CARBOHYDRATES IN ONIONS (ALLIUM CEPA)

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ABSTRACT

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By

George Menelaus Lolas

Carbohydrates make up the bulk of dry-matter of the onion bulb. This study was made to determine qualitatively the type of sugars present in the following varieties of onion: Spartan Banner, Spartan Era, Granada, Ruby and MSU (2935x2879)4535.

The techniques of paper chromatography and thin-layer chromatography were used for the separation and identification of the sugars. Besides glucose, fructose and sucrose nine oligosaccharides were separated by paper chromatography.

Using n-butanol : ethyl alcohol : water = 5:3:2 by volume as solvent and multiple and overrun irrigation, two trisaccharides, three tetrasaccharides, two pentasaccharides and two unidentified oligosaccharides were well separated by paper chromatography.

Despite the fact that these oligosaccharides are found in very small quantities attempts were made to determine the structures of the three tetrasaccharides from the products of their partial hydrolysis.

The Seliwanoff reaction of fructose and the enzymatic/colorimetric determination of glucose (glucose oxidase) were used for the estimation of the ratio of fructose : glucose in the oligosaccharides.

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George Menelaus Lolas

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DEDICATION

To my Father and Mother Menelaus and Anastasia

for their patience and encouragement

over the years.

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INTRODUCTION

Considerable information about the onion has been accumulated since about 1900. Some of this has given us a new insight into disease control, adaptation, cultivar improvement, and the commercial possibilities of manufactured products.

A great deal is known about the general physiology of the plant. Initial investigations gave the first information about the specific effect of day-length on plants, and showed that bulbing in the onion was dependent upon a suitable length of day. Temperature also exerts a profound influence on bulbing and flower initiation. However, relatively little of the detailed chemistry of the plant has been worked out. This is not surprising for, until the development of new analytical methods, especially, of chromatography in its various forms, searching for all but the most obvious components of the onion was almost an impossible task.

On the basis of nutrient content per pound of fresh vegetable, onions ranked about midway among 31 vegetables considered. On the nutrients per-acre basis, late onions ranked 3 (exceeded only by mustard greens and white potato) and early onions, 9. On the basis of nutrients

per man-hour of labor for production, late onions ranked 6 and early onions 9 or 10 (22).

Apart from these comparisons, it should be noted that onions, compared with other fresh vegetables, are relatively high in food energy, intermediate in protein content, and rich in calcium and riboflavin (22).

In this study the free sugars of the onion were identified and attempts were made to characterize some of the oligosaccharides present.

Unfortunately, the quantities of the isolated oligosaccharides were not large enough to allow definite structural identification by the technique of complete methylation.

REVIEW OF THE LITERATURE

Methods in the Determination of Sugars

Colorimetric tests for reducing sugars and polysaccharides have been known for a considerable time. Reagents such as 1-naphthol (41) for carbohydrates in general; benzidine for pentoses and uronic acids (21); naphthoresorcinol (46), and resorcinol disulfonic acid (34) for ketoses are well known examples of colorimetric tests that may be carried out in acid solution. Such tests as these and modifications of them using aromatic amines and phenols (6,19,47) have recently gained added importance because of the extensive development of partition chromatography for the separation and characterization of minute amounts of sugars and their derivatives (1,6,16,17,19,20, 44,63). Polyols and carbohydrates with a reducing group may be detected by the Tollens silver reagent (46,68), perhaps one of the best reagents in the art of chromatography. Reducing sugars are also detectable by picric acid (7,66), o-dinitrobenzene (16), and methylene blue, while diazouracil is said to be specific for sucrose as well as oligosaccharides and polysaccharides containing the sucrose residue (51).

Volumetric procedures involving the use of potassium ferricyanide (18), ceric sulfate (62), copper sulfate (15, 57), and sodium hypoiodite are used to determine small amounts of reducing sugars after separation by partition chromatography. However, experience shows that these methods require considerable skill, are time-consuming, and are sensitive to slight variation in the conditions.

The anthrone (12,42,43) and the 1-naphtholsulfonate (11) reagents are excellent for standard sugar solutions (42), but when applied to the analysis of sugars separated by partition chromatography, the presence of only traces of residual solvent developer may render them useless. Phenol in the presence of sulfuric acid can be used for the quantitative colorimetric microdetermination of sugars and their methyl derivatives, oligosaccharides, and polysaccharides (13). The enzymatic/colorimetric determination of glucose (glucose-oxidase) is unique for exclusively determining glucose.

Methods of Chromatography

Chromatography is a separation technique of great resolving power and considerable complexity.

Paper chromatography (5) may have had its origin with the description by Pliny (23-79 A.D.) of the use of papyrus impregnated with an extract of gall nuts for the detection of ferrous sulfare, or with the studies of

"Kapillaranalyse" by Runge, Schonbein, and Goppelsroeder in the period from 1850 to 1910.

There is little doubt that M. S. Tswett should be given credit for discovering the principle of preferential adsorption (adsorption chromatography) of plant pigments on a large variety of adsorbents packed in a glass tube.

The great popularity of the present-day paper chromatography is due to A. J. P. Martin, R. Consden, A. H. Gordon and R. L. M. Synge (35).

Since Tswett's experiments, chromatography has been employed on a very small or micro scale, on a medium scale, and on a large industrial scale. It has been utilized in various geometric modifications; with one-way or linear flow of solvent in columns, strips, sheets, or thin layers of the sorbent (61); with two-way or transverse flow with two different solvents in succession in sheets or thin layers of the sorbent; and with radial flow from a central zone or point in a sheet or layer of the sorbent (64). It has been utilized with all kinds of liquids as the wash medium (solution chromatography) (5,9,30,56,65). It has been modified through the use of many different sorptive materials such as surface-active liquids as well as solids (9,30,64,65) (adsorption chromatography), ion excange resins (ion-excange chromatography) (9,30), fixed polar liquids (liquid-liquid or partition chromatography), fixed nonpolar liquids (reversed phase partition

chromatography), paper alone (paper adsorption chromatography), paper plus fixed liquids (paper partition chromatography) (9,30,61,64,65), and polymeric gels (gel filtration and gel permeation chromatography).

Variations of the sorptive phase have been accompanied by great variations of the solvents, ranging from nonpolar hydrocarbons (sorption solvents) to polar acids, bases, and aqueous solutions (elution solvents) (30). With all these modifications of sorbents and solvents, chromatography has been readily adopted to the examination of all kinds of substances, from the smallest molecules and ions to large colloids and biological particulates (64,65).

