The Carbohydrates of the Jerusalem Artichoke and other Compositae

By J. S. D. BACON AND J. EDELMAN
Department of Biochemistry, University of Sheffield

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The most fully investigated polysaccharide of the Compositae is inulin (named from *Inula helenium*), first obtained from the tubers of the Jerusalem artichoke, *Helianthus tuberosus* L. (Rose, 1804), but more easily prepared from the dahlia tuber (cf. McDonald, 1946). ('Artichoke' is used to indicate Jerusalem artichoke throughout this paper.) It has long been evident (cf. Tanret, 1893*a, b*) that inulin may constitute only a small fraction of the total carbohydrate present in the artichoke tuber, the remainder resembling inulin in being composed essentially of fructofuranose units, but having a greater solubility in water and in aqueous ethanol. The term 'inulide' has been used by French workers (Wolff & Geslin, 1918) to describe substances of this type, which occur also in the tissues of many other Compositae (cf. Colin, 1919). Lemoigne (1941*a*, 1942) has published a comprehensive review of existing knowledge of the carbohydrates of the artichoke.

In the course of an investigation into the transformations of fructose polysaccharides in the tubers we have made a survey of the carbohydrates present, using new methods, in particular paper partition chromatography. Although it was not our main intention to investigate the composition of the tubers, and some of the techniques employed are perhaps not the most suitable for that purpose, it is clear from the results presented here that existing ideas about the tuber carbohydrates must be modified. A preliminary account of this work has been given (Bacon & Edelman, 1949).

METHODS

Standard solutions of sugars. Glucose and fructose were dissolved in saturated benzoic acid solution (Folin, 1929), sucrose in water, and the concentration checked by polarimetry. No glucose could be detected by glucose oxidase in the fructose sample used.

Polarimetry. The optical rotations of solutions were read in 2 dm. tubes using a sodium lamp as light source.

Dry weight determinations. Tubers were cut out into quarters and thin slices taken from the cut surfaces. Samples with a fresh weight of 8–12 g. were dried for 24 hr. at 100°; a further 24 hr. drying decreased the weight by only 10–20 mg.

Deproteinization. Routinely, extracts were deproteinized by adding 0.1 vol. of 30% (w/v) lead acetate ($\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$) solution. To the filtrate was added 3% (w/v) sodium oxalate solution in the proportion 2.5:16 ml. of filtrate. Occasionally minimal amounts of each reagent were used.

Estimation of total reducing substances (TRS). The method used was essentially that of Miller & Van Slyke (1936) with slight modifications (cf. Bacon & Bell, 1948). Reducing sugar in easily hydrolysed combination was determined by TRS estimations before and after hydrolysis, which was done by adding 0.1 vol. of 5.5% (w/v) oxalic acid solution to the sample and heating the mixture for 30 min. in a boiling-water bath. The concentration of carbohydrate was so chosen that a dilution of 20 times or more was needed before the TRS estimation; under these conditions neutralization of the acid was unnecessary. Contrary to the previous experience of one of us (J. S. D. B.; see Bacon & Bell, 1948) we found that fructose gave 1.06 times the reduction by an equivalent amount of glucose. The correction required was usually about the same as the experimental error because the ratio of fructose to glucose in the whole extracts was approximately 4:1. The TRS is expressed as hexose; the standard used is stated where the discrepancy between glucose and fructose is important.

Estimation of glucose by glucose oxidase. *Aspergillus niger* (National Collection of Type Cultures, No. 594), subcultured on malt-agar slopes, was inoculated into flasks containing a liquid culture medium (0.5% NaNO_3 , 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3% K_2HPO_4 , 10% glucose (w/v)) and the flasks incubated at 30°. The appearance of the enzyme was followed by measuring the O_2 uptake of small portions of the medium in the presence of glucose.

The liquid was poured off, dialysed against running tap water and filtered through a folded filter paper (J. Barcham Green Ltd., Hayle Mill, Maidstone, no. 9044). The dialysed material was neutralized with NaOH and concentrated *in vacuo* in a water bath at 35–40°. The product was a clear yellow liquid which contained a little invertase activity in addition to the glucose oxidase, indicated by a slow O_2 uptake in the presence of sucrose. Glucose was estimated in 0.2M-acetate buffer (pH 5) either manometrically (Keilin & Hartree, 1948) or by difference in TRS before and after incubation with the enzyme.

Estimation of ketose. This was done colorimetrically using the method of Cole (unpublished; cf. Bacon & Bell, 1948) with a fructose standard. Chromatographic evidence suggested that fructose was the only ketose present in the extracts. The method gave the expected amount of fructose when applied to sucrose; it gave the same ketose value with the tuber extracts before and after acid hydrolysis, but this cannot be taken as evidence that it measures accurately fructose combined as in inulin.

Paper partition chromatography

The apparatus used was similar to that of Consden, Gordon & Martin (1944) and Partridge (1948). Strips of sponge rubber were clipped round the tops of the drainpipes, and a sheet of plate glass resting on this gave a satisfactory airtight seal. The pipes stood in lead or aluminium trays con-

taining the aqueous phase. Large sheets of paper were run in wooden tanks lined with lead (for phenol and collidine). Butanol-acetic acid mixtures rapidly corroded the lead, and an aluminium tank lagged with cotton wool was used for this solvent. The troughs were constructed of stainless steel. Strips (15 × 60 cm.) and sheets (60 × 60 cm. or 57 × 46 cm.) of Whatman no. 1 filter paper were used. The solvent was removed from the paper by heating it in a current of air in a specially constructed oven.

Solvents. In addition to the solvent mixtures described by Partridge (1948), isopropanol-aqueous NH_3 (9 : 1, v/v), *n*-butanol saturated with *N*-aqueous NH_3 and mixtures of varying proportions of *n*-butanol-acetic acid-water were used. Of all mixtures investigated, that composed of 40 ml. *n*-butanol, 10 ml. acetic acid and 50 ml. water (called hereinafter 'butanol-acetic') (Partridge, 1948) was found to give the most satisfactory results with the tuber carbohydrates, and was used for most of the qualitative and all the quantitative chromatography.

