The Specific Determination and Detection of Glucose as a Probable Constituent Radical of Certain Fructosans by Means of Notatin

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For some time reports have appeared that fructosans isolated from a number of different plant species contain small proportions of an aldohexose, usually D-glucose, as an integral part of their structures. The whole question is in some confusion. In the present work the specific action of glucose oxidase ('notatin') has been applied to this problem as this enzyme can be expected to yield unequivocal evidence, both qualitative and quantitative. The polysaccharides examined were (*a*) inulin, (*b*) levan isolated from grasses and that synthesized by *Bacillus subtilis* from sucrose, and (*c*) irisin. It must be noted that the inulins used in this work are the polysaccharides isolated from subterranean storage organs of certain Compositae and purified by means of their insolubility in cold water, and not material obtained by simple ethanolic precipitation of aqueous solutions (cf. Jackson & McDonald, 1930).

Most previous investigators of the suspected aldose content of fructosans have worked on inulin, isolated in various ways and not always purified according to modern standards. Further work, carried out in France, includes investigations of other fructosans of widely different types from monocotyledonous plants. These substances do not lend themselves to purification so readily as does inulin. Fructosans from *Narcissus* (Belval, 1937; Belval & de Grandchamp, 1949), from *Asphodelus microcarpus* (Belval & de Grandchamp, 1949), from the bulbs of *Scilla maritima* (Colin & Chaudin, 1933*b*) and from *Lycoris squamigera* (Belval, 1931) have been found to contain glucose. Finally, glucose has been found by Belval & Delaporte (1947) to exist in the levan synthesized by *Phytobacterium vitrosum* from sucrose. Phlein, obtained from the roots of *Phleum pratense* by Schlubach & Sinh (1940), is stated by Belval & de Grandchamp (1949) to contain no glucose.

The usual procedure has been first to subject the polysaccharide to mild acid or enzymic hydrolysis; in the latter case crude enzyme preparations were often employed and more than one enzyme may have been present. The aldose content of the hydrolysate so obtained was then determined by oxidation with hypoiodite or by polarimetry, neither method being specific for glucose. In a few instances crystalline derivatives of D-glucose have been isolated; e.g. Schlubach & Elsner (1929) isolated α-pentaacetyl D-glucose from the acetolysis products of triacetyl inulin, but it is not clear how these authors purified the inulin used for this experiment. In addition, treatment of hydrolysates of fructosans with methanol in the presence of emulsin (Bourquelot & Bridel, 1921) has led to the isolation of β-methyl D-glucoside in a number of instances. Colin & Chaudin (1933*a*) have, by this method, obtained qualitative evidence of the presence of glucose radicals in synanthrin (Tanret, 1893), which is the most ethanol-soluble fructosan fraction isolated from tubers of *Helianthus tuberosus*. Qualitative evidence of the presence of glucose was similarly found by the workers quoted above in hydrolysates of the fructosans from *Scilla maritima*, *Lycoris squamigera* and *Phytobacterium vitrosum*. In all this work, however, there is always the possibility that the glucose may have originated in contaminating material of a non-fructosan nature.

Polarimetric examinations of hydrolysates can afford only approximate values for the amount of glucose present, since the calculations used take no account of optically active components other than glucose and fructose. For instance, with inulin, such calculations are complicated by the presence of difructose anhydrides (Jackson & Georgen, 1929). Attempts to determine small amounts of glucose in the presence of large amounts of fructose by means of oxidation of the aldose with hypoiodite are also unsatisfactory. Bailey & Hopkins (1933) have shown that fructose is undoubtedly oxidized by hypoiodite, the degree of oxidation being difficult to control as it depends, among other factors, on the rate of addition of the alkali, especially when sodium hydroxide is used. Reduction of hypoiodite by ketoses has also been found by the author to occur when the alkali employed is sodium carbonate (Macleod & Robison, 1929), or phosphate (Ingles & Israel, 1948). With both systems it has been found that, for example, 1:3:4:6-tetramethyl D-fructose consumes an appreciable amount of hypoiodite. Furthermore, mixtures of crystalline methylated glucoses and fructoses in

proportions of the order existing in fractions of hydrolysed methylated inulin (Hirst, McGilvray & Percival, 1950) reduce more hypoiodite than corresponds to the amount of aldose present. That the hypoiodite consumption of our purest 1:3:4:6-tetramethyl D-fructose, which cannot be obtained crystalline, is not due to the presence of furfural or its derivatives was shown by measurement of the ultraviolet absorption of the material by Mr P. D. Mitchell of this laboratory (cf. Hirst & Young, 1938). The quantity of furfural derivatives present was negligible. Further, the complete absence of glucose derivatives in the material was confirmed by paper chromatography.