Chromatography has been refined by the use of inert gases to carry mixtures of gases or vapors through sorption columns (gas chromatography). In these modifications, the sorbents are usually surface active solids (gas adsorption chromatography) or fixed, nonvolatile liquids (gas partition chromatography or gas-liquid chromatography). Gas chromatography is useful with all kinds of gaseous and volatile compounds.

Partridge (46,48), employing many of the methods and solvents used in the resolution of amino acid mixtures on paper, introduced the paper chromatography of carbohydrates.

Reviewing the basic methods of paper chromatography of sugars, Kowkabany (29) gave solvents and spray reagents suitable for carbohydrate analysis.

Column processes are the more useful chromatographic methods for the separation of pure compounds, in quantity, from mixtures. Separation of the various solutes from a mixture occurs in an appropriate system as a result of differential migration of bands through a column of stationary phase.

Mixtures of sugars (31) and their derivatives have been separated by adsorption chromatography using a wide variety of adsorbents, for example, magnesol, alumina, clays (32), charcoal (69) and silicic acid. Certain of these methods possess merits which ensure their continued use, especially for the separation of such closely related compounds as anomers and certain diastereoisomers. Nevertheless, these methods are subject to the unpredictability of adsorption chromatography and are difficult, expensive, and time-consuming.

Partition on celite columns, using both elution and extrusion techniques, has been found to be a convenient and satisfactory chromatographic method for preparative or analytical work involving carbohydrates and their derivatives.

Another technique closely related to column chromatography is the thin-layer chromatography. Both

involve the same types of chromatography: adsorption and partition. The major difference lies in the location of the stationary phase. In thin-layer (TLC) the stationary phase is in a thin layer on a glass plate, and in column chromatography it is in a column held in a glass tube.

Thin-layer chromatography now is considered an indispensable tool in many laboratories, especially those engaged in research on lipophilic natural products.

In 1938 Izmailov and Shraiber described the basic principle underlying the process of TLC, and they used the method for separating plant extracts.

Martin and Synge (36,37) developed a method of partition chromatography for separating amino acids and their derivatives.

Consden, Gordon and Martin (10) in 1944 started using filter paper, in order to carry out partition chromatography on a microscale.

Kirchner (27) in 1950 attempted to separate lipophilic mixtures using adsorption chromatography on impregnated filter paper, and later glass fiber paper coated with silicic acid or alumina.

Kirchner and Miller (28,38,39) proceeded to investigate methods of separating terpene derivatives on thin adsorbent plates.

It is astonishing that the method of thin-layer chromatography remained in obscurity until 1956 when

Stahl described equipment and procedures for the preparation of chromatoplates and demonstrated the potential usefulness of TLC in the fractionation of substances other than terpenes.

Pastuska (49) described a procedure for the quantitative titrimetric determination of sugars in zones scraped off thin-layer chromatograms on kieselguhr G. The method is based on a wet combustion with potassium dichromate-sulphuric acid and titration of the unused dichromate.

Sugars in Onions

Sugar content of onion bulbs has been measured in numerous physiological studies (22). Bennett (4) in 1941, reporting two year's work, measured reducing sugars (as glucose) and non-reducing sugars (as sucrose) of "Ebenezer" onions when they were placed in storage and after 3 1/2 months of storage. In the first year the bulbs contained, on a dry-weight basis at the beginning of storage, 60 per cent of non-reducing sugars and 5.2 per cent of reducing sugars; in the second year, the contents were 42 per cent and 17 per cent, respectively. The percentage loss of total sugars varied from 3.0 to 8.1 per cent, depending on storage conditions. However, reducing sugars increased in all storages, especially at low temperatures (33 to 34 F), where they rose from 5.2 to 21 per cent the first year and from 17 to 29 per cent the

second year. Karmarkar and Joshi (24) in 1941 also found that reducing sugars increased during low-temperatures storage, and this was again observed by Yamaguchi et al. (70) (1957), working on "Southport White Globe." These latter workers also found, contrary to Bennett's observation, that reducing sugars exceeded non-reducing sugars at the start of storage.

Some investigators (50,55) have reported arabinose, xylose, ribose, rhamnose, lactose and raffinose in the onion bulb, but most workers say that the onion bulb contains only glucose, fructose, sucrose and higher oligofructosides (2,3,8,14,33,40,58,67).

Bacon (2,3) found glucose, fructose, sucrose, and a series of water-soluble, mainly non-reducing oligosaccharides whose chain length did not exceed eight residues. No fructose polysaccharide was found. The oligosaccharides are distributed unevenly in the mature onion bulbs, being absent from the outer scales, while in the inner ones they may constitute more than half the soluble carbohydrate. The oligosaccharide is concentrated more at the base of the scales than at the top and considerable variations occur from point to point round the circumference of each scale. Bacon (3) identified two trisaccharides, fructose : glucose ratio 2:1, as 1^{F}-β-fructosylsucrose and 6^{G}-β-fructosylsucrose. These were

followed by the first tetrasaccharide, fructose : glucose ratio 3:1.

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 other Monocotyledon species shows that they occur as a result of normal physiological processes.

Sugars of onions and other Monocotyledons have been studied extensively, in an attempt to find the mechanism of fructosan biosyntehsis in plants (53,54).

MATERIALS AND METHODS

Preparation of Sample for Analysis

The onions for this study were commercial onions from the fields around East Lansing, Michigan. They were planted in April, 1971 and harvested in September, 1971. Maleic hydrazide had been used as pre-harvest treatment to prevent sprouting. After harvesting they were stored in a refrigerator at 0-3°C. The following varieties were investigated: Spartan Banner, Spartan Era, Granada, Ruby and MSU (2935 x 2879)4535 (3-way).

In the first experiments two onion bulbs of each genotype were cleaned from the outside dry material, divided into representative pieces and one hundred grams were weighed out (representative pieces of bulb), chopped into smaller pieces, covered with 80% hot ethyl alcohol and heated on a steam bath for one hour. The solution was decanted and the onion pieces were comminuted into a Waring blender with 80% ethyl alcohol. The comminuted material from blender was again heated for half an hour on a steam bath.

The mixture was filtered through a Buchner funnel and the filtrate, combined with the previous decanted solution, was transferred to a Buchler flash evaporator and concentrated to about 100 ml at 38°C. The concentrate

was centrifuged at 10,000 r.p.m. for 15-20 minutes. The clear syrup was stored at 0°F to be used later for chromatographic work.