Reagents for detection of carbohydrates. The developed and dried chromatograms were sprayed with the appropriate reagents and heated in the oven. (i) *Phloroglucinol* (Horrocks & Manning, 1949). Spots containing free or combined ketose show pink when heated at 100–110° with this reagent. Glucose is not detected. (ii) *Naphthoresorcinol* (Partridge, 1948) and similar reagents made with resorcinol and orcinol were tried. Resorcinol gave a delayed colour production during a day at room temperature following the usual heating in the oven; orcinol gave green spots on a pink background. (iii) *Benzidine* (Horrocks & Manning, 1949). Spots containing free aldose groups show a chocolate brown colour when heated at 100–120°, but glucose in easily hydrolysed combination gives a delayed colour production after several days at room temperature. Fructose, either free or combined, is detected in relatively large quantities only (about 100 μg . or more). (iv) *Benzidine-trichloroacetic acid*. 0.5 g. Benzidine (A.R.) in 10 ml. acetic acid, 10 ml. 40% (w/v) aqueous trichloroacetic acid, 80 ml. ethanol. This was found to be the most useful reagent for the materials studied. It is very sensitive to both free and combined glucose, giving a more intense brown colour with this sugar than the benzidine reagent described above, and will detect less than 1 μg . A yellow colour is developed with free or combined fructose, but the reagent is appreciably less sensitive to this sugar. (v) *Aniline acid phthalate* (Partridge, 1949).

Treatment with invertase on the paper. Sugars which are attacked by invertase can be treated with this enzyme on the paper. Chromatograms run in one direction in butanol-acetic were sprayed with a 4% (v/v) dilution of 'invertase concentrate' (British Drug Houses Ltd.) in water and hung in the oven at 50° over a free water surface for 15–30 min. The products of hydrolysis were separated in the usual way by running the chromatogram at right angles to the original direction. This treatment hydrolysed completely up to 200 μg . of sucrose and did not interfere with R_f values or colour development.

Photography of chromatograms. After colour development the chromatograms were photographed: a sheet of Kodak Reflex Contact Document paper was placed face downwards in close contact with the chromatogram, the two being held between pieces of plate glass, and illuminated by a 40 W. electric lamp 35 cm. above the chromatogram. With exposures ranging from 20 to 40 sec. satisfactory negatives were obtained from which positives (cf. Fig. 2) were made by

transmitted light, often showing better contrast than the original chromatogram. The procedure was used routinely because the background colour of the chromatograms intensified with keeping, and made faint spots almost invisible, particularly with the phloroglucinol reagent.

Quantitative estimation. Quantities (5 μl .) of the solution to be analysed were put on to strips or sheets of filter paper using an 'Aglā' micrometer syringe (Burroughs Wellcome and Co.); the measurement has an accuracy of $\pm 1\%$. The paper was run for a suitable time (usually 2 days) in butanol-acetic and dried at 60–80°. Every second or third strip, each corresponding to one application of the solution, was cut out and sprayed with an appropriate reagent. The positions of the spots in the unsprayed strips were then deduced and rectangles from sufficient strips (usually two to four) to give enough material to estimate were combined for a single estimation. The carbohydrate was extracted by heating the paper to 75–80° for 20 min. in a known volume of water in a glass-stoppered tube, and shaking the tube vigorously to disintegrate the paper. The resulting suspension was filtered. The estimation was carried out on a sample of the filtrate. This procedure was found satisfactory for ketose estimation, as blanks on the paper gave density readings of less than 0.01 in nearly every case; recoveries of 95–100% were obtained with sucrose, but lower recoveries, namely 85–95%, were found with tuber extracts. Estimation of TRS was not as accurate or convenient. The estimation is less accurate than that used for fructose for amounts of material less than 80 μg ., so more material had to be taken. Blanks were of the order of 10 μg /spot, varying with the area of paper, and were not eliminated by washing the paper with cold water before application of the solution to be estimated. Combined reducing sugar was hydrolysed after extraction from the paper by adding 0.5 ml. 0.5 *N*-HCl to 4.0 ml. of the filtrate contained in the tube in which the estimation was to be carried out, and heating it in a boiling-water bath for 30 min. The tubes were cooled in water and the acid neutralized by adding 0.5 ml. 0.5 *N*-NaOH, after which alkaline ferri-cyanide was added and the estimation completed by the normal procedure.

RESULTS

CARBOHYDRATES OF THE ARTICHOKE TUBERS

Sources of material. At first, small lots of tubers were purchased as they were required, during November 1948, but later a sackful was bought and stored in a moderately cool cellar (7–8°). Although bought at different times these tubers were probably all from the same batch, variety unknown. During 1949 a crop was raised from some of these tubers, which were planted in a sunny position on the outskirts of Sheffield. The plants grew 3–3.5 m. high, and although flower buds formed they did not open. A few tubers were taken out of the ground in mid-September, when they were still very small, but the majority were left there until April 1950 when the bulk were harvested and some small tubers replanted. In the autumn of 1949 another sackful of tubers, indistinguishable from the previous year's batch, was purchased; these were named 'Bedfordshire Pinks' and had probably been grown in the Midlands.

Extraction of tubers. The tubers were thoroughly scrubbed in running cold water, cut into pieces and disintegrated in a Waring blender with about half their weight of water. The proportion of water required depended to some extent on the losses of water which the tubers had suffered in storage. When about 10 % water had been lost the tubers became rubbery in consistency and disproportionately large amounts of extra water were needed to get satisfactory blending. The dry weight of tubers was approximately 20 % of the fresh weight (see Table 1). During the blending, and afterwards, the mash darkened, presumably through the action of phenol oxidases, but this could be largely prevented either by adding potassium cyanide to a final concentration of about 0.005 M, or by removing the outer layers of the tubers. A combination of these two procedures gave almost colourless preparations. The mash obtained was squeezed through gauze or madapolam, yielding an opalescent liquid. A few extracts, which are mentioned below, were made in other ways.

Examination of extracts

pH. The pH of all extracts was about 6.5, irrespective of the maturity and age of the tubers. The addition of calcium carbonate to the tubers during the extraction process to protect the carbohydrates from hydrolysis was therefore not necessary. Its presence when the mash was boiled with water

seemed in our experience to be a disadvantage, since it encouraged the extraction of gelatinous material insoluble in 50 % (v/v) aqueous ethanol.