In the work now to be described the polysaccharides were hydrolysed by 0·05N-sulphuric acid at 100° and glucose in the hydrolysates was estimated by the use of notatin (Keilin & Hartree, 1948). Control experiments on samples of pure fructose and sucrose were carried out simultaneously; in the case of fructose the measurable oxygen uptake of the system was only very slight. In hydrolysates of most of the fructosans examined D-glucose was found to the extent of 2–3 %, but where the molecule is undoubtedly of considerable size, as with irisin and bacterial levan, the glucose content was much lower.

EXPERIMENTAL

Isolation and purification of polysaccharides and sugars

Dahlia inulin. This was kindly provided by Dr C. S. Hanes, F.R.S., who isolated it according to McDonald (1946). Further purification was effected by recrystallization from water containing a trace of NH_4OH. During recrystallization the inulin was fractionated into rapidly and slowly crystallizing fractions, the former being collected on a filter and the latter by centrifugation.

Inulins from Taraxacum officinale *and* Inula Helenium. These samples were isolated by Dr D. J. Bell according to Onslow (1929), deproteinization of neutral extracts of the storage organs being effected by lead acetate, and the inulin precipitated from a neutral aqueous solution with an equal volume of ethanol. Further purification was effected by the author by recrystallization of each sample three times from water, the inulin being frozen out in each case.

Levan from grasses. This polysaccharide is contained in the leaves of certain grasses. Samples of dried grass of authentic strain were provided by the co-operation of Dr J. A. B. Smith of the Hannah Dairy Research Institute, and levan isolated according to the following method, evolved in 1947 by Dr J. S. D. Bacon in this laboratory.

$HgCl_2$ solution (1 l. of 0·1 % w/v) was percolated through 200 g. of the dried grass, previously mixed with 5 g. of finely powdered $HgCl_2$, packed in a glass column of length 1 m. and diameter 3·5 cm., using slight suction (30 cm. Hg). The aqueous extract (500 ml.), which could be shown to contain all the water-extractable carbohydrate, after adjustment to pH 8, was then treated with a solution containing 33 % (w/w) barium acetate monohydrate. The precipitate formed was collected by filtration on a layer of Hyflosupercel. To the filtrate was added 150 ml. of hot (100°) saturated $Ba(OH)_2$ solution and the resulting precipitate, which contained the barium-polysaccharide complex, allowed to settle overnight. The solid was then collected on a hardened filter paper (Whatman no. 50) and washed with cold, saturated $Ba(OH)_2$ solution until the washings were colourless. The solid complex, suspended in warm water, was decomposed by CO_2, the process being complete when the pH had fallen to 8. The residual solid was removed by filtration on a layer of charcoal, and the filtrate (pale yellow) concentrated at 40° under reduced pressure, any barium ions remaining being removed by judicious addition of saturated K_2SO_4 solution. The barium-free solution was finally treated with charcoal, filtered, and the polysaccharide precipitated by the addition of ethanol to a concentration of 80 % (v/v). The polysaccharide was then redissolved in a minimum volume of water and reprecipitated. The final product was dehydrated with absolute ethanol, acetone and light petroleum (b.p. 40–60°), and dried in a high vacuum over P_2O_5 and solid NaOH.

The levan so obtained is a white powder, soluble in water but insoluble in organic solvents. $[\alpha]_D^{18°} - 40·8°$ in water (*c*, 4·0). A typical yield from 200 g. dried Italain rye-grass was 6–7 g.

For the experiments described in this paper further purification was effected by two to ten additional reprecipitations. In some cases the material was fractionated by precipitation at concentrations of 60, 70 and 80 % (v/v) ethanol, from which were precipitated 87, 8 and 5 % of the material respectively.

