DISCUSSION

The enzyme described has three properties in common with the cellulase of *Helix* and those of many moulds, it reduces the viscosity of a solution of sodium carboxymethylcellulose, it splits cellulose, and its optimal pH range is between 5 and 6. It has not been possible to show unequivocally that it is a cellulase, because it has not been got in a concentrated enough state to produce detectable breakdown of a completely unsubstituted cellulose. If it is, in fact, a cellulase, then it may be concerned in the breakdown of the fine cellulose fibrils of the primary cell wall of growing cells, some evidence for which has been supplied by Frey-Wyssling (1949). To explain occurrence of the enzyme in relatively higher concentrations in roots, where elongation and

renewal of root hairs is a continuous process, and in young leaves where growth is still going on, and the very low concentrations of enzyme got by extracting whole organs, it is reasonable to postulate a localization of the enzyme to the very small proportion of cells in which primary cell-wall modification is in process.

SUMMARY

1. An enzyme that can depolymerize sodium carboxymethylcellulose, and split reprecipitated cellulose has been found in tobacco plants.

2. The enzyme, which is similar in properties to animal and fungal cellulases, is present in very low concentrations. It is suggested that this may be explained by its localization in cells in which reconstruction of the thin primary cell wall is occurring as a concomitant of cell growth.

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A Colorimetric Method for the Determination of Pentoses in the Presence of Hexoses and Uronic Acids

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A method for the determination of pentoses in the presence of large amounts of hexoses and uronic acids has been developed for use in the examination of extracts of leaf fibre after enzyme treatments. Methods previously described are not satisfactory, owing to interference by hexoses or uronic acids or to lack of sensitivity. The survey by White & Green (1932) of colour tests for sugars and their derivatives in urine indicated that their qualitative 'aniline' test for pentoses might be developed. In this test, the sugar solution with an equal volume of glacial acetic acid and a few drops of aniline is heated to boiling, allowed to stand for 2 min., cooled and the colour extracted with chloroform. A quantitative modification of this test is now described, in which the interference by glucose and other sugars is reduced by allowing the reaction to take place at room temperature. It has the advantage that little manipulation or attention is required.

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EXPERIMENTAL

Method

100 ml. glacial acetic acid, 10 ml. 5% (w/v) aqueous oxalic acid, 24 ml. water and 16 ml. colourless aniline are mixed and stored in a dark-glass bottle. The reagent should be stored out of direct light and not be used more than a week after preparation. Reagent (6 ml.) is added from a burette to each of a series of test tubes (75 × 15 mm.) matched for use in a photoelectric absorptiometer (the Evans Electro-selenium Portable model was used). The solution to be tested (containing 10–80 µg. pentose) is added, together with enough water to bring the final volume to 8 ml. and the contents of the tube well mixed. At the same time a blank (2 ml. water + 6 ml. reagent) and two standards of xylose (10 and 50 µg.) are set up. If it is known that the pentose in the solution is not xylose, standards of that pentose are employed. Standard solutions of pentose containing 50 µg./ml. are conveniently made up in saturated benzoic acid solution. These standards do not deteriorate at room tem-

perature over long periods. The tubes are then kept for 20–24 hr. in the dark at room temperature. The colour developed is measured with the absorptiometer using Ilford filter 622, the reagent-water blank being used for setting the instrument. The amount of pentose present is then calculated from the readings obtained on the standards by direct proportion, for the relation between instrument reading and the amount of pentose present is linear over the range used.

Factors influencing colour development

In examining the effect of varied conditions on the specificity and sensitivity of the test, glucose and galactose were tested in addition to pentoses, as preliminary tests had shown that these two sugars were the most important interfering substances. Sensitivity was determined by calculating the scale reflexion/mg. sugar produced on the absorptiometer using filter 622, and interference by calculating the deflexion/mg. as a percentage of that produced by xylose under the same conditions.

Fig. 1. Variation of specificity and sensitivity with final aniline concentration. Interference as scale deflexion/mg. sugar as percentage of that for xylose under same conditions. Sensitivity as scale deflexion/mg. xylose under conditions shown. Times are period of colour development at room temperature.

Amount of aniline. Colour formation at room temperature during a period less than 48 hr. increases with the amount of aniline present in the test solution over a range of 5–12% (v/v). After a concentration of 8% is reached, the increase for xylose is slower than the increase with glucose and galactose. 8% was therefore chosen for the final concentration of aniline in the test solution as it gives nearly maximal colour with xylose and higher or lower concentrations increase interference by glucose and galactose (Fig. 1).

