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# The Trisaccharide Fraction of Some Monocotyledons

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In those plants which accumulate fructosans (chiefly members of the Compositae and Campanulaceae, and the monocotyledons), oligosaccharide fractions of a similar character are always present. Relatively little is yet known about these fractions, despite the increased attention which has been given in recent years to the structure of the polysaccharides.

Dedonder (1952) and Bacon & Edelman (1951) suggested that the trisaccharide fraction of the tubers of *Helianthus tuberosus* contained *O*-α-D-glucopyranosyl-(1→2)-*O*-β-D-fructofuranosyl-(1→2) β-fructofuranoside ($1^F$-β-fructosylsucrose). Later work (Allen & Bacon, 1956) confirmed this, and also demonstrated the virtual absence of other trisaccharides. In unpublished work, the author has recently shown that the rampion (*Campanula rapunculus* L.) resembles the composite in this respect.

The use of paper chromatography has shown the presence of fructose-containing oligosaccharides in various monocotyledons: for example, in the leaves and stems of wheat (Bradfield & Flood, 1950; Lopatecki, Longair & Farstad, 1957), in onion bulbs (Egyptian 'Silvers'; Dr J. Edelman, personal communication, 1951), in leaves and stems of barley (Porter & Edelman, 1952) and in the seeds of cereals (MacLeod, 1951; White & Secor, 1953), but in very few cases have the substances been identified. Schlubach, Lübbers & Borowski (1955) have reported that *O*-α-D-glucopyranosyl-(1→2)-*O*-β-D-fructofuranosyl-(6→2) β-D-fructofuranoside ($6^F$-β-fructosylsucrose; 'kestose') is the main trisaccharide of perennial ryegrass (*Lolium perenne* L.).

It seemed that the use of chromatographic methods which have permitted the isolation of the products of fructose transfer by invertases (Edelman, 1956; Bacon, 1954) might help to characterize the trisaccharide fraction of monocotyledons. These trisaccharides are of interest not only because they serve as substrates for digestive enzymes, and for microbial attack above and below the ground, but also because their structures may act as pointers to the mechanism of fructosan biosynthesis (cf. Feingold, Avigad & Hestrin, 1956).

In the experiments reported here these methods were applied to the onion, *Allium cepa* L. (a preliminary report has appeared: Bacon, 1957), the leek, *A. porrum* L., and two grasses: Italian ryegrass (*Lolium multiflorum* Lam.) and tall oatgrass (*Arrhenatherum elatius* L., J. and C. Presl, var. *bulbosum*, 'onion couch').

Two trisaccharides, $1^F$-β-fructosylsucrose and *O*-β-D-fructofuranosyl-(2→6)-α-D-glucopyranosyl-(1→2) β-D-fructofuranoside ($6^G$-β-fructosylsucrose have been identified in all four species; $6^F$-β-fructosylsucrose is probably present, but in smaller amounts, in both grasses.

## MATERIALS

*Onions and leeks.* These were grown in 1956–57 in the gardens of this Institute, the varieties of onion being 'Crosslings' and 'Golden Mammoth', and of leek 'Scottish

Musselburgh'. Other samples of onions of unknown origin were purchased locally from time to time.

*Grasses.* Italian ryegrass, strain S22, was grown in the greenhouse at about 18° in pots of garden soil.

Tall oatgrass was collected in hedgerows round Dittisham, South Devon, during July 1957. By this time the plants had lost their seeds and were almost air-dry. Only the basal nodes and lower part of the stem were kept.

*Sugars.* The trisaccharides used as reference standards were obtained from plant material ($1^F$-β-fructosylsucrose) or were the products of invertase action ($6^G$-β-fructosylsucrose and $6^F$-β-fructosylsucrose). Dr B. H. Howard of the Rowett Institute (see Howard, 1959) kindly supplied us with a sample of the last-named.

## METHODS

*Extraction of plants.* This followed closely that described by Bacon & Dickinson (1957). All material, with the exception of that from *A. elatius*, was extracted with boiling water as soon as possible after its removal from the plant, the pots of ryegrass being brought into the laboratory for sampling. After the addition of an equal volume of ethanol and removal of any precipitate by filtration, the extract was evaporated to dryness under reduced pressure and dissolved in water. At this stage, and during further fractionation, the soluble sugars were examined by paper chromatography.

*Paper chromatography.* Most of the solvents and spraying reagents used have already been described (Bacon & Dickinson, 1957); additional solvents are mentioned where their use is described below.