Deproteinization. When these experiments were begun, the use of lead acetate and sodium oxalate was thought sufficient both to remove protein and to destroy any enzymic activity, but it was discovered that the optical rotation of fresh extracts cleared in this way fell slowly when they were kept at room temperature; simultaneously the free TRS increased. If the extracts were boiled before deproteinization they showed no such change in rotation. The change could not be ascribed to a reaction between cyanide and free reducing sugar in the extracts; a 2 % (w/v) solution of fructose in 0.005 M-potassium cyanide showed no change in rotation when kept for 2 days at room temperature. It seems certain that a hydrolytic enzyme system was responsible, because later experiments (Edelman & Bacon, 1950) demonstrated that the hydrolytic system in the tubers was relatively stable in the presence of lead acetate. All extracts to be used for analyses were accordingly heated in a boiling-water bath for 10 min. as soon as possible after their preparation, usually reaching 80° within 10–15 min. of the beginning of blending.

Optical rotation. The rotations of deproteinized extracts varied considerably; Table 1 shows typical results, with details of the maturity and age of the tubers.

Table 1. *Composition and optical rotation of tuber extracts*

(All extractions were carried out as described under Methods, with the exception of that dated 8 Feb. 1949 which will be described later (Edelman & Bacon, 1950). Where dry weight determinations had not been made, the calculations of g. TRS after mild hydrolysis/100 g. fresh tuber were made assuming 80 % water.)

Date bought	Date dug up	Date extracted	Dry matter (g./100 g. fresh tuber)	Total reducing substances (TRS) after mild acid hydrolysis (g./100 g. fresh tuber)	Ketose as percentage of TRS after hydrolysis	Optical rotation corrected to undiluted juice (2 dm. tube)	$[\alpha]_D$ calculated on TRS after hydrolysis
8 Nov. 1948	—	17 Nov. 1948	—	15.5	—	-4.03°*	-10.4°*
22 Nov. 1948	—	23 Nov. 1948	—	—	—	-0.62°	—
1 Dec. 1948	—	1 Dec. 1948	—	15.7†	—	—	—
18 Dec. 1948	—	18 Dec. 1948	—	14.8	78	-0.78°	-2.52°
3 Feb. 1949	—	3 Feb. 1949	—	—	—	+1.64°*	—
8 Feb. 1949	—	8 Feb. 1949	—	12.3	79	+1.59°	+5.15°
8 Feb. 1949	—	19 Mar. 1949	—	—	—	+3.28°	—
8 Feb. 1949	—	4 Apr. 1949	20.6	14.4	78	+0.13°	+0.38°
8 Feb. 1949	—	9 May 1949	20.3	14.2	78	+1.02°	+2.85°
—	28 Sept. 1949	6 Oct. 1949	18.4	14.4	91	-7.78°	-22.1°
—	28 Sept. 1949	7 Nov. 1949	21.1	16.0	86	-4.15°	-10.3°
—	28 Sept. 1949	24 Nov. 1949	25.0	19.4	85	-3.54°	-6.84°
—	24 Nov. 1949	24 Nov. 1949	16.6	14.4	73	+1.02°	+2.94°
24 Nov. 1949	—	24 Nov. 1949	22.0	18.9	83	-3.87°	-8.03°
—	5 Apr. 1950	1 May 1950	15.3	10.6	85	-0.36°	-1.43°
—	5 Apr. 1950	1 May 1950†	14.9	10.9	83	+0.05°	+0.19°

* These extracts were not boiled before deproteinization, but the rotations were read immediately afterwards.

† The $[\alpha]_D$ of the sugar liberated by mild acid hydrolysis was -64.8°.

‡ This extract was made by throwing diced tubers into boiling water and blending the whole when cold.

It is probable that some enzymic hydrolysis had taken place during extraction, causing a small change of rotation in the negative direction. Experiments in which the time of extraction in the Waring blender was varied indicated that differences of the order of -0.5° (calc. for undiluted juice) might be ascribed to this effect, which is thus small compared with the seasonal variations. Alternative methods of extraction, e.g. by first throwing diced tubers into boiling water, and later blending them, gave solutions with more positive rotations but otherwise very similar in composition (cf. the two extracts made 1 May 1950). It is by no means certain that the whole of the optical activity of extracts is due to carbohydrate.

On hydrolysis the rotation always became more negative, and the 'differential' $[\alpha]_D$ calculated from the change in rotation and the change in TRS for complete hydrolysis with oxalic acid at 60° was -63.5° ; that calculated for inulin ($[\alpha]_D - 40.0^\circ$) is -53.4° , and for sucrose -83.0° .

The $[\alpha]_D$ of the mixture of sugars produced by mild acid hydrolysis of one extract was -64.8° , which corresponds to a mixture of fructose and glucose in the ratio 4.3 : 1. This calculation does not take into account the probable formation of difructose anhydrides during hydrolysis (Jackson & Goergen, 1929).

Spontaneous precipitates. Although 'inulin' is almost insoluble in cold water it can be extracted from plant tissues without the use of heat, and under these conditions it later appears as a dense white precipitate on the walls of the vessel holding the extract. No such precipitation was noticed with extracts made by the procedure described above during the period from November 1948 to the summer of 1949. An extract made by the method of Green (1888), i.e. by boiling diced tubers with water and concentrating the extract in an evaporating basin, deposited a small rather flocculent precipitate in the course of 2 months. The precipitate was centrifuged off, washed with water, and dissolved in water by warming to about 80° . On freezing, the material reprecipitated; it could be presumed to be inulin (ketose was present), but insufficient was available to characterize it further.

Material resembling inulin was obtained from extracts to which ethanol had been added: an equal volume of ethanol was added to fresh extract and the precipitate allowed to settle overnight before being filtered off. The clear filtrate, left for several months at room temperature, slowly deposited a translucent layer of carbohydrate on the walls of the flask. When an attempt was made to dissolve this material in a little cold water it did not dissolve completely, and the insoluble fraction proved to have the properties of inulin, i.e. it was a white, ketose-containing, substance, soluble in water at 80° , but slowly separating as a compact precipitate after the solution had been frozen and left in the refrigerator.

It would seem from these and similar experiments that 'inulin' was not present in significant amounts in extracts of the tubers obtained in November 1948. It is possible that the solubility of inulin in cold water (in itself not a very well defined property, cf. Lemoigne, 1941*b*) is increased by the presence of inulides, but in a qualitative experiment it was found that a commercial sample of inulin dissolved in tuber extract would still precipitate when the solution was frozen and kept cool.