No other clarification or preparation was necessary.

In later experiments in which there was an interest in securing greater quantities of oligofructosides which normally are found in the center of the bulb the following procedure was employed.

The four outer scales of the bulbs were discarded and the remaining scales, one kilogram in all, underwent the same treatment as before. A concentrated extract syrup of 200 ml was obtained.

Paper Chromatography

Separation by paper chromatography is based upon differences in the partition coefficients of the sugars. Separation of optical enantiomorphs such as DL-sugars is not possible by paper chromatography. The process involves countercurrent partition between a stationary cellulose-water complex, filter paper which contains 20% water and a great number of fibers, and a mobile phase composed of an organic solvent, or mixtures of solvents, containing some quantity of water.

The choice of solvent is very important and particularly the water content is a critical factor, since the solubility of a sugar in the mobile phase will define its

rate of movement on the paper chromatogram and thus affect the degree of separation of a mixture of sugars.

The technique of descending chromatographic procedure was employed using Whatman No. 1 chromatographic paper. The onion syrup was applied on a line 2.5 inches from the top of the paper. Known sugars were also applied at one inch intervals across the paper. The standards had a concentration of 1% and were prepared in 10 per cent aqueous iso-propyl alcohol and stored in the refrigerator. After application the spots were dried with a cold air current from a hair drier. A micropipette was used to apply the samples.

A number of solvent systems were used in an effort to separate as many of the sugars as possible. Table 1 shows the composition of the solvent systems tested.

TABLE 1.--Composition of solvent systems by volume.

1.	n-Butanol : acetic acid : water = 4:1:5
2.	n-Butanol : ammonia : water = 4:1:5
3.	n-Butanol : ethyl acetate : water = 4:1:5
4.	n-Butanol : acetic acid : ethanol : water = 8:1:1:10
5.	n-Butanol : ethyl alcohol : water = 5:3:2
6.	n-Butanol : iso-propyl alcohol : water = 3:5:2
7.	n-Butanol : pyridine : water = 6:4:3
8.	Benzene : acetic acid : methanol = 1:1:3
9.	n-Propyl alcohol : ethyl acetate : water = 6:1:3
10.	n-Propyl alcohol : water = 7:1
11.	iso-Propyl alcohol : water = 4:1
12.	iso-Propyl alcohol : acetic acid : methanol = 7:2:1

The solvent system which showed the best resolution was #5, n-butanol : ethyl alcohol : water = 5:3:2, by volume.

This solvent system is monophasic. Generally, monophasic solvent systems are considered superior of biphasic solvent systems because the composition of the biphasic solvent mixtures renders them temperature-dependent and a constant-temperature room for chromatography and solvent equilibration should, therefore, be used to avoid separation of the solvent mixture into two phases during development.

Monophasic systems generally consist of water, a water-soluble organic solvent and an organic solvent which is immiscible with water. The amount of the water-soluble organic solvent (ethyl alcohol in the present case) determines the amount of water which may be added to the mixture if it is to remain monophasic and this is important, because it is the water content which in practice controls the mobility of the carbohydrates.

The techniques of multiple and overrun development were used for the separation of onion carbohydrates.

Multiple chromatography includes procedures in which development is repeated either in the same direction as in the first run (multiple development) or in a direction perpendicular to the flow of the first system (two-dimensional chromatography). This can be done by using the

same solvent or a different kind of solvent system. In the present case the same solvent system was used (except in a few cases where two different solvent systems were used) and always in the same direction.

Overrun development is employed when the R_F values of the components of the mixture are too low. In such cases, especially where the mixture does not contain components with high R_F values, it is possible to allow the solvent front to run off the paper (overrun development). In this technique, the development does not end when the front of the solvent system reaches the lower edge of the chromatogram, but it is allowed to continue with the solvent freely dripping from the paper. In order to prevent any irregularities in the flow of the solvent, the lower edge of the descending chromatogram was cut in saw-tooth fashion (each tooth about one inch wide and one inch long).

The papers and the chamber were saturated with solvent placed in two large plates on the bottom of the chamber for about four hours prior to the introduction of the rest of the solvent to the troughs of the chamber for the irrigation to start.

The development was continued for 48 hours, and for the separation of higher oligosaccharides the following technique was employed. The chromatograms were removed from the chamber, dried in the chromatographic oven at 40°C and were put again into the chromatographic chamber

for a new development in exactly the same way as before. This was repeated a third time, so that, the whole procedure took about seven days.

After drying, the papers were sprayed with the spray reagent and heated to 105°C for ten minutes or until colored spots appeared. The spray most often used was the benzidine spray (56). This was made up of:

a. Benzidine, 1 g in glacial acetic acid 40 ml
   Trichloroacetic acid . . . . . 30 g 1 vol.
   Water . . . . . 40 ml
b. Acetone 9 vol.

The benzidine is dissolved in the acetic acid, the trichloroacetic acid is dissolved in the water and the two solutions are then mixed. The resultant monophasic solution is stable in the refrigerator although it slowly darkens in color; it is diluted with acetone immediately before use.

Spot-localization is either based on the reducing properties of sugars and sugar alcohols (alkaline silver oxide, periodic acid, dinitrosalicylate), or on the condensation of acid degradation products of sugars with either a reactive phenol (naphthoresorcinol or phloroglucinol), or an aromatic amine (aniline, diphenylamine, benzidine or di-anisidine) to give colored derivatives.

Other spot-localization reagents tried were the naphthoresorcinol reagent, specific for ketoses (46), the

ammoniacal silver nitrate solution for reducing sugars (48) and the periodate-permanganate reagent useful for the detection of reducing substances (31).

Quantitative Analysis

In order to quantify the sugars by paper chromatography the following procedure was used.

Two lines were drawn lengthwide 2.5 inches from the edge of the paper. Two more lines were drawn 2 inches and 2.25 inches from the top. The application of the sample was made by transverse streaking in the zone between 2 and 2.25 inches from the top. The chromatograms were developed as in the qualitative analysis. A blank paper was also developed with the same way to serve as a control. Then two strips containing the unknown sugars, sucrose, and stachyose, used as location agents since stachyose corresponds with the fourth oligosaccharide following sucrose and sucrose is followed by the first oligosaccharide, were cut from the paper and sprayed with the benzidine spray to locate the distance that the sugars had travelled.