A useful reagent for free ketoses was brought to our notice by Dr D. J. D. Hockenhull of Glaxo Laboratories Ltd., Greenford, Middlesex; it was developed there for the detection of steroidal primary and secondary ketols (Brooks *et al.* 1958).

For use with sugars, 20 mg. of 2:5-diphenyl-3-*p*-styrylphenyltetrazolium chloride (May and Baker Ltd., Dagenham, Essex; M & B 1767) is dissolved in 10 ml. of ethanol and immediately before spraying is mixed with an equal volume of 0·1 N-NaOH. This reagent is more reactive than those based on 2:3:5-triphenyltetrazolium chloride (Trevelyan, Procter & Harrison, 1950; Wallenfels, 1950). After a few minutes at room temperature, or with slight warming, free ketoses and oligosaccharides having a ketose-reducing group appear as purple spots on a yellow background; 5 μg. of fructose per spot is detectable. Aldoses do not appear to react at all, unless the paper chromatogram is heated strongly during the removal of solvent. (This spraying reagent is referred to below as the 'TSTZ spray'.)

*Column chromatography.* The extracts were further fractionated on charcoal–Celite columns (cf. Bacon & Dickinson, 1957). Where the oligosaccharide component was small a preliminary separation on charcoal–Celite was carried out with water followed by 25% (v/v) ethanol, the ethanol eluate then being subjected to gradient elution from a similar column. Extract containing $x$ mg. of total trisaccharide was eluted from columns of about $2x$ g. of charcoal–Celite (1:1, w/w) with a gradient produced by 50% (v/v) ethanol dropping into $20x$ ml. of water. This usually separated the trisaccharides into three fractions, but did not always separate them completely from accompanying di- and tetra-saccharides. For this reason each trisaccharide fraction was further purified by partition chromatography on Celite (Lemieux, Bishop & Pelletier, 1956). As much as 50 mg. of sugar was applied to 10 g. of Celite no. 535 packed in a column 19 mm. diam. and 10–11 cm. high, and eluted with butan-1-ol saturated with water. No special precautions were taken to control temperature but a trap was inserted between the main reservoir and the column to remove any droplets of water. The trisaccharides emerged after about 600–700 ml. had passed through the column and were contained in about 400 ml. of butanol. Evaporation to dryness under reduced pressure always left a greasy residue from which the sugar could be extracted with water. Attempts to remove the contaminating material by a preliminary extraction of the Celite with acetone, or with butanol in a Soxhlet apparatus, were unsuccessful, as also was a preliminary fractional distillation of the butanol.

*Characterization of trisaccharides.* Indications of the nature of the trisaccharides were given by their $R_F$ values and response to various spraying reagents on paper chromatograms, and by their position in the effluent from charcoal columns (cf. Bacon, 1954). After separation the optical rotation was measured where the total amount available permitted, and analyses of fructose (Seliwanoff reaction; Bacon & Bell, 1948) and glucose (glucose oxidase; Huggett & Nixon, 1957) were made on oxalic acid hydrolysates.

The Raybin test (Raybin, 1933) was applied in solution or on paper (Breuer & Bacon, 1957). In the latter case it was found better to apply 0·5–1·0 mg. of sugar to paper previously soaked in 5% (w/v) $MgCl_2$ and dried, and to spray with a 1% (w/v) solution of diazouracil in 0·1 N-NaOH, freshly prepared at 0°. After 3 min. in an oven at 80° a positive reaction was discernible and the blue intensified when the papers were left for a day or two at room temperature.

The samples of $1^F$-β-fructosylsucrose and raffinose isolated from Celite columns were crystallized without difficulty; $6^G$-β-fructosylsucrose was converted into a dry powder by precipitation with ether from solution in methanol. The infrared spectra of these materials were examined and compared with those of authentic specimens.

Additional evidence for the presence of $6^G$-β-fructosylsucrose was obtained by partial acid hydrolysis (Bacon, 1954), which produces $6^G$-β-fructosylglucose, a reducing disaccharide with $R_F$ between that of the trisaccharide and that of sucrose.

