- Drury, H. F. (1948). *Arch. Biochem.* **19**, 455.
 Gomori, G. (1942). *J. Lab. clin. Med.* **27**, 955.
 Johanson, R. (1953). *Nature, Lond.*, **171**, 176.
 Kamen, M. D. & Spiegelman, S. (1948). *Cold Spr. Harb. Symp. quant. Biol.* **13**, 151.
 Kreger, D. R. (1954). *Biochim. biophys. Acta*, **13**, 1.
 Meibbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
 Meyerhof, O. & Schulz, W. (1936). *Biochem. Z.* **287**, 206.
 Myrbäck, K. (1949). *Ergebn. Enzymforsch.* **10**, 168.
 Northcote, D. H. (1953). *Biochem. J.* **53**, 348.
 Northcote, D. H. & Horne, R. W. (1952). *Biochem. J.* **51**, 232.
 Salkowski, E. (1894). *Ber. dtsch. chem. Ges.* **27**, 497.
 Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
 Sevag, M. G., Cattaneo, C. & Maiweg, L. (1935). *Liebigs Ann.* **519**, 111.
 Trevelyan, W. E., Forrester, R. S. & Harrison, J. S. (1952). *Nature, Lond.*, **170**, 626.
 Trevelyan, W. E. & Harrison, J. S. (1952). *Biochem. J.* **50**, 298.
 Trevelyan, W. E. & Harrison, J. S. (1954). *Biochem. J.* **57**, 556.
 Trevelyan, W. E. & Harrison, J. S. (1956a). *Biochem. J.* **62**, 177.
 Trevelyan, W. E. & Harrison, J. S. (1956b). *Biochem. J.* **62**, 183.
 Volkin, E. & Cohn, W. E. (1954). *Methods of Biochemical Analysis*, vol. 1, p. 287. Ed. by Glick, D. New York: Interscience.
 Whistler, R. L. & Smart, C. L. (1953). *Polysaccharide Chemistry*. New York: Academic Press.
 Yemm, E. W. & Folkes, B. F. (1954). *Biochem. J.* **57**, 495.

A Study of the Nature of Plant Lignin by Means of Alkaline Nitrobenzene Oxidation

BY F. E. ROADHOUSE AND D. MACDOUGALL
 Ontario Agricultural College, Guelph, Canada

(Received 12 September 1955)

Studies on the development and nature of lignin in plant tissues have been greatly hampered by the limitations of existing methods for its determination. The difficulty encountered is due to inadequate means for the characterization of lignin and for its isolation from other plant constituents. Two types of error may occur during the separation of lignin: losses during the pretreatment and isolation procedures, and contamination with interfering material arising from the action of the extracting reagents on other plant constituents. It is generally conceded that the advantages of the pretreatment extractions outweigh the disadvantages. Furthermore, the nature of both the lignin and the potential interfering substances changes with increasing age, so that a procedure which is adequate at one growth stage may be quite inadequate for some other growth stage.

In order to evaluate the various errors in the problem outlined above and, at the same time, to obtain more reliable information on the formation of lignin in plants, it was decided to oxidize plant materials of different species and ages with alkaline nitrobenzene and to determine the amounts of vanillin, *p*-hydroxybenzaldehyde and syringaldehyde formed. An attempt was made to determine lignin losses due to the pretreatment extractions with ethanol-benzene, water, and 1% hydrochloric acid by subjecting the residues from each step to alkaline nitrobenzene oxidation.

The alkaline nitrobenzene technique was used by Freudenberg, Lautsch & Engler (1940). These workers found that sulphonated lignins, isolated

lignins, and wood itself gave substantial yields of vanillin when oxidized with this reagent. Creighton, McCarthy & Hibbert (1941) applied the technique to a large number of materials (jute, rye straw, corncobs, bamboo, sassafras, etc.). They obtained sufficient vanillin and syringaldehyde from angiosperms to account for 25-45% of the Klason lignin. In later work (Creighton & Hibbert, 1944) vanillin and syringaldehyde were isolated after oxidation of dicotyledonous plants. From monocotyledons *p*-hydroxybenzaldehyde was obtained in addition to the other two aldehydes. Bondi & Meyer (1948) used the same techniques to investigate the lignins from grasses, legumes and sheep faeces.