The strips were then matched with the center portion and the center was cut into sections corresponding to the location of the sugars. Each section was again cut into small pieces about one square centimeter and transferred to 250 ml beakers. Enough deionized water was added to cover the paper clippings. The beakers were covered and stored overnight in the refrigerator. During this time

the sugars became equally distributed in the free liquid and that between the cellulose fibers. The eluate was then passed carefully through a fine sintered glass filter to retain the cellulose lint of the paper.

The filtrates were concentrated by a Buchler flash evaporator (vacuum) to 5 ml at 40°C. These were hydrolysed by dilute hydrochloric acid at 70°C for two hours in a water bath. After hydrolysis, the solutions were neutralized by dilute sodium hydroxide and the necessary dilutions were made for the determination of glucose and fructose in order to find the ratio of fructose : glucose in the oligofructosides.

The reason sintered glass filter was used is that if cellulose fibers are present in the solution of oligosaccharides these could be hydrolysed to glucose and a considerable error would be introduced in the determination of glucose. The purpose of the blank paper was to check the presence of cellulose in the filtrate and the influence of solvents in the determination of both the glucose and fructose.

The enzymatic/colorimetric determination of glucose (25,26) was used, according to SIGMA Technical Bulletin No. 510 published by SIGMA chemical Company (3500 De Kalb St., St. Louis, Mo. 63118, U.S.A). In each tube of blank, standard and unknown solutions were added in the order indicated, 0.1 ml of color reagent solution (50 mg of

o-dianisidine dihydrochloride, Stock No. 510-50 plus 20 ml water), 5.0 ml enzyme solution (contents of one PGO enzymes capsule plus 100 ml water in amber bottle). To the blank was added 0.5 ml water, to the standard 0.5 ml of a 20-fold dilution of glucose standard solution, concentration 1 mg/ml, Stock No. 635-100 (1 part solution and 19 parts water) and to the tubes of the unknown solutions 0.5 of each unknown solution, respectively. The tubes were mixed well, incubated at 37°C for 30 ($\pm$5) minutes, and at the end of this period, the tubes were removed from the water bath and the absorbance of standard and unknown solutions, using the blank as reference, was read at 450 mμ ($\pm$25mμ). The reading of the brown color was completed within an additional 30 minutes that the method required.

According to the SIGMA bulletin no calibration curve is required because all instruments with which they have prepared a calibration curve produced practically a straight line (Beer's law). To verify the above statement of the Company two solutions of glucose containing 25γ and 50γ per ml were used and their absorbance was measured. The absorbance of the second glucose solution was found to be almost exactly twice that of the first one (0.230 against 0.117).

For the determination of fructose the reaction with resorcinol and hydrochloric acid (52) was employed.

To 1 ml of a solution containing 10-50γ of fructose was added 4 ml of a reagent prepared by mixing 7 parts of 30% hydrochloric acid (5 volumes of conc. hydrochloric acid plus 1 volume of water) with 1 part of a reagent prepared by dissolving 0.1 g of resorcinol and 0.25 g of thiourea in 100 ml of glacial acetic acid. The reaction mixture was heated 10 minutes in a water bath at 80°C with frequent shaking and then cooled in running water. The appeared purple color is measured at 515 mμ.

Lowering the temperature of the water bath from 100°C to 80°C reduced somewhat the intensity of color produced but increased the specificity of the reaction. Thus glucose had a negligible effect at a concentration of 1 mg/ml.

Absorbance for both glucose and fructose was measured with a Beckman D U Spectrophotometer.

Thin-Layer Chromatography

Many attempts to separate sugar mixtures by thin-layer chromatography have been made since Stahl's (59,60) first experiments. However, results obtained with all known adsorbent mixtures have proved unsatisfactory (49), and identification of many of the most important sugars was highly dubious.

Ovodov *et al*. (45) have stressed that poor separation of some of the more common sugars and the low

capacity of the chromatoplates are responsible for the lack of general acceptance of thin-layer chromatography in research work on carbohydrates.

Pre-coated plates were mostly used. It is generally considered that pre-coated plates surpass those made in the lab in strength during multiple development employed for the increase of the separating power or for the purification of the layer prior to certain quantitative assays.

Despite the difficulties of separation of carbohydrates on thin-layer chromatograms the following materials were used.

Pre-coated TLC plates (5X20 cm) with silica gel F-254, layer thickness 0.25 mm distributed by Brinkmann Instruments, Inc. (Westbury, N.Y. 11590). The plates were used after impregnation with 0.1 N boric acid or in the state they were obtained (49).

Pre-coated polyamide F-254 (20X20 cm) plates purchased from the same company, and uniplate pre-coated TLC plates with Avicel, thickness 0.25 mm of Analtech, Inc. (100 South Justison St., Wilmington, Del. 19801) were also used. The latter one was impregnated or not with 33 mM of K_2HPO_4 (23) and was used more frequently since it gave the best results, especially after the impregnation with K_2HPO_4. The plates, after impregnation, were dried for 24 hours at room temperature.

Solvents used were some of those employed for paper chromatography but the best results were obtained by the solvent systems: n-butanol : ethyl alcohol : water = 5:3:2, and n-propyl alcohol : ethyl acetate : water = 6:1:3 by volume.

The cabinet was saturated with the solvent and then the plates were introduced. The chromatograms were routinely developed three times in the same solvent (multiple thin-layer chromatography) until the solvent front was about 1 1/2 inches from the top. The plates were dried with warm air before the next development.

The spray reagent of benzidine was used for the development of the spots. The presence of sugars was indicated by the appearance of dark brown spots (except the fructose spot which was yellow) on a light yellow-brown background. The identification was made as in paper chromatography.

RESULTS AND DISCUSSION

Paper chromatographic studies of onion extracts from all five varieties showed the presence of glucose, fructose, sucrose, two trisaccharides, 1^F-β-fructosylsucrose and 6^G-β-fructosylsucrose, three tetrasaccharides and two or probably four pentasaccharides.

Figure 1 shows that in onion only glucose, fructose, sucrose and higher oligosaccharides occur. When the chromatogram was irrigated for about 24 hours the R_F values of xylose, rhamnose, ribose and arabinose are higher than the R_F values of the sugars present in the onion. It should be noticed that only one trisaccharide has been separated. If multiple and overrun chromatography is applied more oligosaccharides will be separated, and the differences in the partition coefficients of xylose, rhamnose, ribose and arabinose will be much greater than those of the sugars of the onion. These results are in conflict with the results of some other workers (50,55) who reported that they found these sugars in the onion bulb.

