- Fredga, A. (1942). *Ark. Kemi*, 15B, 1.
- Greenstein, J. P. & Winitz, M. (1961). *Chemistry of the Amino Acids*, New York: John Wiley and Sons Inc.
- Grobelaar, N., Pollard, J. K. & Steward, F. C. (1955). *Nature, Lond.*, 175, 703.
- Gross, D. (1961). *J. Chromat.* 5, 194.
- Levintow, L., Meister, A., Hogeboom, G. H. & Kuff, E. L. (1955). *J. Amer. chem. Soc.* 77, 5304.
- Linko, P. & Virtanen, A. I. (1958). *Acta chem. scand.* 12, 68.
- Lipmann, F. & Tuttle, L. C. (1945). *J. biol. Chem.* 159, 21.
- Marcus, A., Feeley, J. & Shannon, L. M. (1963). *Arch. Biochem. Biophys.* 100, 80.
- Nakayama, S. & Kaneko, T. (1957). *J. chem. Soc. Japan*, 78, 1216.
- Rydon, H. N. & Smith, P. W. G. (1952). *Nature, Lond.*, 169, 922.
- Smith, I. (1960). *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 279. London: W. Heinemann and Co. Ltd.
- Steward, F. C., Wetmore, R. H. & Pollard, J. K. (1955). *Amer. J. Bot.* 42, 946.
- Tschiersch, B. (1962). *Phytochemistry*, 1, 103.
- Virtanen, A. I. & Berg, A.-N. (1955). *Acta chem. scand.* 9, 553.
- Wailes, P., Whiting, M. C. & Fowden, L. (1954). *Nature, Lond.*, 174, 130.
- Zacharius, R. M., Pollard, J. K. & Steward, F. C. (1954). *J. Amer. chem. Soc.* 76, 1961.

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Trans- β -glucosylation by Alfalfa-Seed Enzymes

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An early comparison between the glycosidase system of alfalfa (lucerne, *Medicago sativa*) seeds and sweet almonds (Hill, 1934) revealed a similar distribution of activities except that alfalfa 'emulsin' contained only traces of β -glucosidase. The various activities were assayed with synthetic phenyl glycosides and naturally occurring phenolic glycosides such as salicin and amygdalin, i.e. glycosides with non-carbohydrate aglycones. A survey of the disaccharidases of alfalfa, however, has now shown (D. H. Hutson & D. J. Manners, unpublished work) that β -glucosidases are present in substantial amounts, although only weak activity was displayed towards phenyl β -D-glucopyranoside, salicin and amygdalin. Moreover, of the common glycosidases found in the seed extracts, this activity was the only one that showed trans-glucosylation properties when incubated with a 1% solution of substrate for 24 hr.

Plant and fungal β -glucosidases, in general, appear to possess a group rather than an absolute specificity (Peat, Whelan & Hinson, 1952; Manners, 1960), although only a few fractionation studies have been reported (e.g. Krishna-Murti & Stone, 1961; Anderson, Cunningham & Manners, 1963). Investigations of the trans- β -glucosylation action of various plant and fungal preparations on cellobiose have been carried out by several workers (e.g. Barker, Bourne, Hewitt & Stacey, 1955; Buston & Khan, 1956; Crook & Stone, 1957; Anderson & Manners, 1959) and the acceptor specificities of the enzyme preparations determined. In these experiments, transglucosylation occurred predominantly

on the C₆-hydroxyl group, but was also observed on the C₃-, C₃'- and C₄-hydroxyl groups of D-glucopyranose and D-glucopyranosides. There appear to be, however, certain minor differences in acceptor specificity in these systems.

Trans- β -glucosylation by alfalfa-seed extracts has now been examined with a view to determination of the acceptor specificity pattern with cellobiose as substrate. Since β -(1 $\rightarrow$ 6)- and β -(1 $\rightarrow$ 3)-linkage synthesis is usually encountered, it was pertinent to study the behaviour of gentiobiose and laminariobiose under similar conditions.