Samples of levan from Italian rye-grass were prepared by the author, and samples of levan from the grass leafy cocksfoot, *Dactylis glomerata*, were kindly supplied by Prof. J. Beattie.

Irisin from wild Iris pseudacorus. Irisin was isolated from the rhizomes of wild *I. pseudacorus* according to the method of Euler & Erdtmann (1925) in the laboratory of Prof. J. Beattie, who kindly provided samples.

Even after acetylation and deacetylation the polysaccharide contained some material interfering with the action of notatin; this was removed by dialysis for 1 week against repeated changes of glass-distilled water.

Levan synthesized by Bacillus subtilis *from sucrose.* This was prepared by Mr V. W. Leonard of the Sub-department of Chemical Microbiology, Cambridge, according to Hibbert & Brauns (1931). Purification was not effected by electrodialysis, as described by these authors, but the material was first dialysed by Dr D. J. Bell against glass-distilled water containing a trace of thymol until the dialysate contained no fructose detectable with resorcinol. The polysaccharide was isolated as a dry solid by freeze-drying as all attempts to precipitate it by ethanol produced intractable gums.

D-*Fructose.* This was a commercial sample purified by recrystallization several times from ethanol-ether.

Sucrose. British Drug Houses Ltd. Analytical reagent.

Hydrolysis of fructosans

About 0·7 g. of each of the purified fructosans, previously dried to constant weight in a high vacuum over P_2O_5 and solid KOH, were hydrolysed with 12 ml. of 0·05N-H_2SO_4 at 100° for 10 min., the completion of the hydrolysis being checked polarimetrically. The solution was then cooled, neutralized (pH 7·0) with N-NaOH solution and, after filtering, the volume adjusted to 20 ml.

Table 1. *Results of estimations of glucose in mild acid hydrolysates of some fructose polysaccharides, calculated from the oxygen uptake of samples of these hydrolysates in the presence of notatin and catalase*

(The manometer vessels contained: phosphate buffer (0·25M, pH 5·6), 0·5 ml.; catalase* (0·15–0·2 mg. haemin/ml.), 0·1 ml.; notatin (5 mg. crude enzyme/0·2 ml.), 0·2 ml. in the side bulb; standard glucose solution (3 mg./ml.), 1·0 ml. or hydrolysate, 2·0 ml. Water was added to adjust the total fluid volume in each manometer vessel to 3·3 ml. O_2 uptake at 15° was followed until complete (4 hr.). Internal controls contained 0·5 ml. standard glucose together with 1·0 ml. hydrolysate.)

Fructosan examined (1)	Weight of fructosan in sample treated with notatin (g.) (2)	O_2 uptake by 3 mg. glucose (x) (μl.) (3)	O_2 uptake by sample of hydrolysate (y) (μl.) (4)	O_2 uptake in internal control (μl.) (5)	Theoretical O_2 uptake in internal control $\frac{1}{2}(x+y)$ (μl.) (6)	No. of mol. glucose/ 100 mono-saccharide mol. in hydrolysate (7)	No. of mol. fructose/ 100 mono-saccharide units in fructosan (8)	Total yield (9)	No. of mono-saccharide units in fructosan mol. assuming mol. con-tains only one glucose unit (10)	Mol. wt. of fructosan calculated from column 10 (11)
D-Fructose	0·075	180	6·7	94	93·3	0·15	100	100	—	—
Sucrose	0·0043	185	132	160	158·5	50	49	96	2	352
Taraxacum inulin	0·075	178	121	152	150	2·6	89	92	39	6,300
Dahlia inulin, rapidly crystallizing fraction	0·076	171	118	149	145	2·4	93	95	42	6,800
Dahlia inulin, slowly crystallizing fraction	0·079	183	119	150	151	2·2	94	96	45	7,290
Inula inulin	0·074	181	115	147	148	2·4	88	90	42	6,800
Inula inulin, after acetyl-ation and deacetylation	0·053	188	77	128	132	1·9	99	100	53	8,580
Irisin, dialysed	0·073	190	25	104	107·5	0·3	98	99	300	48,600
Levan, Italian rye-grass, pptd. in 80% (v/v) ethanol ten times	0·066	188	128	156	158	2·7	96	99	37	6,160
Levan, leafy cocksfoot, pptd. in 80% (v/v) ethanol twice	0·098	153†	145	148	149	2·7	90	93	37	6,160
Levan, leafy cocksfoot, pptd. in 80% (v/v) ethanol after acetylation and de-acetylation	0·076	183	177	187	180	3·4	91	95	29	4,700
Levan, Italian rye-grass, pptd. in 57% (v/v) ethanol	0·079	176	145	172	161	2·8	92	94	36	5,830
Levan, Italian rye-grass, pptd. in 69% (v/v) ethanol	0·071	183	239	233	211	5·0	92	97	20	3,240
Levan, Italian rye-grass, pptd. in 80% (v/v) ethanol	0·044	183	168	187	176	5·2	90	96	19	3,080
Levan synthesized by *B. subtilis* from sucrose	0·078	182	27	103	105	0·4	90	90	233	37,700