## RESULTS

For each plant the isolation procedure followed the outline already given under Methods: from charcoal–Celite columns fractions which would be expected to contain respectively $6^F$-, $1^F$- and $6^G$-β-fructosylsucrose were first obtained, and then, if necessary, were subjected to partition chromatography on Celite columns. A crystalline sample of $1^F$-β-fructosylsucrose was obtained from each of the plants examined. In each case its infrared spectrum was found to be identical with that of an authentic sample, and the Raybin test was negative. Partial acid hydrolysis and the Raybin test

were applied to each sample suspected to be $6^G$-β-fructosylsucrose, with the expected results. Infrared spectra confirmed the identity for *A. cepa*, *A. porrum* and *L. multiflorum*; the sample from *A. elatius* was not examined. Additional quantitative information is given in Table 1, and features peculiar to each plant are considered below.

*Onion* (Allium cepa *L.*)

Paper chromatograms of extracts of the whole bulb showed the presence of considerable amounts of fructose-containing oligosaccharide. With the exception of a sample of Egyptian onions bought in 1957 the onions examined showed no sign of true fructose polysaccharide, all the oligosaccharide moving off the base line when development with butanol–acetic acid (Partridge, 1948) was continued for 4–5 days. Examination of a series of fractions from charcoal–Celite suggested that the largest molecules contained seven to eight monosaccharide residues.

Extraction of the bulb, layer by layer, showed that the oligosaccharide occurred mainly in the inner region; the outer scales often contained nothing but sucrose, fructose and glucose, and occasionally very little water-soluble carbohydrate at all. A typical analysis is given in Table 2. The total sugar and oligosaccharide concentrations varied markedly from top to bottom and round the circumference of each scale.

About 10% of the ketose of extracts was present in the trisaccharide fraction (i.e. about 0·5% on a fresh-weight basis). Raffinose was not seen at any stage of the fractionation. Only two trisaccharides could be detected, $1^F$-β-fructosylsucrose and $6^G$-β-fructosylsucrose (Table 1). The total yield corresponded to about 0·4% of fresh weight of onion taken, the $1^F$-β-fructosylsucrose preponderating slightly.

The pattern of elution of the oligosaccharides from charcoal–Celite was complex, and there were at least two components of each chain length and possibly more. Only $1^F$-β-fructosylsucrose emerged pure; $6^G$-β-fructosylsucrose was accompanied by the first tetrasaccharide to emerge (fructose:glucose ratio, 2·95:1), and in later fractions more overlapping occurred. However, it appears that by a combination of absorption with partition chromatography it should be possible to separate each of the oligosaccharide fractions above trisaccharide into at least two fractions.

Table 1. *Some quantitative observations on the trisaccharide fractions*

Optical rotation was measured in a 2 dm. stainless-steel tube, capacity about 2·5 ml. Fructose and glucose were estimated on samples hydrolysed with 0·5% oxalic acid at 100° for 30 min.

| Suspected structure | Species | Yields of fraction (mg./100 g. fresh wt. of tissue)* | $[\alpha]_D$ Calc. on dry wt. | $[\alpha]_D$ Calc. from sugars in hydrolysate | Ratio, fructose: glucose in acid hydrolysate |
|---|---|---|---|---|---|
| $1^F$-β-Fructosylsucrose | *A. cepa* | 150, 235 | +28° | +28° | 1·90 |
| | *A. porrum* | 45, 75 | +27 | +28 | 1·94 |
| | *L. multiflorum* | 10 | +23 | +24 | 1·95 |
| $6^G$-β-Fructosylsucrose | *A. cepa* | 125, 165 | —† | +21 | 1·80 |
| | *A. porrum* | 65 | +19 | +22 | 1·90 |

* To nearest 5 mg. Yield of $6^G$-β-fructosylsucrose from *L. multiflorum*: 10 mg.; yield of each trisaccharide from dry stems of *A. elatius*: 15–20 mg.

† Not measured.

Table 2. *Composition of individual layers of onion bulb*

An onion (120 g.) was halved and the scales were separated. Each scale was weighed and extracted with twice its weight of water at 100°. The extracts were analysed by quantitative paper chromatography, the ketose being measured in regions corresponding to fructose, sucrose and as many of the higher oligosaccharides as could be separated. The results are expressed as mg. of ketose/g. fresh weight of tissue. The scales are numbered from the outside, 9 being the innermost part of the bulb, including the growing point.