More recently, Stone & Blundell (1951) have described a micromethod for alkaline nitrobenzene oxidation. Stone, Blundell & Tanner (1951) used this technique on wheat plants and observed that the ethanol-benzene extraction removed appreciable amounts of lignin, the water extraction removed traces of lignin, and the 1% hydrochloric acid treatment removed little or no lignin. They also reported that the decreasing order of abundance of the aldehydes in young tissues was *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde. In the mature plant this order was reversed.

MATERIALS AND METHODS

Plant tissues. The plants used in this investigation were winter wheat (Dawson's Golden Chaff, no. 16), alfalfa (O.A.C. no. 1), and meadow fescue (Mefon). These were

Table 1. *Description of plant material*

Growth stage no.	Winter wheat (Dawson's Golden Chaff no. 16)			Alfalfa (O.A.C. no. 1)			Meadow fescue (Mefon)		
	Harvest date	Height (cm.)	Description	Harvest date	Height (cm.)	Description	Harvest date	Height (cm.)	Description
1	19 May	30-36	Plants in rows 20-25 cm. apart	18 May	36-40	Plants in rows 46 cm. apart	23 May	10-12.5	Plants in rows 46 cm. apart
2	6 June	80-92	Heads forming	8 June	80-86	Flower buds showing	6 June	25-30	Heads forming
3	25 June	125-140	Kernels soft and milky	29 June	125-132	Almost full bloom	20 June	38-46	Headed out
4	25 July	125-140	Ripe	26 July	125-132	Ripe	4 July	38-46	Not ripe

harvested by cutting off at approximately 1-2 cm. from the ground on the dates shown in Table 1. All material was spread out on wrapping paper and allowed to dry at room temperature. The alfalfa and wheat plants were cut into 2.5-5 cm. lengths before drying. The meadow fescue was not cut before drying. The wheat was hand-threshed and the grain was not included in the material used for analysis. In the case of the alfalfa it was found impossible to remove the seed from the rest of the plant. Therefore, the whole plant was used. This caused some difficulty in grinding.

All samples were ground in a Wiley Mill (Arthur H. Thomas Co., Philadelphia Pa., U.S.A. 5) to pass a 0.5 mm. screen and were then stored in tightly stoppered bottles. Analyses carried out immediately after drying and after storage for considerable periods showed no differences in results.

Preparation of aldehydes. Vanillin (Eastman Kodak no. 273), crystallized three times from light petroleum (b.p. 100-120°), melted at 81.7-82.2°. *p*-Hydroxybenzaldehyde (Eastman Kodak no. 985), crystallized twice from light petroleum (b.p. 100-120°), melted at 115.8-116°. Syringaldehyde was prepared from vanillin by the method of Pepper & MacDonald (1953), and recrystallized five times from light petroleum (b.p. 80-100°) to yield pale-brown crystals melting at 109.5-110°. This product was dissolved in hot water and treated with decolorizing carbon. After boiling, filtering and cooling, a colourless product crystallized (m.p. 109.6-110°). A mixed m.p. determination, carried out with a sample of syringaldehyde supplied by Dr J. M. Pepper of the University of Saskatchewan, showed no depression.

Moisture. This was determined on all samples by drying at 100° for 24 hr.

Lignin. Pretreatment procedures used involved extraction with ethanol-benzene (1:2, by vol.), hot water, and 1% (w/w) hydrochloric acid according to A.O.A.C. (1950). Klason lignin was isolated by the 72% (w/w) sulphuric acid method of Manning & DeLong (1941). Lignin for methoxyl determinations was isolated by filtering in sintered-glass crucibles, with naphthalene as filter aid (Meuller & Herrmann, 1926).