Extracts made from the batch of tubers dug up in August 1949 showed a deposition of inulin almost at once; solutions prepared for polarimetry and left overnight had to be heated to dissolve the hazy precipitate which had formed even at room temperature. Fresh extracts showed a precipitation when frozen, but after having been heated to boiling point to destroy the hydrolytic enzyme system they showed a more delayed deposition of polysaccharide. It seems possible that the 'inulo-coagulase' effect of Wolff (1916) may be due to the addition of nuclei of aggregated polysaccharide to boiled extracts.

Extracts made from tubers grown under the same conditions, but dug up in a more mature condition several months later, showed a slight precipitation of inulin, but extracts made from the tubers harvested in April 1950 resembled those used in the winter of 1948-9 in giving no precipitate.

In the absence of any method of determining 'inulin' these observations may be taken to confirm earlier observations (Thaysen, Bakes & Green, 1929) that the amount of inulin in the tubers decreases during the autumn and winter, both relatively and absolutely.

Precipitation by ethanol. Although many experiments were carried out with a view to preparing polysaccharide by ethanol precipitation, to serve as a substrate for metabolic studies, it was clear that fractionation by this means was at best very unsatisfactory. Addition of ethanol to a concentration greater than 50% (v/v) leads to the appearance of a heavy flocculent precipitate, but after this has been removed by filtration or centrifugation, precipitation continues for some months at room temperature. For example, of 23.4 g. of total ketose in an extract only 6.6 g. was precipitated by the addition of an equal volume of ethanol; but later the filtrate deposited a precipitate from which a substance resembling inulin was prepared (see above). This slow 'after precipitation' appears to be characteristic of the carbohydrates of the tuber (cf. discussion of the depolymerization of inulin, Lemoigne, 1941*c*), and would seem to make it impossible to define any fractions as being soluble or insoluble in a given concentration of ethanol (but see, nevertheless, Tanret (1893*a, b*), who also notes the considerable effects of temperature on the solubility of fractions).

Precipitation by acetone. The addition of 7–20 vol. acetone to extracts produced an immediate flocculent precipitate, and an opalescence which persisted for several days. Chromatographic analysis (see below) of the precipitates and supernatant fluids indicated that many substances are common to the two fractions.

Precipitation by aqueous baryta (cf. Tanret, 1893*a*). Aqueous baryta added in excess precipitated 15 and 2 %, respectively, of the total ketose of fresh extracts, and of the material soluble in 50 % (v/v) ethanol.

Free TRS. The free TRS of deproteinized extracts amounts usually to only 2–3 % of the total TRS after mild acid hydrolysis. Since reducing sugar is liberated from the carbohydrates by a hydrolytic enzyme system in the extracts (Edelman & Bacon, 1949) the exact value of the free TRS is probably not of much significance. Values have been found ranging from 0.25 to 0.75 g./100 g. fresh weight of tubers. In some extracts there has been chromatographic evidence for the presence of monosaccharides, but in others it seems that the bulk of the free TRS must either be non-carbohydrate in character, or alternatively due to reducing end groups on some of the polysaccharide present.

Combined TRS. The greater part, possibly all, of the combined reducing sugar is in a form liberated by mild acid hydrolysis. An experiment was carried out to discover whether more drastic acid hydrolysis would liberate further glucose in the extracts (see Table 2). From these results it seems that only an insignificant amount of the glucose can be present in combination with pyranosidic linkages, or with non-carbohydrate residues.

After mild acid hydrolysis the ketose content of the extracts is unchanged, and corresponds to 80–85 % of the TRS, the exact percentage depending on the maturity and age of the tubers (see Table 1). The remaining TRS liberated was accounted for as glucose by the use of glucose oxidase (see Tables 2 and 3).

It is evident from these data that the carbohydrates consist essentially of non-reducing saccharides, in which the majority of linkages are of the furanosidic type.

Paper partition chromatography

Qualitative. The extracts showed a series of spots, all detected with either phloroglucinol or benzidine-trichloroacetic sprays (see Fig. 1). Free hexoses, when

Fig. 1. Typical chromatogram of extract of artichoke tuber. Run in butanol-acetic for 3 days; sprayed with benzidine-trichloroacetic acid. The position of spot 1 corresponds with that of sucrose (see Fig. 3). (Second positive print.)

Table 2. Comparison of effects of weak and more drastic acid hydrolysis on liberation of glucose from artichoke-tuber extract

(2.0 ml. extract heated with 2.0 ml. of 10*N*-H₂SO₄ in boiling-water bath for 2 hr. Cooled, and neutralized with 10*N*-NaOH. Further 2.0 ml. heated with 0.4 ml. 5.5 % (w/v) oxalic acid in boiling-water bath for 30 min. Both hydrolysates diluted to 25 ml. with water. 2.0 ml. of each solution incubated at 40° in Warburg cups with 1.2 ml. 0.6*M*-acetate buffer (pH 5), 0.3 ml. glucose oxidase and 0.1 ml. catalase solutions until O₂ uptake ceased. Values calculated per ml. original extract.)

	O ₂ at N.T.P. (μl.)	Glucose calculated (mg.)	TRS (mg.)	Ketose (mg.)	Glucose (by difference) (mg.)
Weakly hydrolysed extract	1510	23.4*	97.5	74.5	22.6
More drastically hydrolysed extract	1520	23.6*	—	—	—

* 1 mg. glucose under the conditions of the experiment gave O₂ uptake of 64.5 μl.

Table 3. *Estimation of glucose in hydrolysed artichoke-tuber extract by glucose oxidase*

(1.0 ml. hydrolysed extract (diluted $\times 20$) incubated at 40° in Warburg cup with 1.0 ml. 0.2M-acetate buffer (pH 5), 0.2 ml. glucose oxidase and 1 drop catalase (horse-kidney extract), until O_2 uptake ceased. Estimations done on contents of cup diluted to 50 ml. Values calculated per ml. of original extract.)