* Prepared from horse liver according to Keilin & Hartree (1945) and kindly supplied by Dr E. F. Hartree.

† In this experiment the solution of notatin used had been kept for 4 days resulting in a slow uptake of O_2.

Analytical methods

Determination of glucose. This was done in Warburg manometers at room temperature according to Keilin & Hartree (1948) using a notatin preparation, supplied by Sir Jack Drummond, F.R.S., of Boots' Pure Drug Co., in the presence of catalase. The amount of glucose in the hydrolysates was calculated from the O_2 uptake of these solutions in the presence of notatin and catalase compared with the O_2 uptake of a standard solution of glucose. Control experiments were carried out in which no notatin was present, and no O_2 uptake was observed either with the standard glucose solutions employed or with the hydrolysates. Further control experiments in which both the standard glucose solution and a sample of the hydrolysates were present together were performed and in every case a quantitative recovery of the glucose was obtained.

Determination of fructose. The amount of fructose in the hydrolysates was determined colorimetrically with resorcinol in the presence of conc. HCl according to Cole, Hanes, Jackson & Loughman (1948).

RESULTS

The results of determinations of the glucose content of certain fructosans are recorded in Table 1. The figures given for fructose recovery in column 7 are based on the colorimetric determination of fructose which measures free fructose liberated on hydrolysis of the polysaccharide with dilute acids and also, in the case of inulin, fructose existing in the form of difructose anhydride (cf. McDonald, 1946) produced under these hydrolysis conditions. When pure fructose was subjected to the hydrolysis procedure employed for the polysaccharides a 100 % recovery was obtained by this colorimetric method, but in the instances of the polysaccharides examined the combined glucose and fructose recoveries obtained from the hydrolysates was not always 100 % of the theoretical value, calculated from the weight of polysaccharide taken. Lower values were probably due to incomplete drying of the fructosan (cf. in the case of inulin, Irvine & Steele, 1920), despite the fact that all were dried in a high vacuum, over phosphoric oxide to constant weight. The figures for glucose recorded in column 6 of Table 1 are therefore expressed in the form of the number of molecules of glucose contained in 100 molecules of monosaccharides as estimated in the hydrolysates, and are not presented as a percentage of the measured weight of fructosan hydrolysed. Further, the figures for glucose, as presented, have been corrected for the slight oxygen uptake observed with pure fructose when treated similarly to the polysaccharides examined.

In column 9 are given the numbers of monosaccharide units in the fructosan molecules calculated on the assumption that each molecule contains only one glucose unit; and molecular weights of the fructosans estimated from these figures are shown in column 10.

Purified samples of inulin from different plant sources have glucose contents of 2·2–2·6 %, and this glucose, at any rate in the case of inulin isolated from *Inula Helenium*, persists even after acetylation and deacetylation of the polysaccharide. Dahlia inulin was fractionated during recrystallization from water; both fractions, however, contain very similar amounts of glucose (cf. Jackson & McDonald, 1930) and on this basis, therefore, do not differ appreciably in chain length. Levans from grasses have a slightly higher glucose content, which increases in the fractionated material as the solubility in ethanol increases, whereas the one sample examined of a levan synthesized by a bacterium from sucrose had a much smaller, although still definite, glucose content of the same order as that of irisin. This levan and irisin both consist, apparently, of molecules large compared with those of grass levan, for neither, unlike grass levan, will pass through a cellophan dialysis sac. Examination of some bacterial levans on the ultracentrifuge by Ingelman & Siegbahn (1944) revealed very large particles. Examination of irisin by Dr A. G. Ogston showed the material to be apparently homogeneous in the ultracentrifuge and to have a molecular weight of 22,000–24,000.