| | Scale no. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sugars | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Pentasaccharide and higher | — | — | — | — | 2·7 | 3·3 | 6·4 | 8·5* | 12·0 |
| Tetrasaccharide | — | — | — | — | 4·7 | 4·6 | 4·0 | 8·7 | 10·1 |
| Trisaccharide | — | — | — | 0·5 | 6·2 | 7·4 | 7·4 | 9·5 | 4·7 |
| Sucrose | 4·7 | 4·6 | 5·2 | 5·6 | 10·4 | 11·4 | 9·6 | 11·1 | 12·3 |
| Fructose | 16·4 | 15·3 | 14·6 | 14·2 | 11·0 | 12·2 | 12·3 | 9·1 | 4·9 |
| Wt. of scale (g.) | 28·0 | 22·5 | 32·9 | 14·0 | 8·0 | 4·2 | 2·1 | 1·0 | 1·1 |

* Pentasaccharide 3·5; hexasaccharide and higher 5·0.

All the oligosaccharide was hydrolysed into glucose and fructose by yeast invertase, or by dilute oxalic acid at 100°.

*Leek* (Allium porrum *L.*)

Although onion and leek have basically the same structure, in the mature leek plant the leaves remain green and are less sharply demarcated from the bulbous portion. The water-soluble carbohydrates increase in concentration towards the base as the green decreases. When compared with the onion bulb, the leek bulb was found to contain similar concentrations of fructose, sucrose and lower oligosaccharide, but five to ten times as much higher oligo- and poly-saccharide. The proportion of the total ketose comprised in the higher polymers thus exceeds even that in the innermost layer of the onion analysed in Table 2, and was only approached (in our limited experience) by extracts of layers of the Egyptian onions mentioned above. The green portions of the leaves contained little oligo- or poly-saccharide, and raffinose could be seen to be a major component of the trisaccharide fraction. The distribution of oligo- and poly-saccharide through successive sheaths, judged qualitatively, resembled that in the onion bulb. Extracts of the bulbous portion were taken for an investigation of the trisaccharide fraction.

The polysaccharide did not interfere with fractionation on charcoal–Celite, but the first preparations of $1^F$-$\beta$-fructosylsucrose would not crystallize. More careful examination of these effluents showed that the trisaccharide was immediately preceded by a substance of slightly greater $R_F$ (butanol–acetic acid), containing only fructose and reducing the TSTZ spray. When these fractions were excluded, the $1^F$-$\beta$-fructosylsucrose crystallized without difficulty. $6^G$-$\beta$-Fructosylsucrose was isolated from later fractions of the effluent. A mixture of a tetrasaccharide and raffinose was present in the intervening fractions. The raffinose was isolated and crystallized, and its identity confirmed by examination of the infrared spectrum.

As in the onion, the oligosaccharide fraction was very complex, additionally so from the presence of raffinose and its higher homologues. Some evidence for the presence of the latter was obtained by subjecting extracts to mild acid hydrolysis and comparing the resistant fructose-free oligosaccharides with those present in a mild acid hydrolysate of psyllid honeydew from the ash tree, *Fraxinus excelsior* (Bacon & Dickinson, 1957). Stachyose was present (as judged by this incomplete evidence) in extracts from all parts of the plant, but verbascose could be detected, and there in very small amounts, only in the fleshy roots. The latter contained smaller amounts of sucrose and oligosaccharide than the leaves.

*Italian ryegrass* (Lolium multiflorum *Lam.*)

Examination of young plants (five to eight leaves) showed the presence of fructosan but very little oligosaccharide. Raffinose was conspicuous in the green part of the leaves but present only in traces in the sheaths. Since the latter have a higher content of water-soluble carbohydrate, extracts were made from plants from which all but the newest leaf had been severed at the junction of leaf with sheath. The material extracted was therefore essentially the sheaths of the older leaves and the whole of the youngest, perhaps 30–40 % of the weight of the plant (minus roots) and half of the total ketose.

After preliminary separation of the oligosaccharide fraction from mono- and poly-saccharide, quantities of extract corresponding to 35–45 g. fresh weight were applied to columns containing 20–40 g. of charcoal, and three portions of effluent were collected corresponding to the three fructosylsucroses. Corresponding fractions from several such fractionations, representing a total of 125 g. of grass, were combined for further examination.

The first fraction showed on paper chromatograms two main spots, resembling sucrose and $6^F$-$\beta$-fructosylsucrose in $R_F$ and colour reactions. The total amount of $6^F$-$\beta$-fructosylsucrose in this fraction was very small, comparison with standards on paper chromatograms suggesting not more than 2 mg. The portion also contained smaller amounts of raffinose, and of two substances reducing the TSTZ spray, one having the same $R_F$ as the substance found in leek extract.