	O_2 uptake (μ l. at N.T.P.)	Glucose calculated from O_2 uptake (mg.)	Ketose (mg.)	TRS (mg.)	Non-ketose (by difference) (mg.)	Glucose calculated as disappearance of TRS (mg.)
Untreated	—	—	79.5	101	21.5	—
Treated with glucose oxidase	1510	22.5*	80.5	78.2	—	22.8

* 1 mg. glucose under the conditions of the experiment gave O_2 uptake of 67.5 μ l.

Table 4. *Distribution of ketose among the components of the tuber carbohydrate*

(Where the dates correspond these analyses refer to the extracts described in Table 1. The figures for individual components are in some cases the results of a single ketose estimation, but in others represent the mean of several, e.g. spots 1, 2 and 3 for 8 Feb. 1949 are each the mean of ten estimations.)

Date bought	Date dug up	Date extracted	TRS after hydrolysis (g./100 g. fresh tuber)	Ketose as per- centage of TRS after hydrolysis	Ketose as % total ketose recovered from chromatogram						
					Fruc- tose spot	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Spot 6
8 Feb. 1949	—	8 Feb. 1949	12.3	79	—	10.8	10.5	11.7	10.9	10.8	—
—	16 Sept. 1949	16 Sept. 1949	—	—	—	2.2	2.5	2.5	—	—	—
—	28 Sept. 1949	6 Oct. 1949	14.4	91	1.5	3.7	3.0	2.8	2.8	3.0	—
—	28 Sept. 1949	17 Oct. 1949	13.3	86	4.2	5.4	3.3	4.4	4.1	4.6	—
—	28 Sept. 1949	24 Oct. 1949	14.2	90	2.2	7.2	5.3	6.6	3.8	4.3	4.9
—	28 Sept. 1949	31 Oct. 1949	15.3	82	2.6	6.3	3.9	5.0	5.3	5.8	—
—	28 Sept. 1949	7 Nov. 1949	16.0	86	1.3	6.6	4.2	6.3	5.5	5.9	—
—	28 Sept. 1949	24 Nov. 1949	19.4	85	—	7.7	5.0	6.5	6.6	6.7	—
—	27 Oct. 1949	23 Nov. 1949	—	—	—	7.3	7.3	8.4	8.0	—	—
—	24 Nov. 1949	24 Nov. 1949	14.4	73	—	9.4	8.7	10.7	9.9	—	—
24 Nov. 1949	—	24 Nov. 1949	18.9	83	—	4.9	4.7	6.6	6.3	6.2	—
24 Nov. 1949	—	4 Jan. 1950*	—	—	—	7.9	7.1	9.1	—	—	—
24 Nov. 1949	—	14 Feb. 1950*	—	82	1.3	8.8	9.3	10.5	—	—	—
24 Nov. 1949	—	3 Mar. 1950*	—	—	1.4	9.2	7.9	9.2	—	—	—
24 Nov. 1949	—	15 Mar. 1950*	—	—	2.1	9.9	8.8	8.8	—	—	—
—	5 Apr. 1950	1 May 1950	10.6	85	—	11.5	9.9	10.4	—	—	—
—	5 Apr. 1950	1 May 1950	10.9	83	—	11.5	10.1	10.6	—	—	—

* Tubers peeled before extraction.

present, occupied positions below sucrose, cf. Fig. 2. It is convenient to refer to the spot in the sucrose position as 'spot 1', to the one above it as 'spot 2' and so on, the spot with the lowest R_f being referred to as 'spot n '. There was very little monosaccharide, none of it pentose. Spot 1 had an R_f indistinguishable from that of sucrose in all solvent mixtures used. It gave the brown colour characteristic of free or combined glucose with benzidine-trichloroacetic. Using this reagent the colour of the higher spots was more and more yellow as the series was ascended to $R_f = 0$, suggesting a higher fructose to glucose ratio.

To test the homogeneity of the spots, two-dimensional chromatograms were run. Using butanol-acetic in both directions the spots were found to lie in a straight line, and there was no resolution of any spot into two or more components. This excludes the possibility that the R_f 's of the spots are influenced by the presence of other members of the

series. With butanol-acetic and phenol no evidence was found for heterogeneity; this is confirmed by the behaviour of fractions of higher R_f (mentioned below) originally separated by the use of phenol, which showed the same number of components whether run in that solvent or in butanol-acetic. These observations do not exclude the possibility that the spots represent mixtures of similar substances, particularly those of lower R_f , which must be assumed to consist of higher oligosaccharides. The spots occupy the same relative positions in phenol as in butanol-acetic.

Quantitative. The ketose content of the spots was estimated in a number of extracts (see Table 4).

The results demonstrate the even distribution of ketose among the components of higher R_f , independently of their contribution to the total ketose. The most noticeable deviation in this respect is spot 3, which seems always to have a higher ketose

content than spots 2 or 4. Estimations of ketose and of combined TRS in spots 1-3 were made as described under 'Methods' (see Table 5 for some typical results with one extract). It will be seen that the ratios of fructose to glucose in spots 2 and 3, calculated on the assumption that the substances are non-reducing, vary according to the basis of calculation. Small errors in the calculation of combined TRS have relatively large effects on the ratio, but an alternative explanation of the discrepancies may be found in slight hydrolysis during the drying process. On the other hand, the assumption that these spots are non-reducing may be incorrect; they may represent mixtures of reducing and non-reducing sugars.

action of purified preparations of invertase cannot be taken as evidence for the presence of sucrose in the extracts (cf. Colin, 1919).

Partial separation of the carbohydrates. A streak of 1.00 ml. of boiled extract, 45 cm. long, was applied 9.5 cm. from one edge of a sheet of filter paper (57 × 47 cm.). This was done by hand in four successive applications from a graduated pipette with a finely drawn tip, the applications being of 0.225, 0.33, 0.375 and 0.07 ml. Guide spots were placed at each end of the streak. The sheet was run for 92 hr. with phenol saturated with water, and the guide strips cut out and sprayed with orcinol. Because of the large quantity of material put on (101 mg. TRS)

Table 5. *TRS and ketose determinations on individual components of the tuber carbohydrate*

(An extract made on 14 Feb. 1950 from tubers bought 24 Nov. 1949, deproteinized with Pb, was used. 5 or 7 μ l. spots were placed on chromatograms, and these were run together in groups A, B and C, all in butanol-acetic for 3 days. The extract was analysed using ordinary pipettes, and also using the 'Agl' syringe: 81.8 (ordinary), 81.1 (Agl) mg. TRS/ml. after hydrolysis; 67.2 (ordinary), 65.7 (Agl) mg. ketose/ml. All results are expressed in μ g./50 μ l. original extract; TRS is calculated as μ g. hexose on basis of a fructose standard.)