DISCUSSION

The presence of D-glucose in mild acid hydrolysates of certain fructosans has been confirmed. The actual amount, as determined by notatin, is rather lower than that reported by earlier workers, probably due to reasons suggested in the introduction. It is interesting to note that in the French work the levan synthesized by *Phytobacterium vitrosum* has a much lower glucose content than other fructosans examined by this group of workers, in accordance with the fact that bacterial levans, in general, appear to consist of molecules of considerable size.

The question that arises from the undoubted presence of glucose in hydrolysates of many fructosans is whether this glucose is, in fact, an integral part of the fructosan molecule, or whether it arises from a separate glucose-containing polysaccharide which is difficult to separate from the fructosan. To support the former view, the amount of glucose present in fructosan hydrolysates does seem to be related to the molecular weight. In early work in this field Tanret (1893) found that the glucose content of his fructosan fractions isolated from tubers of *Helianthus tuberosus* increased as the fructosans became more soluble in ethanol; presumably as the molecular weight decreased. Colin & Chaudin (1933*a*) compared the glucose content as calculated by Tanret from the rotation of mild acid hydrolysates with the molecular weights of the fructosan fractions determined cryoscopically. Whether cryoscopic determinations of molecular weights of fructosans

give accurate values is not definitely known. In view of later work by Thaysen, Bakes & Green (1929) and Bacon & Edelman (1951), who have shown that the true inulin content of the Jerusalem artichoke varies with the time of year, it seems possible that the polysaccharide inulin, as defined in the introduction of this paper, exists *in vivo* in equilibrium with shorter-chain fructosans representing stages in the synthesis and/or breakdown of inulin. Also it may well be that, even after repeated recrystallization from water, the inulin is not perfectly homogeneous, but consists of a mixture of similarly constituted fructosans of slightly differing chain lengths. In the samples of fructosans examined with the help of notatin, the glucose content of fractionated grass levan increases as the solubility in ethanol increases, in a similar manner to the fructosans isolated by Tanret (1893) from *H. tuberosus*. In unfractionated samples of grass levan, the molecular weight, as calculated from the glucose content, assuming that one molecule contains only one glucose unit, is of the same order as that determined by Dr A. G. Ogston for Italian rye-grass levan from ultracentrifuge data (Bell & Palmer, 1949). This levan was found to be definitely non-homogeneous with a mean molecular weight of 5400. In another experiment the material was dialysed for 3 weeks against distilled water, whereupon some of it passed through the dialysis sac. The remaining material was homogeneous and had a molecular weight of 8700. Further, the glucose contents of levan synthesized by *Bacillus subtilis* and of irisin are much less than that of grass levan in agreement with the fact that molecules of these fructosans are large compared with inulin or grass levan.

Further evidence to support the hypothesis that glucose present in hydrolysates of certain fructosans is contained in the fructosan molecule itself is as follows. If the glucose does arise from contaminating glucosans these polysaccharides must be very different from glucosans already known in that they are rapidly hydrolysed by 0·05N-sulphuric acid. Adams, Richtmeyer & Hudson (1943) hydrolysed purified dahlia inulin with purified yeast invertases. Glucose was detected in the hydrolysates; the amount as determined by hypoiodite oxidation was admittedly less than has been found by similar determinations on acid hydrolysed inulin, but the authors point out that 'the glucose must, if not an integral part of the inulin molecule, be an integral part of an associated molecule which is hydrolysed at about the same rate as inulin'.

If the glucose does arise from a glucosan, different samples of the same fructosan would be expected to contain different amounts of glucose depending on the degree of purification. From Table 1, however, it can be seen that levan from Italian rye-grass, precipitated in 80% (v/v) ethanol ten times, contains the same amount of glucose as levan from leafy cocksfoot, precipitated only twice. Again all samples of inulin examined, prepared by different workers from different plant sources, contain approximately the same amount of glucose.

Finally, fructosans containing glucose are widely distributed in nature, are isolated by different chemical methods and have different chemical structures. Plant sources occur both in monocotyledonous and dicotyledonous families, and glucose-containing fructosans are found in both leaves and storage organs, and glucose exists even in bacterial levans, synthesized from sucrose.