MATERIALS AND METHODS

Enzyme preparation. Alfalfa seeds (var. de Puits, 300 g.) were milled and sieved to remove husk material to yield a fine flour (170 g.). This was extracted at room temperature with 0.4M-sodium citrate buffer, pH 6.8 (final volume 800 ml.), and stored at -20° overnight. The thawed extract, after filtration through muslin, was centrifuged and the supernatant solution dialysed against 2 l. of 0.5M-sodium citrate buffer, pH 5.8. Protein was precipitated from the centrifuged dialysis residue by the addition of ammonium sulphate to 70% saturation (at 0°). The precipitated protein was redissolved in 0.01M-sodium citrate buffer, pH 5.8 (140 ml.), and dialysed against the same buffer at 0°. The resultant preparation was stored at 0° and used within 1 week.

Incubation conditions. All digests were incubated at 37° under toluene. One-quarter of the digest volume consisted of 0.2M-disodium hydrogen phosphate-0.1M-citric acid buffer (Mollvaine, 1921).

Chromatography and paper electrophoresis. Paper chromatograms were developed with the following solvents:

(a) ethyl acetate-pyridine-water (10:4:3, by vol.); (b) ethyl acetate-acetic acid-formic acid-water (18:3:1:4, by vol.). Sugars were located generally with a silver nitrate-sodium hydroxide reagent (Trevelyan, Procter & Harrison, 1950). Aniline-diphenylamine spray reagent was used to indicate reducing sugars and, specifically, 4-*O*-substituted reducing end groups of oligosaccharides, which gave a blue coloration (Schwimmer & Bevenue, 1956). Ultrasorb S.C. 120/240, obtained from British Carbo-Norit Union Ltd., Grays, Essex, was used in the charcoal-Celite column for preparative fractionation of the oligosaccharides (Hughes & Whelan, 1958). Paper electrophoresis was carried out in molybdate solution at pH 5.0 (Bourne, Hutson & Weigel, 1961) at 20 v/cm. in an apparatus similar to that described by Gross (1959). Mobilities are expressed relative to sorbitol (M_s values).

Quantitative sugar measurements. Total hexose content was determined by the phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Glucose was determined specifically by using a glucose oxidase-peroxidase-o-toluidine system as described by White & Subers (1961). The reagent showed no β -glucosidase activity.

Reduction of oligosaccharides. Approximately 1% sugar solutions were treated with an equal volume of 1% potassium borohydride for 18 hr. at 20° and the resulting solutions deionized with Amberlite IR-120 (H^+ form) resin followed by four evaporations with methanol. The resulting oligosaccharides were then purified by chromatography on Whatman no. 3MM paper with solvent (a).

Acid hydrolyses. Complete acid hydrolysis was effected by heating the oligosaccharides in 0.25 *N*-sulphuric acid at 100° for 3 hr. The same conditions, but with a 1 hr. heating time, were found suitable for partial hydrolyses. Barium carbonate was used for neutralization.

Degree of polymerization of oligosaccharides. This was determined by the method of Peat, Whelan & Roberts (1956), involving the determination of hexose content before and after reduction with potassium borohydride. However, it was found more convenient to use the phenol-sulphuric acid method (Dubois *et al.* 1956) rather than the recommended anthrone reagent for the determination of hexose content (cf. Timell, 1960).

RESULTS

Preliminary experiments. Cellobiose (20 mg./ml.; 2 ml.), buffer, pH 4.5 (1 ml.), and enzyme solution (10 mg./ml.; 1 ml.) were incubated for 18 hr. Chromatographic analysis with solvent (a) indicated extensive hydrolysis of the disaccharide to glucose with the concurrent production of higher oligosaccharides.

Similar digests, but containing cellobiose at an overall concentration of 2.5 mg./ml. and McIlvaine (1921) buffer at various pH values, were prepared and, after 24 hr. incubation, 2 ml. portions were diluted to 5 ml. with water and assayed for glucose. The resulting relationship between degree of hydrolysis of cellobiose and pH indicated an optimum pH of 5.5 for cellobiase activity. All digests described below were carried out, therefore, at pH 5.5.