Methoxyl determinations. These were carried out by the Viebock & Schwappach modification of the Ziesel method, as described by Clark (1939). Samples were weighed out in gelatin capsules (size no. 4) and a suspension of red phosphorus was used in the scrubber (Samsel & McHard, 1942).

The hydriodic acid was prepared as described by Clark (1938).

Nitrogen determinations. These were carried out by the micro-Kjeldahl method. The digestion mixture recommended by Campbell & Hanna (1937) was used. The ammonia was distilled into a 4% solution of boric acid.

Oxidation with alkaline nitrobenzene. The 5 ml. reaction bombs were made of stainless steel (18% Cr, 8% Ni) and, except for size, were similar in construction to high-pressure hydrogenation bombs. A tight seal was effected by the use of lead gaskets which flowed into coincident grooves in both the bomb and plug cap.

In the oxidation procedure accurately weighed samples (10-50 mg.) were placed in the bombs along with exactly 2 ml. of 2N-NaOH and 0.12 ml. of nitrobenzene. The bomb was closed tightly and then shaken to ensure proper mixing. Six small bombs were wrapped in cheese cloth and placed inside a 2.5 l. hydrogenation bomb. Water was placed in the large bomb, which was then sealed. The bomb was heated to 160° and held at that temperature for 2.5 hr. with shaking. At the end of this time the bomb was cooled to 50° by plunging into cold water. After opening the small bombs the reaction mixtures were poured into small test tubes which were corked and centrifuged at 800g for 30 min. This is essentially the procedure described by Stone & Blundell (1951).

Chromatography. The chromatographic procedure was similar to that described by Stone & Blundell (1951). Sheets of Whatman no. 1 filter paper (57 cm. × 46.5 cm.) were used. Two 0.1 ml. samples of the supernatant liquid from the reaction mixture were streaked along about 15 cm. portions of the base-line. Test spots were placed at each end and at the centre of the base-line between the 15 cm. streaks. The spots on the paper were neutralized by carefully spraying with cold acetic acid. After spraying, the papers were allowed to dry for 30 min. at room temperature before being chromatographed. The chromatography was carried out at 29-31° in large insulated chromatographic chambers (Chromatocarb Research Equipment Corp., Oakland, California, U.S.A.) with water-saturated light petroleum (b.p. 100-120°)-*n*-butyl ether (6:1, by vol.). It required 13 hr. to separate the aldehydes. On removal from the cabinet the papers were dried at room temperature and the test strips were cut out of the large sheets. The positions of the aldehydes were determined by spraying the test strips with a saturated solution of 2,4-dinitrophenylhydrazine in 1% (w/w) HCl. The unsprayed portions of the

paper holding the aldehydes were extracted in small Soxhlet extractors (30 ml. cap.) with aldehyde-free 95% ethanol. The extracts were diluted to 50 ml. with 95% ethanol and the aldehyde concentrations were determined spectrophotometrically.

Determination of aldehydes. A standard curve was prepared for each aldehyde from pure samples. Absorption measurements were made with a Beckman DU spectrophotometer. A straight-line relationship was found between optical density and concentration in each case. The slopes of these lines were determined and used for calculation of the aldehyde concentrations. The absorption of *p*-hydroxybenzaldehyde was measured at 335 m μ ., of syringaldehyde at 368 m μ ., and of vanillin at 352 m μ .. Concentrations of each aldehyde of from 0.2 to 6.0 μ g./ml. could be determined quite satisfactorily by this method.

RESULTS AND DISCUSSION

Recovery experiments

At the outset it was decided to determine whether or not the aldehydes were destroyed during the oxidation procedure. Recovery experiments were run in which known amounts of each aldehyde were placed in the bombs and oxidized in the usual way. The results of these experiments are shown in Table 2.