Spot	Chromatograms	TRS before hydrolysis (p)	TRS after hydrolysis (q)	Ketose (r)	Glucose as 1.06 (q - p - r) (s)	TRS of blanks from same sheet corrected for area (t)	Glucose as 1.06 (q - r - t) (u)	Ratio fructose/glucose from (s)	Ratio fructose/glucose from (u)
1	A	—	602	270	—	76	271	—	1.0
	B	147	658	316	199	—	—	1.6	—
	C	178	668	260	245	166	258	1.1	1.0
2	A	—	494	282	—	67	156	—	1.8
	B	125	521	328	68	—	—	4.8	—
	C	158	542	284	106	124	141	2.7	2.0
3	A	—	489	322	—	56	118	—	2.7
	B	118	533	356	60	—	—	5.9	—
	C	113	532	315	111	104	121	2.8	2.6

Treatment with invertase. When two-dimensional chromatograms of tuber extracts were treated with invertase between the first and second developments, spots in the glucose and fructose positions were obtained from every component except the material with R_F approximately zero. In this case fructose appeared, but glucose could not be detected with certainty. This was not unexpected owing to the small proportion of glucose in this material (see Table 6). Faint spots also appeared with R_F values higher than that of the original spot from which they derived, but similar to original components with higher R_F . Thus, for example, spot 2 gave rise to a very faint spot in position 1, and spot 5 to spots in positions 4 and 3. Many such 'daughter spots' were slightly retarded in position when compared to original components with similar R_F , and, although it appears that invertase produces shorter-chain oligosaccharides from each component, further evidence is necessary to prove them identical with those occurring in the tuber extract. It is obvious that the appearance of reducing sugar through the

only the spot in the sucrose position had separated cleanly and this occupied the region between 220 and 290 mm. from the point of application. The remaining streak was arbitrarily divided into four zones by cuts 9, 45, 155 and 220 mm. from the starting line. The corresponding areas from the unsprayed sheet were then cut out. The strips were extracted with water *in vacuo* at 40-45° in a Soxhlet extraction apparatus, each being extracted six to nine times. The volume of water was such that only about 1 ml. was left in the flask at the last evaporation. The final liquid remaining in the extraction chamber was tested for fructose by the Seliwanoff method with negative or only very slightly positive results, indicating a loss of less than 1% of the total fructose at this stage of the recovery. The concentrated extracts, which were yellow in colour, were each made up to 5 ml. Chromatograms showed that fraction A (R_F corresponding to that of sucrose) was pure, that B contained two spots, and that C, D and E were probably more heterogeneous. An analysis of these fractions is given in Table 6.

Table 6. *Analysis of fractions separated chromatographically from tuber extract*

(For details of experiments see text, p. 120. Values calculated/ml. original extract.)

Fraction	TRS* after hydrolysis (mg.)	Ketose (mg.)	Glucose (by difference $\times 1.06$) (mg.)	Glucose as % total monosaccharide after hydrolysis
A	15.8	7.7	8.6	52.5
B	20.75	13.0	8.2	38.7
C	28.15	22.35	6.15	21.6
D	23.25	21.0	2.4	10.3
E	10.9	10.8	0.1	(1)
Total recovered	98.85	74.85	25.45	—
Total in original extract	101	80.0	22.0	—
Recovery	98 %	94 %	—	—

* Calculated as hexose on basis of fructose standard.

CARBOHYDRATES IN OTHER PARTS OF THE
ARTICHOKE PLANT

Source of material. Plants raised from the tubers described above were used. Leaves were taken to the laboratory as quickly as possible in a cooled vacuum flask; the stems, which were long and heavy, were cut into short lengths.

Extraction and deproteinization. The stems and roots were extracted and the extract deproteinized by essentially the same procedures as were used for tubers. Leaves were extracted and deproteinized simultaneously in the Waring blender. A typical extraction was as follows: 50 g. of lamina were blended with 100 ml. of 35 % (v/v) aqueous ethanol (containing potassium cyanide) and 35 ml. of chloroform, *N*-hydrochloric acid being added cautiously drop by drop to make the mash react green with bromocresol green. The whole mash was centrifuged and the upper layer (yellow with a slight tinge of green) was filtered with the addition of a little filter aid (Hyflo-Supercel, Johns-Manville Co. Ltd., London). Such extracts gave no precipitate with trichloroacetic acid. They were adjusted to pH 7 before concentration *in vacuo* with a bath temperature of 50° or less.

Roots. These showed the same series of components as the tubers when chromatographed.

Stems. The stem, particularly in the lower parts, contained a substantial pith, which could be cut out without much difficulty (Thaysen *et al.* 1929). Chromatographic examination showed a series of components similar to those of the tuber, both in extracts of the pith, and of the upper stem (see Fig. 2). As judged qualitatively there was more of the components of low R_F , and inulin separated from extracts kept in the refrigerator.

Inner and outer regions of immature tubers. The young tubers broke naturally into an outer layer and an inner core. There was no qualitative difference between them in respect of carbohydrate (Fig. 2).

Leaves. The amounts of fructose in the upper parts of the stem, and in the petioles and leaves, were lower than those in the other parts of the plant, so that concentration of the extracts was necessary as a preliminary to chromatographing them. The concentrated extracts failed to give satisfactory chromatograms, the whole of the carbohydrate remaining as a blob or streak, with few signs of differentiation. This effect (cf. Consden *et al.* 1944) could be reproduced artificially to some extent: 7 μ l. spots of *m*-potassium dihydrogen phosphate solution were placed on the starting line of a strip of paper, and after they had dried, 5 μ l. spots of a control mixture of sugars and of a tuber extract known to give satisfactory chromatograms were applied at the same point. When run in butanol-acetic the chromatograms showed retardation and distortion of the spots, though not as pronounced as that seen with the leaf extracts. Application of 7 μ l. spots of 30 % (w/v) lead acetate ($\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$) and 40 % (w/v) barium acetate ($\text{Ba}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot \text{H}_2\text{O}$), which it was thought might precipitate interfering substances, had no effect on the R_F 's of the control mixture of sugars, nor of the tuber extract. It would thus seem that it is not salt concentration alone that is responsible for the failure to achieve satisfactory separation (cf. Partridge, 1948).