Optimum conditions for transglucosylation. Previous studies show clearly that transglycosylation is favoured at higher substrate (acceptor) concentrations (for a review, see Manners, 1960). This is in accordance with the current view that transglycosylation is the general reaction of glycosidases, and that hydrolysis is a special example resulting from the transfer of a glycosyl unit from an aglycone to water. In view of the evidence of transglucosylation in even 1% cellobiose solutions it was decided to use 5% solutions in the present studies. A digest containing cellobiose (5%), buffer, pH 5.5 (1 ml.), and seed extract (1 ml.) was incubated at 37° and the reaction followed at suitable time-intervals by paper chromatography with solvent (a). Transfer products in the trisaccharide region of the chromatogram (R_f 0.30) appeared after 1 hr. and steadily increased in concentration up to 24 hr. At about 6–8 hr. a slow-moving disaccharide-type compound (R_f 0.45) appeared and steadily increased in concentration until, after 3 days, it appeared to be the only significant oligosaccharide present. After 20 hr. the trisaccharide diminished relative to the disaccharide. For a large-scale digest, therefore, a 20 hr. incubation time was regarded as suitable.

Large-scale digest and isolation of products. A digest containing cellobiose (10 g.), standard buffer diluted four times (100 ml.) and seed extract (100 ml.) was incubated for 20 hr. The digest was heated to 100° for 5 min. and the coagulated protein removed by filtration; the solution was placed on a charcoal column (3.9 cm. $\times$ 50 cm.) and washed with water (10 l.). The fractionation of the sugars with increasing concentrations of aqueous ethanol is described in Table 1.

Identification of oligosaccharides. Fraction 1. This sugar had the same R_f as D-glucose and reacted as D-glucose to the specific glucose oxidase reagent.

Fraction 2. This fraction moved as gentiobiose in solvent (a). After reduction with borohydride, the resulting alcohol migrated, during ionophoresis in molybdate solution, at M_s 0.8. This suggests a (1 $\rightarrow$ 6)-linkage but does not rule out the possibility of a (1 $\rightarrow$ 2)-linkage (Bourne *et al.* 1961). Fraction 2 (50 mg., dried over phosphorus pentoxide) was added to 30 mg. of fused sodium acetate in hot acetic anhydride (2 ml.). The initial temperature was held at 140° for 5 min. and then lowered to 110° over 1 hr. The mixture was then cooled and poured into water, and the liquid removed by decantation. The dried chloroform solution of the remaining solid was evaporated and dissolved in hot ethanol. Crystals appeared on cooling and refrigeration. The material was recrystallized five times from ethanol, and had m.p. 185–187°. The mixed m.p. with an authentic sample of β -gentiobiose octa-acetate (m.p. 188–190°) was 184–187°. Fraction 2 is therefore gentiobiose.

Table 1. *Charcoal fractionation of oligosaccharides synthesized from cellobiose*

Experimental details are given in the text.

Eluent	Volume (l.)	R_G of sugar in solvent (a)	Yield (g.)	Fraction no.	Identity
Water	10.0	1.00	approx. 6.0	1	Glucose
Aq. (2%) ethanol	4.5	—	—	—	—
Aq. (4%) ethanol	1.5	—	—	—	—
Aq. (6%) ethanol	4.5	—	—	—	—
Aq. (8%) ethanol	3.0	—	—	—	—
Aq. (8%) ethanol	1.3	0.45	0.16	2	Gentiobiose
Aq. (8%) ethanol	3.5	0.55	3.30	3	Cellobiose
Aq. (10%) ethanol	3.0	—	—	—	—
Aq. (10%) ethanol	6.0	—	—	—	—
Aq. (12%) ethanol	2.2	Mixture	Trace	4	Cellobiose + laminaribiose*
Aq. (14%) ethanol	5.0	—	—	—	—
From aq. (16%) ethanol to aq. (40%) ethanol	20.0	0.15, 0.28, 0.35, 0.42	0.57	5	Trisaccharide fraction

* Laminaribiose (46 mg.) was isolated from this fraction by paper chromatography.

Fraction 4. An oligosaccharide with the same R_G as laminaribiose was isolated by preparative paper chromatography (yield 46 mg.). After reduction it did not migrate (M_s 0) during molybdate ionophoresis, thus indicating a (1→3)-linkage to the original reducing end. The material, twice recrystallized from methanol, had m.p. 195° (frothing at 200°). The mixed m.p. with an authentic sample of α -laminaribiose (m.p. 197°, frothing at 200°), was 195° (frothing at 200°), thus confirming its identity as laminaribiose.