Examination of Table 2 shows that the recovery of aldehydes from the oxidation mixture ranged from 92 to 103%. In view of the many steps involved in the method it was considered that this

indicated that little or no aldehyde was lost during the oxidation procedure.

Recovery experiments were also run in which known amounts of each aldehyde were added to the plant materials before oxidation. It was found that in the presence of plant material the added aldehydes could not be completely recovered. The results for recovery of aldehydes added to the original plant tissues are shown in Table 3. Although the results are quite variable it is evident that in the majority of cases better recoveries of added aldehydes were obtained from the mature than from the young materials.

It was thought that some of the loss of aldehydes during oxidation might have been due to formation of the corresponding acids. This theory was checked by means of paper chromatography. Investigation showed that the acids do not move in the light petroleum-*n*-butyl ether used for isolating the aldehydes. When the origins from several papers, from which the aldehydes had been removed, were re-extracted with 95% ethanol and the extract was rechromatographed in the upper phase of benzene-acetic acid-water (4:2:2, by vol.) no evidence of the presence of the acids was obtained. Samples of the pure acids were run along with the test solutions. The losses of aldehydes observed cannot be explained on the basis of oxidation to the corresponding acids. Investigations are continuing to identify other reaction products in the oxidation mixture.

The fact that added aldehydes cannot be completely recovered from the oxidation mixture was first noted by Freudenberg *et al.* (1940), who were able to recover only 80% of the vanillin added to wood before oxidation. This fact seems to have been overlooked by Stone *et al.* (1951), Bondi & Meyer (1948), and DeStevens & Nord (1953).

Table 2. *Recovery of aldehydes added to the oxidation mixture*

Aldehyde placed in bomb (mg.)	Aldehyde recovered (mg.)		
	Syringaldehyde	<i>p</i> -Hydroxybenzaldehyde	Vanillin
0.200	0.183	0.207	0.205
0.200	0.187	0.193	0.205

Table 3. *Percentage recovery of aldehydes added to plant material before oxidation*

In this experiment 200 μ g. of each aldehyde was added to 50 mg. of plant material. Each value is the mean of at least two determinations. Growth stages are defined in Table 1.

Plant material	Growth stage	Percentage recovery		
		Syringaldehyde	<i>p</i> -Hydroxybenzaldehyde	Vanillin
Alfalfa	1	46.8	57.6	42.6
	2	73.1	65.0	62.8
	3	91.0	81.5	74.5
	4	93.0	75.5	81.0
Wheat	1	61.1	60.3	52.9
	2	47.4	67.9	46.5
	3	59.7	70.4	78.5
	4	59.8	78.3	79.9
Meadow fescue	1	62.8	55.9	44.1
	2	59.9	86.5	78.1
	3	63.5	67.0	71.0
	4	50.4	94.8	87.6

Effect of pretreatments and lignin isolation on amounts of aldehyde formed from plant tissues

In order to discover whether or not the various pretreatment procedures used in the determination of lignin remove aldehyde-forming material it was decided to carry out alkaline-nitrobenzene oxidations on the residues obtained after each successive extraction. Oxidations were also carried out on the Klason lignin isolated from each sample.

In view of the poor recoveries obtained when the aldehydes were added to the original plant tissues similar recoveries were run on all of the materials being oxidized. Recovery of added aldehydes was not improved by preliminary removal of the materials soluble in the various extractants. While the results were somewhat variable they were similar to those shown in Table 3. Therefore these data are not included in the paper. However, all results reported have been corrected for losses of aldehydes which occurred during oxidation.

As there was no apparent relationship between plant age and the effects of the various extractants the results for the four growth stages have been averaged. The results of this study are shown in Table 4.