Figure 2 shows that the five onion varieties examined had the same sugars. The chromatogram was irrigated twice, each time for 48 hours, and the solvent was allowed to run off the paper. In addition to glucose,

fructose and sucrose, six oligosaccharides were separated. When the chromatogram was irrigated for three 48 hour periods, glucose, fructose and sucrose ran off the paper and seven to nine oligosaccharides separate as it is shown in Figure 3. The oligosaccharides are named here for simplicity i,ii,iii, etc. starting with the oligosaccharide which follows the sucrose. The trisaccharide i is the 1^{F}-β-fructosylsucrose and the trisaccharide ii the 6^{G}-β-fructosyl-sucrose, according to Bacon (3). It is to be noticed that stachyose follows the oligofructoside iv in all its movement even after the three successive irrigations. The solvent used was n-butanol : ethyl alcohol : water = 5:3:2 whereas that of chromatogram of Figure 1 was n-butanol : acetic acid : water = 4:1:5. Multiple and overrun chromatography with the latter solvent system did not give equally good results even when continued for longer time. Perhaps, this is due to the biphasic system involved, the composition of which is susceptible to even small changes of temperature. It is also possible that some esterification of butanol with the acetic acid may occur upon long standing. The n-butanol : ethyl alcohol : water = 5:3:2 by vol. solvent system as monophonic is not subject to similar changes.

The chromatogram shown as Figure 4 has been obtained after complete hydrolysis of all the carbohydrates present in the onion bulb. It shows that only glucose and fructose are present in the hydrolysates. These results indicate

that galactose, raffinose and lactose are not present in onions as reported by other investigators (50) since, in that case, after complete hydrolysis, galactose would be present. Also, stachyose cannot be considered to be present in onions despite the clear correspondence with the fourth spot that the onion samples give, because galactose is a part of the stachyose molecule.

Figure 5 shows a thin-layer chromatogram on Avicel. Avicel, in combination with n-propyl alcohol :ethyl acetate : water = 6:1:3 as a solvent, provided the best separation of all sorbents and solvents tried in the thin-layer chromatography. With the help of thin-layer chromatography no more than five oligosaccharides could be separated. This, again, shows the superiority of paper chromatography upon the thin-layer chromatography of carbohydrates. Again the raffinose spot did not correspond to any of the spots of onion sugars and it was located between the spots of 1^F-β-fructosylsucrose and 6^G-β-fructosylsucrose. The plate was developed with the same solvent system three times, each development taking about three hours. The plates were dried with warm air current from a hair drier between each development.

The paper chromatogram of Figure 6 shows the products of partial hydrolysis of tetrasaccharides iii,iv and v. The partial hydrolysis was carried out with 0.005 N hydrochloric acid at 80°C for 30 minutes in a water bath

and after the end of this period the solution was neutralised with 0.005 N sodium hydroxide.

The quantitative determination was done as described previously for the estimation of fructose : glucose ratio. The results, an average of two determinations, are shown in Table 2.

No standard curve was prepared for the determination of fructose, but standard fructose solutions containing 20 and 40γ/ml were included in each set of estimation; these gave absorbancies of 0.168 and 0.340, respectively.

Data in Table 2 show that oligosaccharides iii,iv and v are tetrasaccharides and the vi and vii pentasaccharides. Using the ammoniacal silver nitrate spray all the oligofructosides were found to be non-reducing. The products of partial hydrolysis of tetrasaccharides iii,iv and v (Figure 6) all gave fructose, sucrose and trisaccharides i and/or ii; v is the only one which gave glucose.

In 1950 Blanchard & Albon, and Bacon & Edelman simultaneously found that trisaccharides with a fructose : glucose ration of 2:1 were formed during the hydrolysis of sucrose by ivertases. In the same year a decisive simplification of the adsorption chromatography of sugars on activated carbon was introduced by Whistler and Durso (69), and by its use three trisaccharides, 6^F-β-fructosylsucrose, 1^F-β-fructosylsucrose and 6^G-β-fructosylsucrose, were isolated and characterized; the first two are now known in

TABLE 2.--Determination of fructose : glucose ratio in onion oligosaccharides.

Sample	Glucose determination				Fructose determination				Fructose: Glucose ratio average of two determ.
	Absorb. 450 μm	factor	conc. γ/ml.	conc. correct. for dilution of extract from paper γ/ml	Absorb. 515 μm	factor	conc. γ/ml	conc. correct. for dilution of extract from paper γ/ml	
Std.	0.234	--	50.0	--	0.340	--	40.0	--	--
i	0.264	1.13	56.5	56.5X5=282.5	0.238	0.70	28.0	28.0X20=560.0	1.98
ii	0.224	0.96	48.0	48.0X5=240.0	0.429	1.26	50.4	50.4X10=504.0	2.10
iii	0.154	0.66	33.0	33.0X5=165.0	0.221	0.65	26.0	26.0X20=520.0	3.15
iv	0.240	1.03	51.5	51.5X1= 51.5	0.272	0.80	32.0	32.0X 5=160.0	3.10
v	0.244	1.04	52.0	52.0X1= 52.0	0.281	0.83	33.2	33.2X 5=166.0	3.19
vi	0.113	0.48	24.0	24.0X1= 24.0	0.422	1.24	49.6	49.6X 2= 99.2	4.13
vii	0.057	0.24	12.0	12.0X1= 12.0	0.085	0.25	10.0	10.0X 5= 50.0	4.17

the crystalline form. So far, no other trisaccharide of this general type has been identified. It will be noticed that these are the only three trisaccharides that can be formed by substitution of a primary alcoholic group of sucrose, and all three have been found in plant tissues. Oligofructosides, in general, are presumed to be derived from one or the other of these trisaccharides by addition of further β-fructofuranosyl residues.

Since in the onions tested 6^F-β-fructosylsucrose was absent it may be assumed that some enzyme is responsible for the transfructosidation of C-1 of the fructose residue of sucrose.

Schlubach and Koehn (53), trying to explain the presence of 1^F-, 6^G-β-fructosylsucrose and bifurcose (a tetrasaccharide) in rye, suggested that the simplest explanation is to accept the existence of two different enzymes, one which acts on the 1- and the other on the 6- position of the fructose residue of the sucrose molecule.

