant liquid and stir the residue with distilled water. Centrifuge for 30 sec. and pour off the supernatant liquid. Transfer to an evaporating basin and dry for several hours in an oven at 100°. Pass through the 200-mesh sieve to remove lumps.

Different batches of Decalso may vary in their ability to adsorb aneurin. A sample is suitable for use if 0.1 g. adsorbs over 90% of the aneurin from a solution of 3 μ g. aneurin in 10 ml. of 0.05 M-potassium acid phthalate solution.

(4) 25% (w/v) KCl solution.

(5) 12.5% $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ solution. Dissolve 2.5 g. $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ in distilled water by warming. Cool and dilute to 20 ml. Prepare the solution freshly before use.

(6) 1% HgCl_2 solution.

(7) 0.06 N-NaOH solution. Prepare a CO_2 -free solution and protect from atmospheric CO_2 by means of a soda-lime tube.

(8) Acetone. Distil from an all-glass apparatus before use. Recover acetone from residues by distillation; then re-distil from an all-glass apparatus.

(9) Standard solution of quinine sulphate. To prepare a stock solution dissolve 20 mg. quinine sulphate (dried at 100°) in 0.1 N- H_2SO_4 and dilute to 1 l. Store in the dark. To prepare a standard solution containing 1 μ g./ml., dilute 5 ml. of the stock solution to 100 ml. with 0.1 N- H_2SO_4 solution.

(10) Standard solution of aneurin. Prepare a stock solution by dissolving 50 mg. pure synthetic aneurin in distilled water and diluting to 500 ml. This solution keeps satisfactorily for at least 3 months when stored in a refrigerator. To prepare a standard solution containing 1 μ g./ml., dilute 1 ml. of the stock solution to 100 ml. with 25% KCl solution. This solution must be prepared immediately before use. If results based on moisture-free aneurin are desired, determine the moisture content of the aneurin separately by drying over H_2SO_4 or at 100° and correct the results accordingly.

Procedure. Grind the sample of oats in a mill, taking care to avoid loss of any fraction. Weigh out 1.5 g. and transfer to a pyrex boiling tube. Add 20 ml. phthalate solution, mix and heat in boiling water for 5 min. with frequent mixing. Cool and add 0.1 g. papain, 0.1 g. takadiastase (both measured by volume) and 3 drops of chloroform. Stopper the tube and incubate for 16 hr. at 37°, mixing at intervals for the first few hours. Transfer to a centrifuge tube and, after centrifuging for 5 min. at 3000 r.p.m., transfer the supernatant liquid to a tube marked at the level corresponding to 30 ml. Wash the residue in the boiling tube into

the centrifuge tube with 10 ml. phthalate solution and stir up the residue in the centrifuge tube. Centrifuge for 5 min. and transfer the supernatant liquid to the tube marked at 30 ml. Adjust the combined extract to pH 4.0 by adding N-HCl drop by drop and testing a micro-drop of the extract with bromocresol green. Make up to 30 ml. with phthalate solution, mix and centrifuge for 5 min.

Transfer 10 ml. of the extract to a centrifuge tube containing 0.1 g. Decalso (measured by volume). An 18 ml. round-bottomed tube is suitable. Mix well and centrifuge for 2 min. at 3000 r.p.m. Transfer the supernatant liquid to a second centrifuge tube containing 0.1 g. Decalso, mix and centrifuge for 2 min. Transfer the supernatant liquid to a third tube containing 0.1 g. Decalso and continue this process until five successive adsorptions with 0.1 g. Decalso have been made; then discard the supernatant liquid. Add 5 ml. distilled water to the first tube, washing down the sides and stirring up the Decalso and transfer the Decalso from the second tube to the first with 5 ml. distilled water. Add 5 ml. distilled water to the third tube, washing down the sides and stirring up the Decalso and transfer the Decalso from the fourth and fifth tubes to the third with 5 ml. distilled water in both cases. Centrifuge the first and third tubes for 5 min. and siphon off the supernatant liquid. Transfer the Decalso from the third tube to the first with 10 ml. of 25% KCl solution and heat in a boiling water-bath for several minutes with frequent mixing. Cool, centrifuge for 5 min. and transfer the supernatant liquid to a pyrex boiling tube marked at levels corresponding to 12 and 15 ml. To the residue in the centrifuge tube add 5 ml. of 25% KCl solution, heat in boiling water, cool and centrifuge for 5 min. Transfer the supernatant liquid to the tube marked at 12 and 15 ml. and make up to 15 ml. with 25% KCl solution.

Transfer 3 ml. of the KCl extract to a boiling tube and add 2 ml. of 25% KCl solution. This is the test solution. To prepare standards add 0.05 ml. 0.5 N-NaOH and 0.2 ml. Na_2SO_3 solution to the remaining 12 ml. of KCl extract, mix and heat in a boiling water-bath for 1 hr. Cool, acidify with 0.2 ml. N-HCl and wash down the sides with a little distilled water. Remove SO_2 by evacuating the tube while drawing air through the solution from a fine capillary. Almost neutralize the solution (0.1 ml. of 0.5 N-NaOH), checking the pH of a micro-drop with bromocresol green to make certain that the solution is still acid and make up to 12 ml. with distilled water. Transfer 3 ml. to each of three boiling tubes. Add a different amount of the standard solution of aneurin to each of the three tubes, the amounts varying over the range expected in the test solution. Then add sufficient 25% KCl solution to bring the volume to 5 ml.

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 0.06N-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 Decalco 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

BY K. BAILEY AND E. C. WEBB, *Biochemical Laboratory, Cambridge*

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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