SUMMARY

1. By the use of notatin the presence of glucose has been confirmed, and the amount determined, in mild acid hydrolysates of the purified fructosans inulin, grass levan, irisin and levan synthesized by *Bacillus subtilis* from sucrose.

2. The amount of glucose in hydrolysates of fructosans appears to vary inversely with the molecular size of the polysaccharide.

3. On the assumption that one fructosan molecule contains one glucose radical the molecular weights of these polysaccharides have been calculated.

4. Evidence that glucose is an integral part of the fructosan molecules is discussed.

In addition to those named in the text who gave materials, I am much indebted to Dr T. Mann who instructed me in the technique of estimating glucose with notatin, and to Dr D. J. Bell for encouragement and advice. I wish also to acknowledge a grant from the Agricultural Research Council held during the time in which this work was carried out.

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The Fate of Certain Organic Acids and Amides in the Rabbit

12. AMINOHYDROXYBENZOIC ACIDS

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Our interest in the aminohydroxybenzoic acids arose from the finding that these acids or their amides were excreted after the administration to rabbits of the aminobenzamides (Bray, Lake, Neale, Thorpe & Wood, 1948) and *o*-nitrobenzamide (Bray, Thorpe & Wood, 1949). 2-Amino-3-hydroxy- and 2-amino-5-hydroxy-benzoic acids were isolated from hydrolysed *o*-aminobenzamide urine, 3-amino-6-hydroxybenzoic acid (5-amino-2-hydroxybenzoic acid) from hydrolysed *m*-aminobenzamide urine, 4-amino-3-hydroxybenzoic acid from hydrolysed *p*-aminobenzamide urine and 2-amino-5-hydroxybenzoic acid from hydrolysed *o*-nitrobenzamide urine. It was also suspected that 3-amino-4-hydroxybenzoic acid (or its amide) was present in *m*-aminobenzamide urine and 2-amino-3-hydroxybenzoic acid (or its amide) in *o*-nitrobenzamide urine, although these were not isolated. It was, however, shown later, using paper chromatography, that all the theoretical products of ortho and para hydroxylation are present in aminobenzamide and *o*-nitrobenzamide urines (Bray, Lake, Thorpe & White, 1950).

Two aminohydroxybenzoic acids have recently received attention by other workers. 4-Amino-2-hydroxybenzoic acid ('*p*-aminosalicylic acid', 'PAS') has been used in the treatment of tuberculous lesions in human patients (e.g. Lehmann, 1946; Dempsey & Logg, 1947), and some investigations of its metabolism have been reported (e.g. McClosky, Smith & Frias, 1948; Way, Weiss, Howie & Smith, 1948). In a more detailed study Venkataraman, Venkataraman & Lewis (1948) concluded that 40–60 % of the administered acid was excreted by the rabbit in 24 hr. as the *N*-acetyl derivative. No decarboxylation, glycine conjugation or *O*-conjugation was observed.

2-Amino-3-hydroxybenzoic acid has been shown to be a precursor of nicotinic acid in *Neurospora* (Mitchell & Nyc, 1948; Bonner, 1948) and in the rat (Mitchell, Nyc & Owen, 1948; Heidelberger, Abraham & Lepkovsky, 1948, 1949; Albert, Scheer & Deuel, 1948). It is suggested that this acid may be an intermediate in the conversion of tryptophan into nicotinic acid. No investigations of the metabolic fate of large doses of 2-amino-3-hydroxybenzoic acid appear to have been carried out, though Henderson & Hirsch (1949) reported that rats which had received intraperitoneal injections of this acid excreted quinolinic acid (pyridine-2:3-dicarboxylic acid).

In the present investigation, which is an extension of that previously reported in brief (Bray, Ryman & Thorpe, 1948*b*), we have studied the fate of seven of the ten aminohydroxybenzoic acids and have determined the main metabolic pathways of six of them. 2-Amino-4-hydroxy and 3-amino-5-hydroxybenzoic acids have been prepared, but in yields so small as to render accumulation of the acids for metabolic investigation impracticable. Attempts to prepare 2-amino-6-hydroxybenzoic acid were unsuccessful.