Attempts to remove salts from the leaf extracts by the use of ion-exchange resins (in which we were greatly helped by Dr R. E. Kressman and the Permutit Company) had only limited success in the summer of 1949. The cations were removed readily by Zeo-Karb 217, but the resulting acid solution was brought towards neutrality only slowly, even by the more basic resins of the 'Deacidite' series (up to 'F'). The treated solutions, which had been cycled repeatedly through columns of the two types of resin gave improved, but not entirely satisfactory, chromatograms, in which 'spot 1', glucose and fructose were always seen. In one or two extracts indication was given of material with lower R_F than

sucrose, but in none was there evidence of a series of components resembling the tuber extract. Starch is present in the leaves (Brown & Morris, 1893).

family (cf. Willis, 1931) were represented (see Table 7).

Underground organs (100 g.) (after removal of the

Fig. 2. Chromatograms of extracts of various parts of artichoke tubers and stems. Run in butanol-acetic for 40 hr.; sprayed with benzidine-trichloroacetic acid. Extracts made from plant 16 Sept. 1949 unless otherwise indicated (see Fig. 3). (First positive print.)

CARBOHYDRATES IN OTHER MEMBERS OF THE COMPOSITAE

The storage organs of other arbitrarily selected species of the Compositae were investigated chromatographically. Members of both subgroups of the

fibrous roots) were disintegrated in the Waring blender with an equal weight of 0.01M-potassium cyanide for about 5 min. In most cases some difficulty was experienced in the blending because of the fibrous or woody nature of the material. The most notable exception (except for the dahlia tubers

Table 7. *Details of plants examined*

(All from the Sheffield area.)

Subfamily	Systematic name	Common name	Part of plant examined	Date of collection (1949)
Tubuliflorae	<i>Dahlia</i> sp.	Garden dahlia 'Baby Rose'	Tuber	1 Sept.
	<i>Tussilago farfara</i> L.	Coltsfoot	Rootstock	15 June
	<i>Helianthus tuberosus</i> L.	Jerusalem artichoke	All vegetative organs	—
Liguliflorae	<i>Lactuca sativa</i> L.	Lettuce, 'Tom Thumb'	Taproot	16 August
	<i>Leontodon autumnalis</i> L.	Autumnal hawkbit	Rootstock	{ 20 July 26 August
	<i>Sonchus oleraceus</i> L.	Common sowthistle	Rootstock	20 July
	<i>Taraxacum officinale</i> Weber	Dandelion	Rootstock	27 April
	<i>Tragopogon pratensis</i> L.	Goatsbeard	Taproot	4 July

which resembled artichoke tubers in their ease of handling) was *Leontodon autumnalis* which had crisp, parenchymatous rootstocks with little fibrous material. The extracts obtained after squeezing the material through gauze were light-brown or yellow in colour, of pH about 6.0–6.5 and more or less opaque. They were heated in a boiling-water bath for 15 min. as soon as possible after preparation. These boiled extracts were concentrated *in vacuo* to a thick syrup and taken up in water to give sufficient concentration of carbohydrate to allow of chromatographic investigation, namely 5–10% (w/v) total fructose. When extracts so concentrated were kept in the refrigerator there was precipitation (to a lesser or greater degree) of material, presumably inulin, soluble in hot but not in cold water. The amount of this precipitate was small, except in one case. The whole concentrated extract of *Leontodon autumnalis*, however, set solid, even at room temperature, and a relatively large quantity of polysaccharide was isolated from it, the yield from 70 ml. of original unconcentrated extract being 0.7 g. In spite of considerable salt effects (especially in the case of *Sonchus oleraceus*), it was obvious that a series of carbohydrates comparable to that in artichoke tuber extracts was present in every case (see Fig. 3).

DISCUSSION

The carbohydrates of the artichoke tuber

These results demonstrate the heterogeneity of the tuber carbohydrates; they also provide a basis for the separation and estimation of the components of higher R_F . It is to be noted that the latter constitute a considerable proportion of the total carbohydrate: the five spots with R_F 's ranging from that of sucrose to about 0.025 (in butanol-acetic) contain more than half the fructose of some extracts, and if their higher relative content of glucose is assumed from the qualitative and quantitative data available they would comprise an even higher proportion of the total sugar. The even distribution of fructose among these components in all the extracts examined would seem to contradict the claims of

Tanret (1893*b*) that his 'helianthénine' and 'synanthrine' were chemical entities, since in his analysis these two fractions and sucrose occurred in a ratio of approximately 1 : 8 : 2, the three constituting two-thirds of the total carbohydrate of tubers stored until June.

It is evident that considerable possibilities exist for variation in over-all composition through variations in the relative proportions of the many components. In practice this would seem to be the explanation for the variations in fructose, as percentage of total carbohydrate, and it seems likely that the optical rotation of the mixture will depend on similar variations, the components with higher R_F probably having more positive specific rotations. It is not clear, however, whether any considerable change could occur within a given mixture of inulin, inulides and sucrose without an accompanying change either in the ratio of fructose to glucose, or in the proportion of free hexose present.

It is difficult to avoid the assumption that all the carbohydrates present in the tuber bear a relation to inulin; that they represent a series terminated by inulin at one end, and bounded by sucrose at the other, all members having the typical 1:2-linked fructofuranoside grouping and bearing a glucopyranoside as a non-reducing end group (I). Hirst, McGilvray & Percival (1950) have reported the isolation of 2.2% of tetramethyl glucopyranoside from methylated dahlia inulin, and have postulated a sucrose-like terminal group for the molecule. In addition they found circumstantial evidence for 2.6% of 2:4:6-trimethylglucose, which would indicate the presence of glucose within the chain (cf. Irvine & Montgomery, 1933). The total of methylated glucose agrees well with the percentage of glucose estimated by their chromatographic procedure, i.e. 5.7%, but is considerably higher than the figures given by others (cf. McDonald, 1946; Bell & Palmer, 1949) which lie between 1.5 and 2.5%. We have not been able to trace the source of the attribution by Lemoigne (1941*a*) to Kiliani of a formula for inulin, $(C_6H_{10}O_5)_4C_{12}H_{22}O_{11}$, in which the group $C_{12}H_{22}O_{11}$ is intended to represent sucrose.