It is evident from Table 4 that each successive step in the isolation of lignin causes a marked lowering in the amounts of the aldehydes obtainable from the residue. This is particularly true of the extraction with hydrochloric acid and the final isolation of lignin. In general, the ethanol-benzene extraction does not appear to affect greatly the amount of aldehyde-forming material.

There are two possible explanations for the observed decrease in aldehydes formed from the residue left after each successive step. In the first

place the aldehyde-forming material may have been removed by the various extractants, or it may have been modified in such a way that aldehydes are not formed from it. If the latter be the case, it is difficult, if not impossible, to ascertain whether the original aldehyde-forming material is present in the solvent or in the residue.

In an attempt to throw some light on this problem the solutions from the wheat extractions were examined. These extracts were evaporated to a small volume *in vacuo*. The extract was finally dried to constant weight at 60° at atmospheric pressure. Before evaporating the hydrochloric acid extract it was neutralized with sodium hydroxide. This resulted in the presence of a large amount of sodium chloride in the residues from these solutions. Suitable control experiments showed that this salt did not interfere with either the oxidation or subsequent chromatography. The residues obtained by evaporation were oxidized and chromatographed in the normal way.

In this study it was found that the decreases in aldehyde-forming material caused by the ethanol-benzene and water extractions (Table 4) could be accounted for completely on the basis of the aldehydes formed by oxidation of the residues from the evaporated extracts. This was not the case with the hydrochloric acid extracts, where no aldehyde could be detected in the evaporated residues. From this experiment, it is impossible to judge whether the hydrochloric acid treatment has removed aldehyde-forming material or not. Presumably the very low yields of aldehyde obtained on oxidation of isolated Klason lignin are due to a continuation, by the sulphuric acid treatments, of the processes begun during the hydrochloric acid pretreatment. These results are similar to those reported by

Table 4. *Effect of each step in lignin isolation on yields of aldehydes*

Results are expressed as percentage of aldehyde found in the unextracted plant tissue. Each value is the mean of the results obtained at four growth stages (see Table 1).

Plant material	Treatment*	Percentage of aldehyde		
		Syringaldehyde	<i>p</i> -Hydroxybenzaldehyde	Vanillin
Alfalfa	I	97.8	84.7	87.9
	II	89.1	70.0	72.7
	III	55.0	42.0	39.5
	IV	24.8	21.6	15.5
Wheat	I	100.8	91.5	97.0
	II	70.0	58.4	69.6
	III	59.3	34.8	62.9
	IV	19.7	23.7	18.6
Meadow fescue	I	92.6	63.0	77.1
	II	77.7	57.9	63.7
	III	42.7	30.0	28.1
	IV	18.7	12.5	10.2

*Treatments: I, 30 hr. ethanol-benzene extraction; II, I plus 3 hr. hot-water extraction; III, II plus 3 hr. hot 1% (w/w) HCl extraction; IV, isolated Klason lignin.

Stone *et al.* (1951), except that more aldehyde was recovered from the water extract than they reported. It should be pointed out that they used a cold-water extraction for 30 min. and we used boiling water for 3 hr.

It was thought that an examination of the methoxyl contents of the residues left after the various extractions might shed some light on this problem. Accordingly, methoxyl determinations were carried out on the wheat samples at each extraction stage. The results are shown in Table 5. Sufficient methoxyl was removed by the various extraction steps to account for the loss of aldehydes if they were extracted. Thus the methoxyl data neither prove nor disprove the point. There is no way of determining whether the methoxyl removed was of lignin or non-lignin origin.

We are inclined to favour the view that the aldehyde-forming material has been so changed by the acid treatments that it does not form aldehydes on subsequent oxidation. There are several reasons for this. Freudenberg & Bittner (1953) have shown that when either lignin or a dehydrogenation polymer formed from possible lignin-building units is treated with acid before methyl-

ation and oxidation little or no isohemipinic acid is formed in comparison with that produced from the material not treated with acid. The losses of methoxyl on extraction with hydrochloric acid and during the final isolation step may be due to removal of non-lignin methoxyl-containing material, to extraction of lignin, or to demethylation of the lignin. Evidence has been presented (MacDougall, 1954) which indicates that treatment with acid may demethylate lignin to some extent.