Fraction 5. This fraction was separated into four components (5A, 5B, 5C and 5D) by paper chromatography with solvent (a); after further chromatographic purification, these fractions were obtained in the proportions 8:70:10:1. The yields are given in Table 2. Complete acid hydrolysis of all four fractions yielded only glucose, and almond emulsin completely hydrolysed each to glucose, confirming the expected β -configuration of the linkages. Reaction with the specific aniline-diphenylamine spray indicated a (1→4)-linkage at the reducing end of each oligosaccharide. On reduction and ionophoresis in molybdate, the migration rates (M_s 0.25, 0.27, 0.27 and 0.22) lay clearly in the region of 4-*O*-substituted trisaccharide alcohols and not that of 3-*O*-substituted (M_s 0) or 6-*O*-substituted (M_s 0.7–0.8) analogues. Linkage analysis of the oligosaccharides were effected by partial hydrolysis of the borohydride-reduced sugars. Co-chromatography with authentic disaccharides in two solvents was used as identification. Solvent (a) distinguished cellobiose and cellobi-itol but solvent (b) was necessary to distinguish cellobi-itol and gentiobiose.

Fraction 5A. This fraction, analysed as described above, yielded laminaribiose and cellobi-itol.

Fraction 5B. This fraction yielded gentiobiose and cellobi-itol, and had $[\alpha]_D^{18} +9.0 \pm 0.8^\circ$ (c 1.28 in water), in close agreement with the values obtained

by previous workers, +9.2° (Crook & Stone, 1957) and +10.2° (Barker *et al.* 1955), for the trisaccharide 6²- β -D-glucosylcellobiose. Attempts to crystallize the trisaccharide from aqueous methanol were not successful.

Fraction 5C. This fraction yielded cellobiose and cellobi-itol, the former also being located as a bright-blue spot with the aniline-diphenylamine reagent. Fraction 5C possessed the R_G values of cellobiose (0.30 and 0.11 respectively) in solvents (a) and (b).

Fraction 5D. This fraction possessed an R_G similar to that of gentiotriose; however, the selective spray reagent and ionophoresis of the reduced material in molybdate indicated a (1→4)-linked reducing end. The only disaccharide-type products produced on partial hydrolysis were gentiobiose and cellobi-itol. A determination of the degree of polymerization of the oligosaccharide (4.3) indicated that the sugar was, in fact, a tetrasaccharide. A partial hydrolysis of fraction 5D with almond emulsin resulted initially in the production of an oligosaccharide with R_G 0.35, identical with fraction 5B.

The above results are summarized in Table 2 together with suggested identity. The presence of 'branched' trisaccharides would have been detected, if present as major components, since these compounds cannot yield reducing disaccharides on partial hydrolysis of the derived alcohols.

Relative rates of hydrolysis of the β -linked disaccharides by alfalfa-seed extract. Cellobiose, gentiobiose and laminaribiose (5 mg. in 2 ml. of water) were separately incubated with 1 ml. of buffer, pH 5.5, and 1 ml. of a fresh seed extract for 20 hr. at 37°, and the liberated glucose was determined with glucose oxidase. The percentage hydrolysis of each substrate (17.9, 25.5 and 34.0) indicated that 'cellobiase', 'gentiobiase' and 'laminari-

Table 2. *Analysis of fraction 5*

Experimental details are given in the text.

Fraction	R_F	Yield (mg.)	M_s of derived alcohol	Suggested linkages at reducing end	Degree of polymerization	Linkages present as indicated by partial hydrolysis of alcohol		Suggested identity
						Non-reducing end	Reducing end	
5A	0.42	36	0.25	(1 $\rightarrow$ 4)	3.0	(1 $\rightarrow$ 3)	(1 $\rightarrow$ 4)	G β -3G β -4G
5B	0.35	320	0.27	(1 $\rightarrow$ 4)	2.9	(1 $\rightarrow$ 6)	(1 $\rightarrow$ 4)	G β -6G β -4G
5C	0.28	46	0.27	(1 $\rightarrow$ 4)	3.2	(1 $\rightarrow$ 4)	(1 $\rightarrow$ 4)	G β -4G β -4G
5D	0.15	4.5	0.22	(1 $\rightarrow$ 4)	4.3	(1 $\rightarrow$ 6)	(1 $\rightarrow$ 4)	G β -6G β -6G β -4G (cellotriose)

Table 3. *Distribution of products after incubation of disaccharide (5% solution) with seed extract*

Experimental details are given in the text. Values in the Table represent relative hexose equivalents as determined by the phenol-sulphuric acid method. Traces of disaccharide were formed but are not measured in this analysis.