Since the onion tetrasaccharides gave among the products of their partial hydrolysis 1^F- and 6^G-β-fructosylsucrose, and since they are non-reducing it could be assumed that they are products of further transfructosidation (transfer of fructose from the molecule of sucrose with the help of enzyme and substitution of the primary alcoholic group of fructose residue of trisaccharide of C-1). It should be also taken into consideration that no

reducing compounds could be detected in the products of partial hydrolysis except glucose and fructose. Three tetrasaccharides would therefore be possible through trans-fructosidation of the two trisaccharides, as it is shown in Figure 7.

At least, tentatively, the structures III,IV and V may be given to the three tetrasaccharides iii, iv and v, respectively. The basis of the given structures is that only tetrasaccharide v gives glucose during partial hydrolysis and the spots corresponding to sucrose and fructose are much more intense than in the case of the other two. Also, this is the only one which has no substitution of C-6 of the glucose, thus differing from the other two. Knowing that the C-6 bond of sugars is hydrolysed with difficulty, it becomes understandable why glucose is absent in the products of partial hydrolysis of tetrasaccharides iii and iv.

Structure III is justified on the basis of the partial hydrolysis products, 1^{F}-β- and 6^{G}-β-fructosylsucrose which give spots stronger than those derived from the other two tetrasaccharides. In addition the fructose spot of the partial hydrolysate of iii is weaker than that of the other two tetrasaccharides.

Structure IV given to tetrasaccharide iv can be justified as follows. iv, hydrolysed, can give the trisaccharide ii and another trisaccharide which differs

from i in that the bond between the glucose and fructose is not between C-1 and C-2 but between C-1 and C-6 of glucose. If this new trisaccharide has the same partition coefficient with i then its spot is understandable on the chromatogram. If this new trisaccharide does not really exist then product i could be derived from iv isomerized to iii during acid hydrolysis. Indeed tetrasaccharide iv upon partial hydrolysis gives a spot corresponding to tetrasaccharide iii.

Although the situation in the two previous cases looks relatively easy to explain, that of tetrasaccharide v is more complicated. The spots corresponding to fructose, glucose, sucrose and trisaccharide i are easy to explain, but not those corresponding to trisaccharide ii, tetrasaccharides iii, iv and the spots in traces between sucrose and i, and i and ii. Glucose here is at the end of the molecule and isomerization by partial breaking and rebinding cannot occur in such a way so that non-reducing products result.

The only remedy in this situation would be to secure greater quantities of these tetrasaccharides so that the methylation technique can work to give the solution to the real structures.

If further transfructosidation is proved to be correct then these tetrasaccharides can give four different pentasaccharides. Thus each one could give:

(iii)	F2-1F2-1G6-2F	F2-1F2-1G6-2F1-2F	(A)
		F2-1F2-1F2-1G6-2F	(B)
(iv)	F2-1G6-2F1-2F	F2-1G6-2F1-2F1-2F	(C)
		F2-1F2-1G6-2F1-2F	(D)
(v)	F2-1F2-1F2-1G	F2-1F2-1F2-1F2-1G	(E)

From the possible five pentasaccharides A and D are identical; therefore only four different pentasaccharides are possible. Two oligosaccharides of the onion had a fructose : glucose ratio 4:1 and are assumed to be pentasaccharides. The other two oligosaccharides appearing when the irrigation is continued for longer time are possibly also pentasaccharides.

Some rough estimates of the quantities of these oligosaccharides based on glucose determination and the quantity of the onion sample applied on paper are shown in Table 3.

From the results in Table 3 one can visualize how difficult it is to deal with these oligosaccharides. Thus, more sensitive techniques of detection should be introduced in every step of the research. Possibly, some kind of column chromatography with the proper adsorbent material and solvent system could be used for the separation of greater quantities of these oligosaccharides.

TABLE 3.--Quantitative observations on oligosaccharides present in the onion (var. 3-Way).

Oligofructoside	Yield in mg/100 g fresh wt. of tissue analyzed
i	186
ii	80
iii	36
iv	11
v	7
vi	3
vii	1

SUMMARY AND CONCLUSIONS

The carbohydrate constituents of five varieties of onions examined were found to be fructose, glucose, sucrose, two trisaccharides, 1^F-β-fructosylsucrose and 6^G-β-fructosylsucrose, three tetrasaccharides and at least two pentasaccharides. Paper chromatography using as solvent n-butanol : ethyl alcohol : water = 5:3:2 by volume has proved to be an excellent method for the separation of minute quantities of sugars present.

Further research is needed for the verification of structures proposed for the tetrasaccharides and pentasaccharides present in the onion bulb.