Time of reaction. At room temperature, maximum colour formation is reached with xylose after about 1 day. The colour is stable for a further day and then begins to fade. With galactose, colour formation continues for about 2 days, and remains steady for a further 3 days or more. Colour formation continues with glucose over a period of many days. Maximum sensitivity and specificity is thus got by reading the colours developed at the end of a period of about 24 hr. (Fig. 2).

Composition of reagent. Adams & Castagne (1948) investigated the effect of salts on the stability of the colour produced by furfural with aniline acetate. Although the colour developed in the present estimation is not due to

furfural (see below, p. 435), the effects of NaCl, Na_2HPO_4 and oxalic acid were investigated, all of which Adams & Castagne found to stabilize and deepen the colour given by furfural. All three substances were added to the original acetic acid-aniline mixture individually and in all combinations. The results showed that only oxalic acid had any effect on colour development. The absorptions given by glucose and galactose using filter 622 were reduced by about 50 and 20% respectively after 24 hr. at room temperature, and the xylose colour was increased by about 50%. Since oxalic acid effected an increase both in specificity and sensitivity it was incorporated in the reagent. Oxalic acid had the further effect of reducing the development of colour in the reagent during storage.

Fig. 2. Variation of sensitivity and specificity with time of development. Standard method, colour development at room temperature. For definitions, see text and Fig. 1.

Additions to the reagent. The addition of ethanol was found to increase the sensitivity of the method, but unfortunately its specificity was reduced. At 24 hr. the presence of ethanol in a final concentration of 12.5% (v/v) had resulted in a 40% increase in the colour given by glucose and xylose, and a 70% increase in that given by galactose. Owing to the increased interference by galactose, ethanol was not incorporated in the reagent. Colour development in Thunberg tubes after de-aeration was found to reduce colour formation by xylose and glucose by 75% and by galactose by 50%. H_2O_2 in a final concentration of 0.2M or continuous aeration during colour development had little effect as compared with development in open tubes.

Light. The amount of colour developed by glucose is unaffected by light. Colour development by xylose and galactose is reduced by light, by amounts depending on the light intensity. The reduction is greater for xylose than for galactose. Development was accordingly carried out in the dark.

Age of reagent. The colour of the reagent deepens with time, when stored in the dark at room temperature. There is little deterioration for the first week, but after longer periods the colour increase due to a given amount of sugar over the blank value decreases markedly.

Temperature. The time for development of maximum colour with xylose is approximately halved by a 10° rise in temperature. Thus times observed at a number of temperatures were: 100° , 6 min.; 45° , 3.5 hr.; 35° , 7 hr.; 25° , 15 hr.

The sensitivity of the reaction with respect to xylose is little affected by increased temperature. There is, however, a fall in specificity. Interference by galactose at 100° , 45° , 35° and 25° was 23, 11, 9.5 and 8.5% respectively, and by glucose at 100° , 45° , 35° and 25° was 9.5, 4.4, 4.1 and 3.7% respectively. Interferences were measured at the time at which maximum xylose colour had been developed.

Amount of sugar. Percentage absorption by xylose is linearly related to the amount of sugar in the sample over the range 10–100 μg . (Fig. 3). Percentage absorption by glucose (0.2–2 mg.) and by galactose and galacturonic acid (0.06–0.6 mg.) was also proportional to the amount present in the test solution.

Fig. 3. Variation in absorption produced with amount of xylose in solution. Standard method, colour development at room temperature.

The nature of the reaction

The colour produced by pentoses under the conditions described is not due to the formation of furfural by the acid followed by the reaction of furfural with the aniline present in the reagent. Heating the pentose at 100° with acetic acid followed by addition of aniline gives a faint pink colour due to furfural, which fades in the course of an hour or so. On keeping the mixture at room temperature, colour development occurs in the normal way and the amount of colour found is the same as that formed by the same amount of pentose treated with full reagent for the same time at room temperature. Similarly, if aniline is added to a pentose-acetic acid mixture, that has been kept at room temperature 24 hr., there is no colour production until a further 24 hr. has elapsed. Furfural (20 μg .) gives an immediate pink colour (sharp maximum absorption at approx. 518 $\text{m}\mu$.) with the reagent, having a density approximately that given by 50 μg . xylose. This colour fades after 2 or 3 hr., and has gone at the end of 24 hr.