Substrate	Glucose	Unchanged disaccharide	Trisaccharide
Cellobiose	17	65	18
Gentiobiose	24	51	25
Laminaribiose	44	33	23

biase' activities were present in the approximate proportions 1.0:1.4:1.9.

Relative amounts of trans- β -glucosylation with β -linked disaccharides. The three disaccharides (25 mg.) were incubated in 5% solution with a freeze-dried seed preparation (2 mg. of protein) in 0.05M-sodium acetate buffer, pH 5.5 (0.5 ml.). The digests were examined by paper chromatography, elution, and determination of the relative hexose contents in the glucose, disaccharide and trisaccharide regions by the phenol-sulphuric acid method. The distribution of products is shown in Table 3. Disaccharide synthesis was apparent from the chromatograms. Gentiobiose was synthesized in low yield from laminaribiose, and traces of laminaribiose appeared in the gentiobiose digest. No cellobiose synthesis was detectable in either digest. The comparatively slow reaction of laminaribiose with the silver nitrate spray reagent excludes the possibility that cellobiose was overlooked.

Transfer from cellobiose to alternative acceptors. Incubation of 4% cellobiose solutions containing 4% of added acceptor under standard conditions indicated transfer to L-arabinose but not to D-xylose or D-ribose. The *p*-anisidine reagent (Hough, Jones & Wadman, 1950) was used in this experiment to locate pentose-containing oligosaccharides.

DISCUSSION

The relative amounts of the various products formed (Table 1) when cellobiose is incubated with an alfalfa-seed extract indicate that the acceptor specificity of this system towards cellobiose is similar to that in the other plant and fungal enzyme systems noted in the introduction. The preponderance of transfer to the primary hydroxyl group is clear from the isolation of relatively large amounts of 6'- β -glucosylcellobiose and gentiobiose. Certain differences in the spectrum of products from that found by other workers are apparent. The yield of gentiobiose is much lower in the present system than in that with barley β -glucosidase (Anderson & Manners, 1959). An examination of the chromatograms of the products of *Aspergillus niger* extracts on cellobiose (Crook & Stone, 1957) also indicates a high yield of gentiobiose. The most likely explanation for this difference lies in the degree of completion of the hydrolysis of cellobiose. The experiments with barley β -glucosidase were carried to a point where only 1.5% of the original cellobiose remained; likewise, a high yield of gentiobiose was obtained with *A. niger* extracts when 8.5% cellobiose remained in the solution (Barker *et al.* 1955). The present conditions were such that 33% of the cellobiose remained unchanged. Since gentiobiose results from the transfer of a β -glucosyl residue from cellobiose to free glucose, its yield will depend on the amount of glucose acceptor available. This, in turn, is a function of the degree of hydrolysis of the cellobiose. The absence of gentiotriose in the alfalfa-extract digest and its isolation by other workers (Anderson & Manners, 1959; Crook & Stone, 1957; Barker *et al.* 1955) are consequences of the relatively low and high concentrations respectively of gentiobiose. The absence of (1 $\rightarrow$ 2)-linkage synthesis is another feature of the present study and may be due either to a different acceptor specificity or to the low substrate concentration and the incomplete reaction.

It was considered of value to use the conditions described, which, though resulting in low yields of products, might give a more exact picture of the specificity of the primary transfer reaction:

in which cellobiose itself is acting as the glucosyl acceptor. The ratio of the trisaccharides might be expected to reflect the true rates of synthesis of the different linkages. These rates lie in the order $(1 \rightarrow 6) > (1 \rightarrow 4) > (1 \rightarrow 3) > (1 \rightarrow 2)$, in agreement with that found at equilibrium by Crook & Stone (1957). However, in the present experiments, the proportions of the products in fractions 5A, 5B and 5C indicate that there is only a slight difference between the apparent rates of synthesis of the $(1 \rightarrow 4)$ - and $(1 \rightarrow 3)$ -linkages.