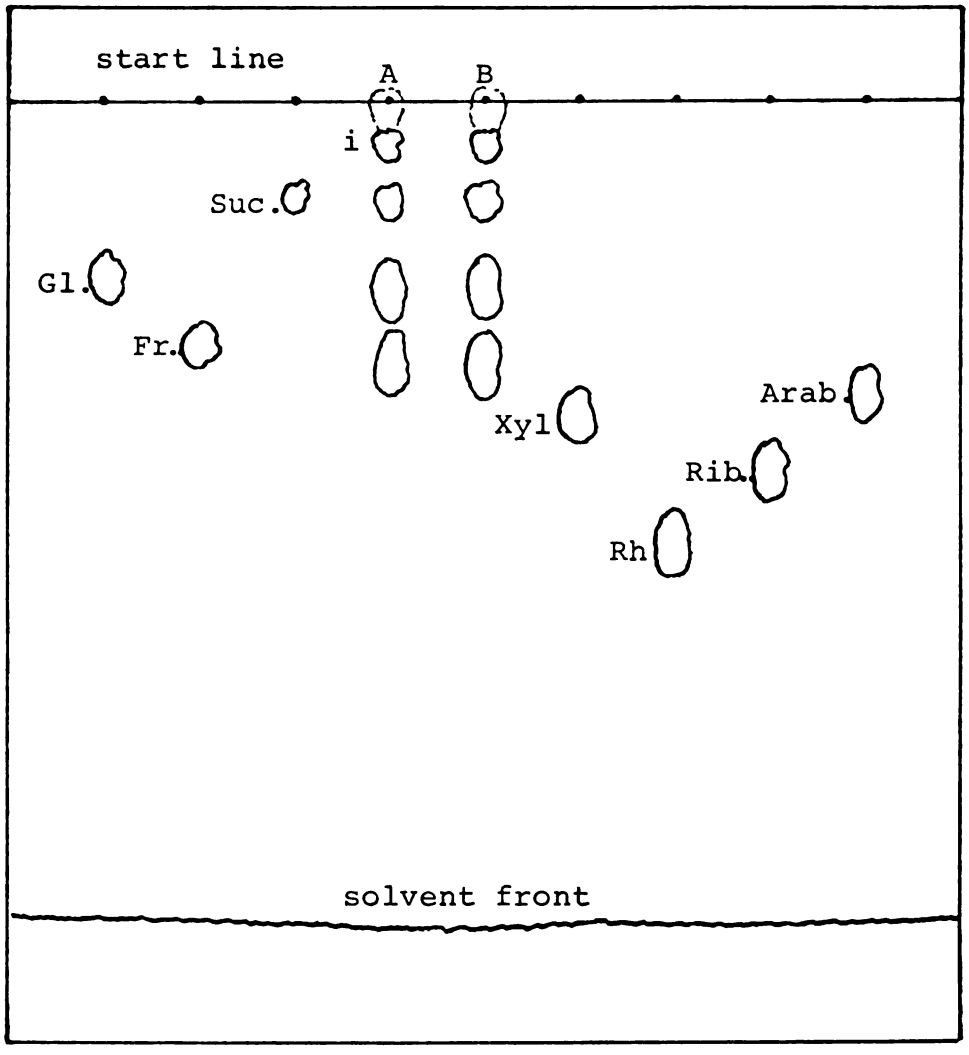


Figure 1.--Descending paper chromatography of the onion carbohydrates with solvent n-butanol : acetic acid : water = 4:1:5 by vol. Gl., glucose; Fr., fructose; Suc., sucrose; Xyl., xylose; Rh., rhamnose; Rib., ribose; Arab., arabinose; i, 1^F-β-fructosylsucrose. Varieties: A, Ruby; B, Spartan Era.

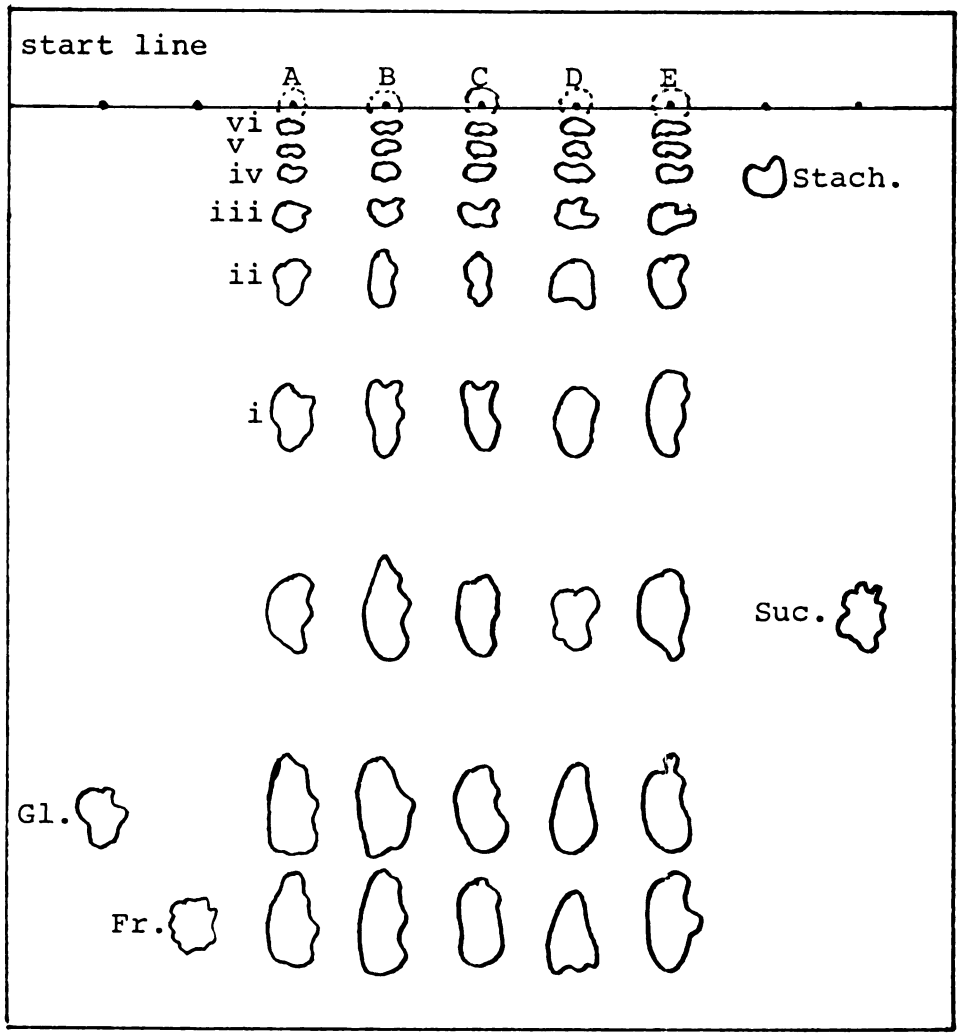


Figure 2.--Multiple descending paper chromatography of five varieties of onions with the solvent n-butanol : ethanol : water = 5:3:2 by vol. Gl., glucose; Fr., fructose; Suc., sucrose; Stach., stachyose; i,ii, trisaccharides; iii,iv,v, tetrasaccharides; vi, pentasaccharide. Varieties: A, Ruby; B, Spartan Era; C, 3-Way; D, Spartan Banner; E, Granada.