Fig. 3. Chromatograms of extracts of subterranean organs of six species of Compositae. Run in butanol-acetic for 6 days; sprayed with phloroglucinol. (First positive print.)

Attempts to support this hypothesis by analysis of spots 2, 3 and 4 in the paper chromatogram have been vitiated by certain difficulties in the estimation.

The carbohydrates in other parts of the artichoke plant

Although no extensive investigation has been undertaken (no flowers or seeds were available), it is evident that inulin and inulides are present in all organs of the artichoke plant, with the possible exception of the leaves; this confirms the observations of Colin (1919). In some instances the effect of interfering substances in the paper chromatogram distorted or delayed the movement of the carbohydrates, and until this difficulty has been overcome a satisfactory quantitative study of the whole plant will not be possible. Until then it must remain an open question, for example, whether inulin is in fact made in the tubers or merely translocated to them, although in view of the homology of stem and tuber it would seem likely that the polysaccharide may be synthesized in both parts.

The carbohydrates of Compositae

The general similarity of the chromatograms made with extracts of other Compositae makes it probable that any conclusions drawn from an investigation of the artichoke tuber may be applied tentatively to all members of this family, and that in this way general information may be gained about the synthesis and breakdown of inulin.

SUMMARY

1. The carbohydrates of the Jerusalem artichoke (*Helianthus tuberosus* L.) and of several other members of the Compositae have been examined by various techniques including the use of qualitative and quantitative paper partition chromatography.

2. The existence has been demonstrated of a series of at least seven non-reducing substances with R_F 's ranging from that of sucrose to zero, consisting chiefly of fructofuranoside residues, but containing glucose residues in proportions decreasing with R_F .

3. These substances are present in the stems, tubers and roots of the Jerusalem artichoke, and in the underground organs of seven other species of Compositae.

4. In all extracts examined fructose residues were distributed evenly among those components with R_F 's sufficiently high for them to be isolated for analysis.

5. In the artichoke tuber the proportion of the total fructose residues contributed by the five components with greatest R_F 's varies from 15% in September to 55% in early spring.

6. Some of the implications of these findings are discussed.

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The Separation of N-2:4-Dinitrophenyl Amino-Acids on Paper Chromatograms

By S. BLACKBURN AND A. G. LOWTHER

Wool Industries Research Association, Torridon, Headingley, Leeds

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The method of identification and estimation of the free amino-groups of proteins, first applied to insulin by Sanger (1945), has since been widely used. The terminal amino-acids of the peptide chains which carry free amino groups are isolated as their N-2:4-dinitrophenyl (DNP) derivatives, which can be separated on silica gel partition chromatograms (Sanger, 1945). Buffered columns may also be employed with a variety of solvent systems (Blackburn, 1949).

A number of attempts to separate DNP-amino-acids on paper chromatograms have met with only limited success due to 'tailing' of the spots (Sanger, 1945; Blackburn, 1949). Phillips & Stephen (1948) separated some of the DNP-amino-acids, particularly the slower moving ones, using a two-dimensional method, but also observed marked 'tailing' in many solvents. In contrast they found that fairly large DNP peptides formed good spots on paper chromatograms. Woolley (1949) also found that some DNP peptides from enzymic digests of DNP-insulin ran as single spots on phenol-ammonia or butanol-ammonia chromatograms.

We have now observed that DNP-amino-acids may be successfully separated on buffered one-dimensional paper chromatograms, compact spots without 'tailing' and possessing characteristic rates (R_F values) being formed in suitable solvents. After the present paper had been read to the Biochemical Society (Blackburn & Lowther, 1950) we received from Prof. C. Fromageot a copy of a paper by Monier & Pénasse (1950), who separated mixtures of DNP-amino-acids on paper using solvents saturated with potassium benzoate solution.

EXPERIMENTAL

The chromatograms were run on strips, approx. 12 × 35 cm., of Whatman no. 4 filter paper which had been previously soaked in buffer solution and dried at room temperature.

Phthalate buffer of pH 6.0 (Britton, 1942, Table 70) proved generally suitable, and was used except where otherwise stated. About 2–5 µg. of a DNP-amino-acid (dissolved in a suitable solvent) was applied to the paper. The papers were then fixed in small stainless steel troughs mounted in glass battery jars, water and solvent being placed in the bottom of the jar, and the top covered with a sheet of plate glass. The closed box was allowed to stand for some time before the solvent (previously saturated at room temperature with the same buffer that was applied to the paper) was introduced into the trough. The movement of the DNP-amino-acids down the paper could be observed during the course of the experiment. Adequate separations were generally obtained in a run lasting several hours, or occasionally overnight.

Solvent systems

Many of the solvent systems tried proved unsuitable, because the DNP-amino-acids ran fast near the solvent front or else 'tailed' somewhat. The solvent systems of general use are described below.

tert.-Amyl alcohol. All the DNP-amino-acids move as well defined spots in this solvent.

Propanol-cyclohexane. A mixture of DNP-leucine, DNP-valine, DNP-alanine and DNP-glycine separates into four clearly defined spots in 30% propanol-cyclohexane (Fig. 1). Occasionally difficulty may be experienced with this solvent, owing to the volatility of cyclohexane, and 'tailing' spots result. This may be overcome by substituting light petroleum (b.p. 100–120°) for the cyclohexane, when similar separations can be achieved.

n-Butanol and benzyl alcohol. The spots formed in these solvents are slightly elongated, but the addition of 10% of ethanol (v/v) before saturation with buffer causes more compact spots to be formed. 10% ethanol-benzyl alcohol is superior to 10% ethanol-butanol, as the differences in R_F values between the different DNP-amino-acids are greater.

Ethyl acetate and 1% butanol-chloroform are not as generally useful as the above solvents. Ethyl acetate may be used for the separation of the slower moving DNP-amino-acids on pH 8 phosphate-citrate buffer paper (Britton, 1942, Table 65). DNP-threonine and DNP-serine can be separated from each other and from DNP-glutamic acid and DNP-aspartic acid, both of which move slowly. The DNP-mono-