The other two fractions (which both contained small amounts of raffinose) were chromatographed on Celite and yielded about 15 mg. each of $1^F$-$\beta$-fructosylsucrose and $6^G$-$\beta$-fructosylsucrose respectively. The specific rotation could not be measured accurately, but the other tests of identity were applied.

*Tall oatgrass*
(Arrhenatherum elatius *var.* bulbosum)

This species was examined because it accumulates fructosan as a reserve carbohydrate. The dry bulbous lower nodes of the stem (100 g.) were sliced into boiling water and the whole was later transferred to a blender to disintegrate the very tough residue. The extract so obtained contained large amounts of polysaccharide, part of which was not removed by precipitation with 50 % ethanol and subsequently contaminated the effluent from charcoal columns. Eventually the greater part was removed by raising the ethanol concentration to 80 % (v/v). The precipitate, corresponding to 63 g. of original stems, weighed 10·8 g. Part of it was

purified by precipitation with $Ba(OH)_2$ (Palmer, 1951), and the product, which had an ash content of less than 0·1 %, had $[\alpha]_D^{21} - 44°$. Analyses of fructose and glucose suggested that it was at least 97 % pure and contained 2·8 % of glucose. Colin & de Cugnac (1926) also found $[\alpha]_D - 44°$ for a fructosan from this species.

The oligosaccharide fraction was by comparison very small, but three fractions were obtained resembling closely those from ryegrass.

The first fraction, which was relatively more abundant than in the ryegrass, was equally complex. It contained an appreciable amount of sucrose. Paper chromatography in several solvents (butanol–acetic acid–water; butanol–pyridine–water, Jeanes, Wise & Dimler, 1951; propan-2-ol–ethanol–water, MacLeod & McCorquodale, 1958) and the application of various spraying reagents provided the following information about the trisaccharide component: (*a*) the main component contained glucose and fructose and had $R_F$ values indistinguishable from those of $6^F$-β-fructosylsucrose; (*b*) a substance reducing the TSTZ spray was also present; (*c*) hydrolysis with dilute oxalic acid left a disaccharide with $R_F$ similar to that of melibiose.

Chromatography of the fraction on Celite confirmed that the main trisaccharide component corresponded with $6^F$-β-fructosylglucose, but small amounts of substances with larger and smaller $R_F$ values were also detected. The reducing substance was distinguishable from melibiose, from the reducing trifructose isolated from the leek extracts and from $6^F$-β-fructosylsucrose by its behaviour in one or other of the solvents named.

Only very small amounts of trisaccharide could be isolated from the other two fractions. From the second, a few milligrams of crystalline $1^F$-β-fructosylsucrose were obtained and from the third a trisaccharide which was not fully characterized, but is probably $6^G$-β-fructosylsucrose.

### *Observations on the metabolism of the onion oligosaccharides*

By dividing a single scale of an onion bulb into many small pieces and selecting groups representative of all positions, it proved possible to obtain comparable samples. Unless these special precautions were taken it was impossible to distinguish metabolic changes from the great local variations in concentration of the carbohydrates already referred to above.

The vacuum-infiltration technique was used to introduce glucose and fructose, in 20 % (w/v) solution, into the bulb tissue. After infiltration the pieces were rinsed in water, blotted on filter paper and left in covered Petri dishes at room temperature for periods up to 3 days. During this time no colonies of micro-organisms were seen. (When dinitrophenol was added to the infiltration medium extensive growth of bacteria occurred in 1 day, suggesting that poisoning of the tissue facilitated their establishment on its surface.) The pieces were then extracted in boiling water and the ketose content of the oligosaccharides was measured by quantitative paper chromatography (Bacon & Loxley, 1952).

Although clearly the variability of the starting material still influenced the results, in the limited number of experiments done (12 with glucose and 2 with fructose) there was a substantial increase in sucrose and often a marked increase of the trisaccharide fraction; Table 3 gives the results of eight experiments of identical design, with glucose infiltration. In experiments in which higher oligosaccharides were also estimated [a total of five infiltrations with glucose, three with fructose and four with 20 % (w/v) sucrose] the ketose of this fraction also increased. Because the designs of these experiments varied it is not possible to combine the results, but the increases were of the order of 50 %, as with the trisaccharide fraction.