The colours produced by pentoses appear to result from a reaction in which the sugar, aniline in great excess, and O_2 take part. The approximate molar ratios of pentose, dissolved O_2 and aniline in the final mixture are 1:14:20,000.

Since aniline must be present in such great excess it seems possible that colour formation is due to the reaction of the pentose, or a derivative of the pentose, with an oxidation product of aniline present only in traces.

Reduction of the amount of dissolved O_2 by evacuation led to a reduction in colour formation, but apparently so little O_2 is used that increasing the O_2 supply by aeration has little effect.

Sensitivity and specificity

Pentoses. The colour developed by ribose is the same as that given by xylose, but only 85% of its density for an equal weight. For arabinose the colour density for an equal

Table 1. *Interference with the colour reaction for pentoses by sugars and related compounds*

(Measurements were made at time of maximum colour development by xylose. Since with most compounds colour development continues after xylose has reached its maximum, interference values vary with time.)

		Absorption/mg. (as percentage of maximum absorption/mg. for xylose)
Combined pentose	Xylan	<1
	Tobacco mosaic virus	<1
	Yeast nucleic acid	<1
Pentoses	Xylose	100
	Ribose	85
	Arabinose	77
Deoxyhexoses	Fucose	1.6
	Rhamnose	<1
	Digitoxose	<1
Aldohexoses	Glucose	1.7
	Mannose	1.3
	Galactose	4.8
Ketohexoses	Fructose	<1
	Sorbose	<1
Related compounds	Glucosamine HCl	<1
	Mannitol	<1
	Dulcitol	<1
	Inositol	<1
	Galacturonic acid	5.0
	Ascorbic acid	<1
Disaccharides	Maltose	<1
	Cellobiose	<1
	Trehalose	<1
	Sucrose	<1
	Melibiose	<1
Oligosaccharides	Melzitose	<1
	Raffinose	<1

weight is 77% of that of xylose. This is similar to the behaviour of pentoses in other colour tests, and in tests dependent on the isolation of furfural by distillation of a pentose with mineral acids. The lower level of 5 μg . xylose/ml. that can be accurately measured by this method may be increased if large volumes of sugar solution are available, by carrying out the colour development in larger volumes, and extracting the colour with CHCl_3 . No colour was given by combined pentose in the form of tobacco mosaic virus, maize xylan, or yeast nucleic acid.

Other sugars. In Table 1 are listed the results obtained by testing a number of sugars. The main sugars likely to interfere are glucose, galactose and galacturonic acid. Inter-

ference by these sugars was also found by Dunstan & Gillam (1949) in a method involving conversion to furfuraldehyde by distillation from 85% H_3PO_4 . The colours given by glucose, galactose and galacturonic acid differ from that given by pentose. Light absorption curves (obtained with a Unicam spectrophotometer) at the time of maximum colour development with xylose are given in Figs. 4 and 5.

Fig. 4. Absorption curves for colour produced by xylose, glucose and galacturonic acid. Measurements made at time of maximum colour development by xylose. 50 μg . xylose, 1 mg. glucose, and 1 mg. galacturonic acid were present in the solutions tested under standard conditions.

The galactose colour is dependent on the amount of aniline in the reagent. With low concentrations of aniline the colour is olive green, with the normal reagent the colour is reddish brown (Fig. 5). The olive-green colour appears to be dependent on the presence of oxalic acid in the reagent, for it does not appear in its absence at low aniline concentrations.

SUMMARY

1. A method using an aqueous solution of aniline, acetic acid and oxalic acid for the colorimetric

estimation of pentoses in the presence of large amounts of other sugars is described. It is suitable for the determination of 10–100 μg . of xylose.

2. Glucose gave about 2% and galactose and galacturonic acid about 5% of the colour intensity from an equal weight of xylose. Interference by other sugars tested was less than 1%.

Fig. 5. Variation in absorption by galactose with final concentration of aniline in solution (shown on figure as % v/v). 0.5 mg. galactose in standard conditions except that composition of reagent varied as shown.

3. The effect of varying conditions on the specificity and sensitivity of the reaction was determined and optimal conditions are described. Increased temperature, time of development and concentration of aniline all effect colour development by the sugars examined, but not equally. The presence of oxalic acid increases the specificity of the test.

4. The colour formed is not due to furfural.

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