Relationship of aldehyde formed to age of plants

The yields of aldehydes obtained from the different plant species are shown in Table 6. It is apparent that age has very little effect on the percentage of *p*-hydroxybenzaldehyde found. It should also be noted that the amount of vanillin formed does not increase as much as might have been expected on the basis of other workers' results (Stone *et al.* 1951). This effect is due to the fact that the correction for loss of aldehydes is greater with the young than with the older tissues. It would appear that the increases in yields of vanillin with increasing age which have been reported by Stone *et al.* (1951) may be due, in part, to failure to make correction for losses of aldehydes during the oxidation. Our experiments indicate that the syringaldehyde is the only one of the three aldehydes which shows a marked increase in yield as plant age increases.

Mention should also be made of the claim by Creighton & Hibbert (1944) that monocotyledons can be distinguished from dicotyledons by the fact that the latter do not yield *p*-hydroxybenzaldehyde on oxidation with alkaline nitrobenzene. This distinction appears to be more arbitrary than real, as in our studies as much *p*-hydroxybenzaldehyde was obtained from the dicotyledon (alfalfa) as from the two monocotyledons (wheat, meadow

Table 5. *Effect of pretreatment extractions on methoxyl content of wheat plants*

Results are expressed as percentage and are all based on the original material. Growth stages are defined in Table 1 and treatments in Table 4.

Treatment	Growth stage			
	1	2	3	4
	Percentage of aldehyde			
(Original material)	1.28	2.08	2.82	3.24
I	1.11	1.64	2.42	3.14
II	0.86	1.37	2.17	3.08
III	0.39	1.17	1.54	2.14
IV	0.35	0.86	1.40	1.91

Table 6. *Aldehyde formed by oxidation of original plant tissues with alkaline nitrobenzene*

Results are expressed as percentage of the dry weight of plant tissue. Growth stages are defined in Table 1.

Plant tissue	Growth stage	Percentage of aldehyde		
		Syringaldehyde	<i>p</i> -Hydroxybenzaldehyde	Vanillin
Alfalfa	1	0.82	0.56	1.80
	2	1.03	0.48	1.87
	3	1.25	0.38	2.12
	4	1.21	0.40	2.06
Wheat	1	0.59	0.49	1.77
	2	1.81	0.52	2.99
	3	2.71	0.57	2.54
	4	3.72	0.55	3.03
Meadow fescue	1	0.60	0.55	1.89
	2	1.02	0.39	1.44
	3	1.60	0.50	2.08
	4	2.46	0.41	1.72

fescue). The claim of Bondi & Meyer (1948) that lignins from succulent plant tissues do not yield syringaldehyde is also refuted.

Relationship between Klason lignin and aldehydes formed on oxidation of plant tissues

The amounts of Klason lignin obtained from the various plant tissues along with the total amounts of aldehyde formed on oxidation are shown in Table 7. Direct comparison of these two sets of data cannot be made, as it has been shown that some of the aldehyde-forming material has been removed by the ethanol-benzene and water extractions, and some doubt exists as to the effect of the hydrochloric acid and sulphuric acid treatments. In spite of this some conclusions can be drawn from these data.