## DISCUSSION

The oligosaccharides of the grasses constitute such a small proportion of the total carbohydrate that it might be argued that they arise as artifacts of the extraction process. The leaf material is so thin that it seems unlikely that any enzymes could survive long enough in the boiling water to bring about any appreciable transformation of the sucrose or polysaccharide, but the high temperature itself might cause some hydrolysis of the fructosan (Laidlaw & Reid, 1951; Schlubach, 1958). If this were so, one might expect to find mainly reducing

Table 3. *Changes in sugars of onion bulb after infiltration with glucose*

A single scale from an onion weighing 150–200 g. was divided into small pieces and three groups were selected (*A*, *B*, *C*), each of about 25–30 pieces (8–10 g.). *A* and *B* were infiltrated with 20 % (w/v) glucose and *C* with water. *A* was extracted at once with water at 100°, *B* and *C* after 3 days in a closed vessel (see text). Ketose was measured in the fructose, sucrose and trisaccharide fractions after separation on paper chromatograms. Results are expressed in mg. of ketose/g. fresh wt. of original tissue; the figures given are the averages of eight such experiments.

| | Infiltrated with water; extracted after 3 days | Infiltrated with glucose | |
|---|---|---|---|
| | | Extracted at once | Extracted after 3 days |
| Trisaccharide | 4·3 | 3·7 | 5·6 |
| Sucrose | 4·7 | 5·8 | 10·5 |
| Fructose | 14·8 | 17·1 | 16·9 |

polyfructose fragments among the oligosaccharides. In fact very little reducing activity could be detected on paper chromatograms, despite the use of a sensitive reagent.

However, it is possible that the reducing substance (probably a trifructose) contaminating the $1^F$-$\beta$-fructosylsucrose fractions from the leek arose from some hydrolysis of polysaccharide, but not necessarily at the initial extraction stage. Extracts of leek tissue made by blending in the presence of mercuric chloride solution at room temperature contained less material reacting with the TSTZ reagent than those made in water at 100°. However, the main reducing substance in boiling-water extracts, although apparently liberated by hydrolytic conditions (e.g. during the removal of butanol–acetic acid from paper chromatograms), does not seem to be a carbohydrate; it has a greater $R_F$ than the trisaccharides and gives little reaction with the benzidine–trichloroacetic acid spray (Bacon & Edelman, 1951). Most of the leek extracts prepared showed little reducing activity associated with the main oligosaccharide series; the one from which the trifructose was isolated was exceptional in this respect, and may have suffered some hydrolysis during concentration.

In the onion, where no polysaccharide fraction is found, there can be no doubt that the oligosaccharides are present as such in the intact bulbs, and it is tempting to argue that the similarity in the trisaccharide fractions of all four species shows that they occur as a result of normal physiological processes. If this is accepted we should expect $1^F$-$\beta$-fructosylsucrose and $6^G$-$\beta$-fructosylsucrose to be the typical trisaccharides of the monocotyledons, whereas $6^F$-$\beta$-fructosylsucrose would occur only occasionally or in relatively small amounts. This picture is confirmed by the paper-chromatographic studies of White & Secor (1953) on the oligosaccharide fraction of the wheat berry ($6^F$-$\beta$-fructosylsucrose was present in very small amounts, insufficient for analysis), but it is not easily reconciled with the results of Schlubach *et al.* (1955). They found only $6^F$-$\beta$-fructosylsucrose ('kestose') in the trisaccharide fraction of *Lolium perenne* L. Their description of their fractionations does not permit one to speculate on the possible presence of other trisaccharides, except in one respect: they state that their trisaccharide gave a positive Raybin reaction. A positive reaction was wrongly attributed to $6^F$-$\beta$-fructosylsucrose by Albon, Bell, Blanchard, Gross & Rundell (1953) (see Bacon & Bell, 1953; Howard, 1959), so that $6^G$-$\beta$-fructosylsucrose, which does react in this test, may have been present in their material. The preponderance of $6^F$-$\beta$-fructosylsucrose apparently shown by their work might be explained by a species difference, or a considerable change in the trisaccharide fraction as the plants mature. We have not attempted to study the physiological influences on the composition of the trisaccharide fraction. The complexity of the fraction makes this difficult, but by no means impossible; estimation by quantitative paper chromatography of the ketose-containing trisaccharide in each of the three fractions from charcoal–Celite would give a good idea of how the amounts of the three main trisaccharides vary during growth and maturity. Such a study might resolve the contradiction between our results and those of Schlubach and his collaborators.