An interpretation of the distribution of products must take into account the relative rates of hydrolysis of the newly synthesized linkages. The rates of hydrolysis of cellobiose, laminaribiose and gentiobiose, determined at an initial substrate concentration of 0.125 % when appreciable transglucosylation does not occur, were in the proportions 1.0:1.9:1.4. These orders of difference are not large but may, in fact, lower the observed amount of $(1 \rightarrow 3)$ -linkage relative to $(1 \rightarrow 4)$ -linkage synthesis.

Ready transglucosylation with gentiobiose and laminaribiose as donor-acceptors is apparent, and the quantitative chromatographic analysis summarized in Table 3 shows that the three disaccharides studied possess approximately the same order of efficiency. The trisaccharides are assumed to be products of $(1 \rightarrow 6)$ -linkage synthesis and their R_G values are in accordance with such a structure. Cellobiose was not synthesized from either gentiobiose or laminaribiose in this experiment, though the latter two disaccharides were synthesized from each of their analogues.

More detailed studies of the relative rates of enzymic hydrolysis and synthesis of oligosaccharide linkages will have to be preceded by analytical investigations of the glucosidase systems. There is no direct evidence to suggest that alfalfa contains only one β -glucosidase with various degrees of affinity towards the different glucoside substrates. Partial and complete fractionations of various β -glucosidases have been reported (e.g. Krishna-Murti & Stone, 1961; Li & King, 1963) and these suggest that the situation is very complex. In alfalfa β -glucosidase, for example, the relatively high activity of laminaribiase may be due, in part, to a component of the powerful laminarinase system found in all parts of the alfalfa plant (D. H. Hutson & D. J. Manners, unpublished work).

SUMMARY

1. Extracts of alfalfa seeds show strong β -glucosidase activity and trans- β -glucosylation activity when disaccharides are used as substrates in 1 % solution.

2. The oligosaccharides produced by trans-glucosylation with cellobiose as substrate have been characterized. Gentiobiose and 6^a-O-glucosylcellobiose were formed together with smaller quantities of cellotriose, 3^a-O-glucosylcellobiose and laminaribiose.

3. The acceptor specificity is similar to that of β -glucosidases from other sources.

4. The rates of hydrolysis of the newly formed linkages are in the order: $(1 \rightarrow 3) > (1 \rightarrow 6) > (1 \rightarrow 4)$.

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REFERENCES

- Anderson, F. B., Cunningham, W. L. & Manners, D. J. (1963). *Biochem. J.* **90**, 30.
- Anderson, F. B. & Manners, D. J. (1959). *Biochem. J.* **71**, 407.
- Barker, S. A., Bourne, E. J., Hewitt, G. C. & Stacey, M. (1955). *J. chem. Soc.* p. 3734.
- Bourne, E. J., Hutson, D. H. & Weigel, H. (1961). *J. chem. Soc.* p. 35.
- Buston, H. W. & Kahn, A. H. (1956). *Biochim. biophys. Acta*, **19**, 593.
- Crook, E. & Stone, B. A. (1957). *Biochem. J.* **65**, 1.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
- Gross, D. (1959). *Chem. & Ind.* p. 1219.
- Hill, K. (1934). *Ber. sächs. Ges. (Akad.), Wiss.* **86**, 115.
- Hough, L., Jones, J. K. N. & Wadman, N. H. (1950). *J. chem. Soc.* p. 1702.
- Hughes, R. C. & Whelan, W. J. (1958). *Chem. & Ind.* p. 884.
- Krishna-Murti, C. R. & Stone, B. A. (1961). *Biochem. J.* **78**, 715.
- Li, L.-H. & King, K. W. (1963). *Appl. Microbiol.* **11**, 320.
- Mellvaine, T. C. (1921). *J. biol. Chem.* **49**, 183.
- Manners, D. J. (1960). *Bull. Soc. Chim. biol., Paris*, **12**, 1789.
- Peat, S., Whelan, W. J. & Hinson, K. A. (1952). *Nature, Lond.*, **170**, 1056.
- Peat, S., Whelan, W. J. & Roberts, J. G. (1956). *J. chem. Soc.* p. 2258.
- Schwimmer, S. & Bevenue, A. (1956). *Science*, **123**, 543.
- Timell, T. E. (1960). *Svensk Papp-Tidn.* **63**, 668.
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
- White, J. W. & Subers, M. H. (1961). *Analyt. Biochem.* **2**, 380.