In the first place it is obvious, on the basis of the methoxyl contents of the lignin and the isolated aldehydes, that the compositions of the two fractions are quite different. If lignin is formed from the type of building units which give rise to the aldehydes then one would expect it to have a considerably higher methoxyl content than is obtained. There are three possible reasons for the relatively low methoxyl content of the isolated lignin. In the first place, it may be contaminated with artifacts formed from other plant constituents during the pretreatment and isolation procedures. In the second place, there may be other lignin-building units of low methoxyl content which are not detected by our oxidation technique. Finally, there may be some demethylation of lignin during its isolation. That the first possibility is partly responsible for the situation is shown by the nitrogen contents of the isolated lignin. On the basis of the ratios of the amounts of methoxyl found in lignin and aldehydes one would expect contamination of the residues to be greatest in the case of the

young tissues. The nitrogen results support this contention. The second possibility cannot be proved or disproved by these data. No definite evidence with regard to the third possibility is given from these results. However, as mentioned above, some demethylation may occur during the acid pretreatment (MacDougall, 1954).

Although only a small fraction of the aldehyde isolable after oxidation of the original tissue can be obtained from the isolated lignin, it is quite probable that much of the original aldehyde-producing material is present in the isolated lignin in a modified form. This point has already been discussed. It was noted that the decreases in aldehyde yields, due to the various pretreatments, were as great with the old as with the young tissues. Thus even though the values for lignin and total aldehyde in Table 7 cannot be compared directly, the losses due to extraction will be about the same in all cases and therefore will not alter the trends. Taking this into consideration it can be seen that the aldehyde formed represents a considerably higher proportion of the Klason lignin for young material than for the older tissues.

It is interesting to speculate on the biological implications of this observation. The reason for these results may be that the aldehyde-forming materials are less highly condensed in young plants than in older tissues. In young material a considerable number of phenylpropyl-type compounds may exist as such. These may be actively engaged in metabolism (Hibbert, 1942). Presumably as the plant matures these compounds condense to form lignin. Whether the unpolymerized substances (if they exist) in the young plants should be referred to as lignin or not is a question which must be decided. A study of the occurrence of phenylpropyl-type compounds in young plant tissues is indicated.

Table 7. *Relationship between Klason lignin and total aldehyde isolated from plant tissues*

Growth stages are defined in Table 1.

Plant tissue	Growth stage	Klason lignin (%)	Methoxyl in lignin (%)	Nitrogen in lignin (%)	Total aldehyde (%)	Methoxyl in aldehyde (%)	Quotient of total aldehyde methoxyl to total lignin methoxyl
Alfalfa	1	5.33	6.83	4.83	3.18	20.4	1.80
	2	8.91	11.8	4.11	3.38	21.5	0.70
	3	11.0	13.0	2.76	3.75	22.9	0.60
	4	12.8	12.1	3.20	3.67	22.5	0.54
Wheat	1	4.54	7.68	4.84	2.85	19.6	1.60
	2	7.66	11.2	3.43	5.32	23.1	1.43
	3	10.3	13.6	2.30	5.82	24.8	1.04
	4	12.4	15.4	1.39	7.30	25.8	0.99
Meadow fescue	1	4.30	5.82	5.93	3.04	19.5	2.36
	2	5.42	7.88	4.65	2.85	22.4	1.49
	3	8.53	10.1	3.88	4.18	23.2	1.13
	4	9.28	10.7	3.80	4.59	25.9	1.20

SUMMARY

1. Considerable losses of vanillin, *p*-hydroxybenzaldehyde and syringaldehyde occur when these aldehydes are added to plant material before oxidation with alkaline nitrobenzene.

2. The recovery of added aldehyde was no better from fully extracted than from unextracted plant tissue.

3. In general, losses of aldehyde during the oxidation were greater with the young than with the older tissues.

4. The ethanol-benzene and hot-water extractions remove appreciable amounts of aldehyde-forming material.

5. The percentage of *p*-hydroxybenzaldehyde obtained on oxidation of plant material does not increase with increasing plant age; the percentage of vanillin formed increases slightly, and the percentage of syringaldehyde produced increases very markedly.

6. The yield of aldehyde in relation to Klason lignin is greater for young than for the more mature tissues.

This paper forms part of a dissertation submitted by F. E. Roadhouse to the Faculty of Graduate Studies, University of Toronto, in partial fulfilment of the requirements for the degree of Master of Science in Agriculture.