Although in the composites there is only one trisaccharide, and this, $1^F$-$\beta$-fructosylsucrose, has the 2:1-fructose–fructose link characteristic of the polysaccharide fraction (inulin, etc.), in the monocotyledons we have examined the situation is more complex. In the first place there are two or three fructosylsucroses, and secondly that one which has the 2:6-linkage characteristic of grass and cereal fructosans is by no means the most prominent, and is seemingly absent in the leek and onion. A parallel to this situation is found in the oligosaccharide fraction present in levansucrase reaction mixtures (Feingold *et al.* 1956; Avigad & Feingold, 1957). Here $1^F$-$\beta$-fructosylsucrose is the most abundant trisaccharide and $6^F$-$\beta$-fructosylsucrose is barely detectable, although the 2:6-linkage is also the predominant linkage of the bacterial levan.

This parallel could be taken to indicate that the grass fructosans are synthesized by an enzyme of the levansucrase type. Two other facts must, however, be taken into account in any discussion of synthetic mechanisms. The first is that $6^G$-$\beta$-fructosylsucrose is also present in appreciable amounts, recalling the transfer products of leaf invertases (Allen & Bacon, 1956). Henderson, Morton & Rawlinson (1959) have recently shown that in all probability the invertase in the pulp of the banana (*Musa cavendishii* Lamb.) is responsible for the appearance of trisaccharide during the ripening process. They identify the trisaccharide as $6^G$-$\beta$-fructosylsucrose, but all their criteria would apply equally well to a mixture of this substance with $1^F$-$\beta$-fructosylsucrose (for example, we have found that the Raybin test is positive on such mixtures), which would be a more likely product of a higher plant invertase (Allen & Bacon, 1956). Such action of invertase *in vivo* could explain the trisaccharide fraction of monocotyledons, but not the oligosaccharide fraction as a whole, because the leaf invertases which have been examined produce mainly trisaccharides, only traces of tetrasaccharide and nothing of higher molecular weight. There is also the consideration that in the onion both the trisaccharide and the higher oligosaccharide fractions increase in amount when the sucrose

content is raised by infiltration of glucose or fructose.

A stronger argument for differences between levansucrase action and fructosan biosynthesis in monocotyledons is the absence of polysaccharide from most of the onions we examined. This might suggest that there are two synthetic processes, one producing oligosaccharide and the other polysaccharide, which sometimes occur together, sometimes separately. A closer study of the synthesis of fructose oligosaccharides in the onion might therefore not provide direct evidence for the mechanism of fructosan synthesis, as we suggested earlier (Bacon, 1957), but could nevertheless help towards a better understanding of fructose metabolism in this very important group of plants.

The work of Hestrin and his collaborators (Hestrin, Feingold & Avigad, 1956) has shown that the low-molecular-weight fructosides discussed here are relatively poor acceptors for transfructosylation by levansucrase. It is therefore necessary to be cautious in suggesting that $6^G$-$\beta$-fructosylsucrose can serve as a basis for fructosan synthesis, but if it did it would give rise to non-terminal glucose residues and might explain the isolation of trimethylglucose from hydrolysates of methylated grass levans (cf. Harwood, Laidlaw & Telfer, 1954).

An extension of the same argument would connect the absence of $6^G$-$\beta$-fructosylglucose from *Helianthus tuberosus* with the apparent absence of non-terminal glucose residues from inulin (Aspinall & Telfer, 1953). Further caution is needed here though, because of the relatively limited range of inulin-type fructosans that has so far been examined in detail. Thus Chollet (1947) has isolated from various *Campanula* species a polysaccharide very similar to inulin but having $[\alpha]_D$ as low as $-29°$ (cf. $[\alpha]_D - 40°$ or even higher for inulin). We have isolated a similar polysaccharide from *Campanula rapunculus* having $[\alpha]_D - 27°$ and a glucose content of 5·2%. Whether such wide differences in specific rotation can be explained by modifications of a structure in which glucose occupies a terminal position must be a matter for further investigation.

The position of raffinose and related oligosaccharides in the monocotyledons examined is of some interest. We could find no evidence for its presence in extracts of any part of the onion plant, whereas it was present throughout the leek tissues. If, as has been suggested, it plays the part of a reserve carbohydrate in some plants (Pridham, 1958) and of a sugar of translocation in others (Zimmermann, 1958), it is possible that in the onion the fructosan-type oligosaccharide and sucrose are themselves able to fulfil these functions.