REFERENCES

- A.O.A.C. (1950). *Methods of Analysis*. 7th ed., p. 109. Washington: Association of Official Agricultural Chemists.
- Bondi, A. & Meyer, H. (1948). *Biochem. J.* **43**, 248.
- Campbell, W. R. & Hanna, M. I. (1937). *J. biol. Chem.* **119**, 1.
- Clark, E. P. (1938). *Industr. Engng Chem. (Anal.)*, **10**, 877.
- Clark, E. P. (1939). *J. Ass. off. agric. Chem., Wash.*, **22**, 100.
- Creighton, R. H. J. & Hibbert, H. (1944). *J. Amer. chem. Soc.* **66**, 37.
- Creighton, R. H. J., McCarthy, J. L. & Hibbert, H. (1941). *J. Amer. chem. Soc.* **63**, 3049.
- DeStevens, G. & Nord, F. F. (1953). *J. Amer. chem. Soc.* **75**, 305.
- Freudenberg, K. & Bittner, F. (1953). *Ber. dtsh. chem. Ges.* **86**, 155.
- Freudenberg, K., Lautsch, W. & Engler, K. (1940). *Ber. dtsh. chem. Ges.* **73**, 167.
- Hibbert, H. (1942). *Annu. Rev. Biochem.* **11**, 183.
- MacDougall, D. (1954). *J. Sci. Fd Agric.* **5**, 103.
- Manning, K. R. & DeLong, W. A. (1941). *Sci. Agric.* **22**, 69.
- Meuller, W. J. & Herrmann, W. (1926). *Papierfabrikant*, **24**, 195. [*Chem. Abstr.* (1926), **20**, 2746.]
- Pepper, J. M. & MacDonald, J. A. (1953). *Canad. J. Chem.* **31**, 476.
- Samsel, E. P. & McHard, J. A. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 750.
- Stone, J. E. & Blundell, M. J. (1951). *Analyt. Chem.* **23**, 771.
- Stone, J. E., Blundell, M. J. & Tanner, K. G. (1951). *Canad. J. Chem.* **29**, 734.

The Fluorimetric Determination of β -Glucosidase: its Occurrence in the Tissues of Animals, including Insects

By D. ROBINSON

Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

(Received 3 November 1955)

In a recent paper from this Laboratory (Mead, Smith & Williams, 1955), a highly sensitive fluorimetric method of assay of β -glucuronidase, in which 4-methylumbelliferone glucuronide was used as the substrate, was described. This method has now been adapted to the determination of β -glucosidase, with 4-methylumbelliferone β -glucoside as the substrate. Previous methods of assay of this enzyme have usually depended on the use of chromogenic substrates, or upon the determination of glucose liberated by the enzyme (Viebel, 1945). The method herein described is claimed to be highly sensitive, and can be readily used for the assay of the small amounts of the enzyme found in the tissues of higher animals. In fact, few studies of the β -glucosidase content of animal tissues have been made owing to the lack of a sufficiently sensitive method of assay. This enzyme has been

shown to occur in the snail (Karrer, 1930), the woodlouse (Newcomer, 1952) and the cockroach (Newcomer, 1954). Few warm-blooded animals have been examined. Neuberg & Hofmann (1935) reported its occurrence in the liver and kidney of horses and cattle, while Steensholt & Viebel (1943) showed its presence in pig intestinal mucosa.

EXPERIMENTAL

Materials

Preparation of 4-methylumbelliferone β -glucoside. This glucoside was prepared according to the general method of Glazer & Wulwek (1924). 4-Methylumbelliferone (11.5 g., m.p. 180°) was dissolved in acetone (140 ml.), and acetobromoglucose (19 g.) added, followed by a solution of NaOH (3.6 g.) in water (90 ml.). After the mixture had been kept at room temp. overnight the acetone was