## SUMMARY

1. The oligosaccharide fractions from *Allium cepa*, *A. porrum*, *Lolium multiflorum* and *Arrhenatherum elatius* have been fractionated by adsorption chromatography on charcoal–Celite and partition chromatography on Celite columns.

2. Two trisaccharides, $1^F$-$\beta$-fructosylsucrose and $6^G$-$\beta$-fructosylsucrose, have been identified in all four extracts. The two grasses also contained smaller amounts of a trisaccharide which was tentatively identified as $6^F$-$\beta$-fructosylsucrose.

3. These findings are discussed in relation to possible mechanisms of fructosan biosynthesis in plants.

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# Protein Interactions and Metabolic Response to Stimulating Agents in Isolated Cerebral Tissues: Histones as Inhibitors

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After keeping slices of cerebral tissues in buffered glucose media at 0° for 5–17 hr., their normal increase in respiration in response to electrical pulses is largely lost; on examining a variety of naturally occurring materials for ability to restore the response, fractions of blood plasma were found most active (Marks & McIlwain, 1959). The restoring agent appeared to be protein in nature and fraction IV-4 of Cohn *et al.* (1946), which contained α- and β-globulins, was most active.

The present investigation began with the supposition that fraction IV-4 might interact at a chemical level with a constituent of cerebral tissues. Though the possible types of interaction are very wide, it was decided first to see whether fraction IV-4 gave an obvious change, as of light absorption or precipitation, with material extracted from cerebral tissues. A material which forms a precipitate with fraction IV-4 has been found in cerebral extracts; it appears to be a histone. Moreover, the cerebral extracts which give the precipitates, and also basic proteins from other sources, have been found to be inhibitors of the response of cerebral tissues to stimulating agents.

These findings enable mechanisms to be suggested for the loss of excitability on keeping the tissue at 0°, and for its restoration, and these mechanisms are explored further below.

## EXPERIMENTAL

*Tissue metabolism.* The procedures described by Marks & McIlwain (1959) were followed in preparing tissues from the cerebral cortex of guinea pigs, in applying electrical pulses and high concentrations of potassium salts, and in measuring respiration and lactic acid formation. Tissues were incubated at 37·5° in an oxygenated medium containing (mM): NaCl, 134; KCl, 5·4; $CaCl_2$, 2·6; $MgSO_4$, 1·3; $KH_2PO_4$, 1·2; glucose, 10 and glycylglycine 30, and adjusted to pH 7·4 with NaOH. This is termed the glycylglycine medium; additions to it are noted in individual experiments below.

*Extracts of cerebral tissues.* Centrifuging was carried out at 0° with either an MSE machine or Spinco model L ultracentrifuge. Extracts (*a*) and (*b*) were made with cold medium as described by Marks & McIlwain (1959) but buffered with glycylglycine, and extract (*c*) was the 'hot extract' of Marks & McIlwain (1959), made with water.

For extracts (*d*) to (*i*), guinea pigs were killed, the whole brain (3 g.) was removed quickly and put into an ice-cooled homogenizer tube, weighed, 6 ml. of 0·9% NaCl was added and the mixture was ground vigorously for 30 sec. Coarse structures were removed by running the mixture through glass wool, and 15 min. later the filtrate was centrifuged for 10 min. at 10 000 *g*, giving a supernatant (*d*). The precipitate was suspended in 12 ml. of water at 0° and after 30 min. centrifuged as before, giving a supernatant (*e*) and a residue which was resuspended as before; centrifuging for 30 min. then gave not a clear solution but a persistent sol (*f*) as supernatant; the remaining precipitate (*g*) was suspended in glycylglycine medium for test.

After preparing extract (*d*) from other guinea pigs, the residue (from 6·6 g. of brain) was suspended in 20 ml. of 0·2 N-$H_2SO_4$ and left at 0° for 60 min., centrifuged for 10 min. at 2000 *g* and the residue was extracted as before. The combined supernatants were tied in Visking cellulose tubing and rotated mechanically in 2 l. of water, renewed at intervals of 1½ hr. for 4½ hr., yielding (*h*), the non-dialysable material. This, already neutral, was evaporated at 10 mm. Hg to a few millilitres for test. Extract (*i*) was prepared in the same way as extract (*h*), but commencing with the residue from extract (*d*) from 7 g. of brain, which by the addition of 5 M-NaCl and water was made to 14 ml., 2·6 M in