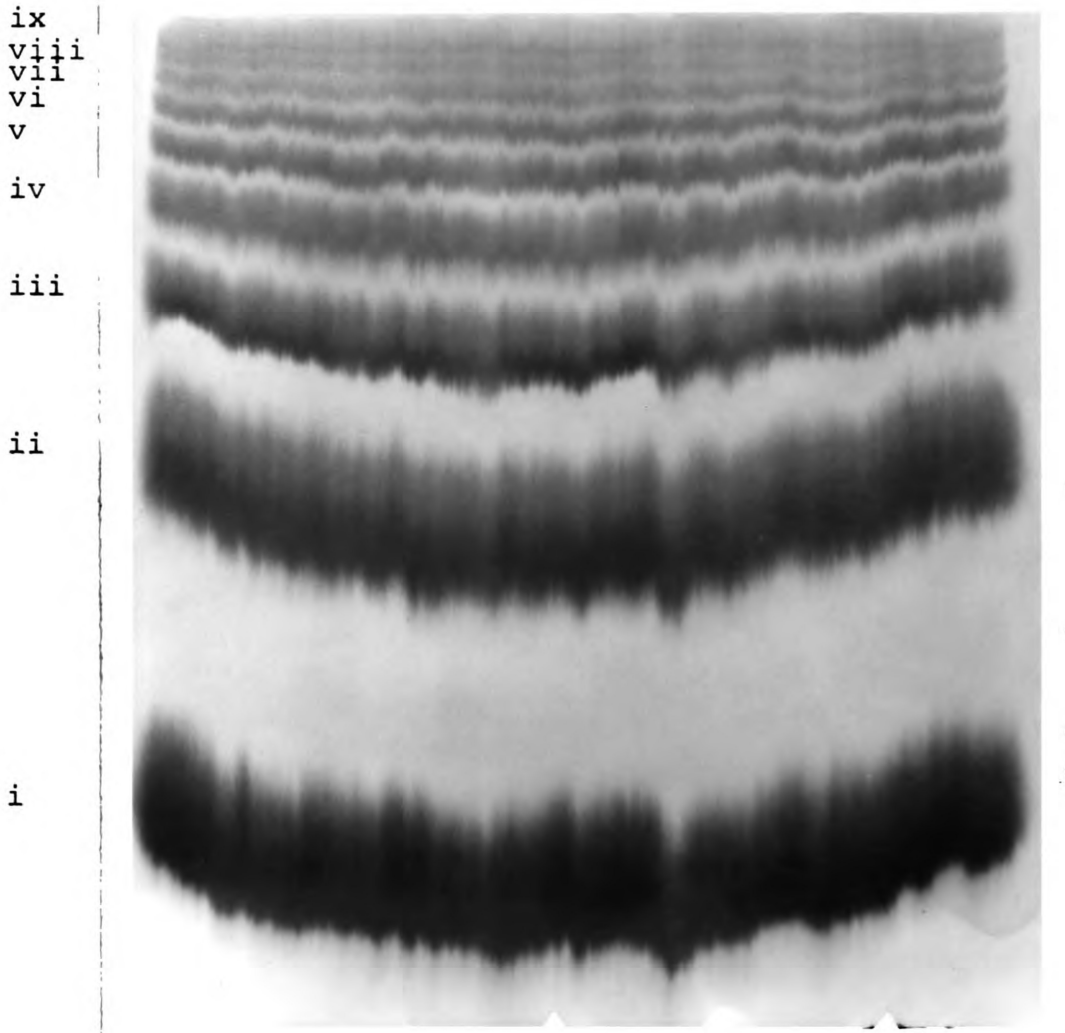


Figure 3.--Multiple descending paper chromatogram of the trisaccharides, tetrasaccharides and pentasaccharides of the onion. Variety: 3-Way. From below, bands i and ii are trisaccharides, bands iii,iv and v tetrasaccharides, vi and vii pentasaccharides and viii and ix unidentified.

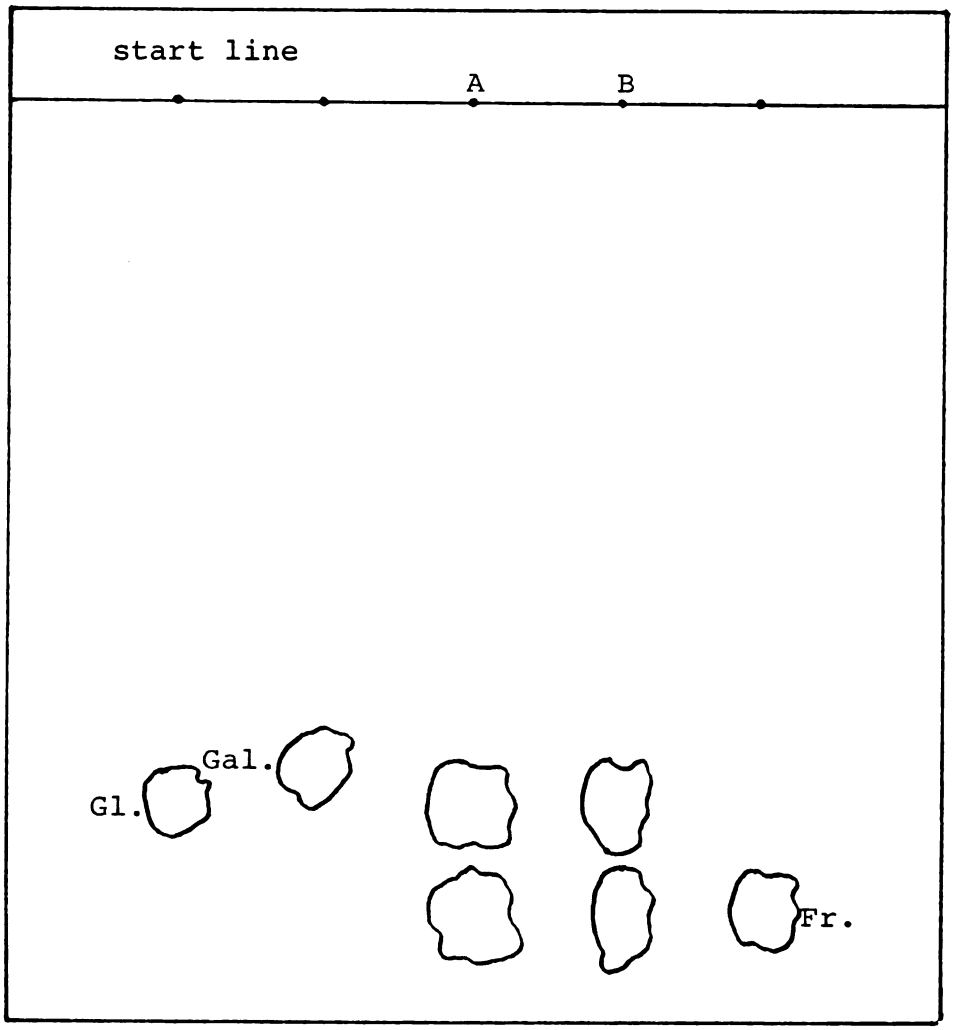


Figure 4.--Multiple descending paper chromatography of hydrolysates (complete hydrolysis) of two varieties of onions: A, Ruby; B, 3-Way, with the solvent n-butanol : ethanol : water = 5:3:2 by vol. Gl., glucose; Gal., galactose; Fr., fructose.

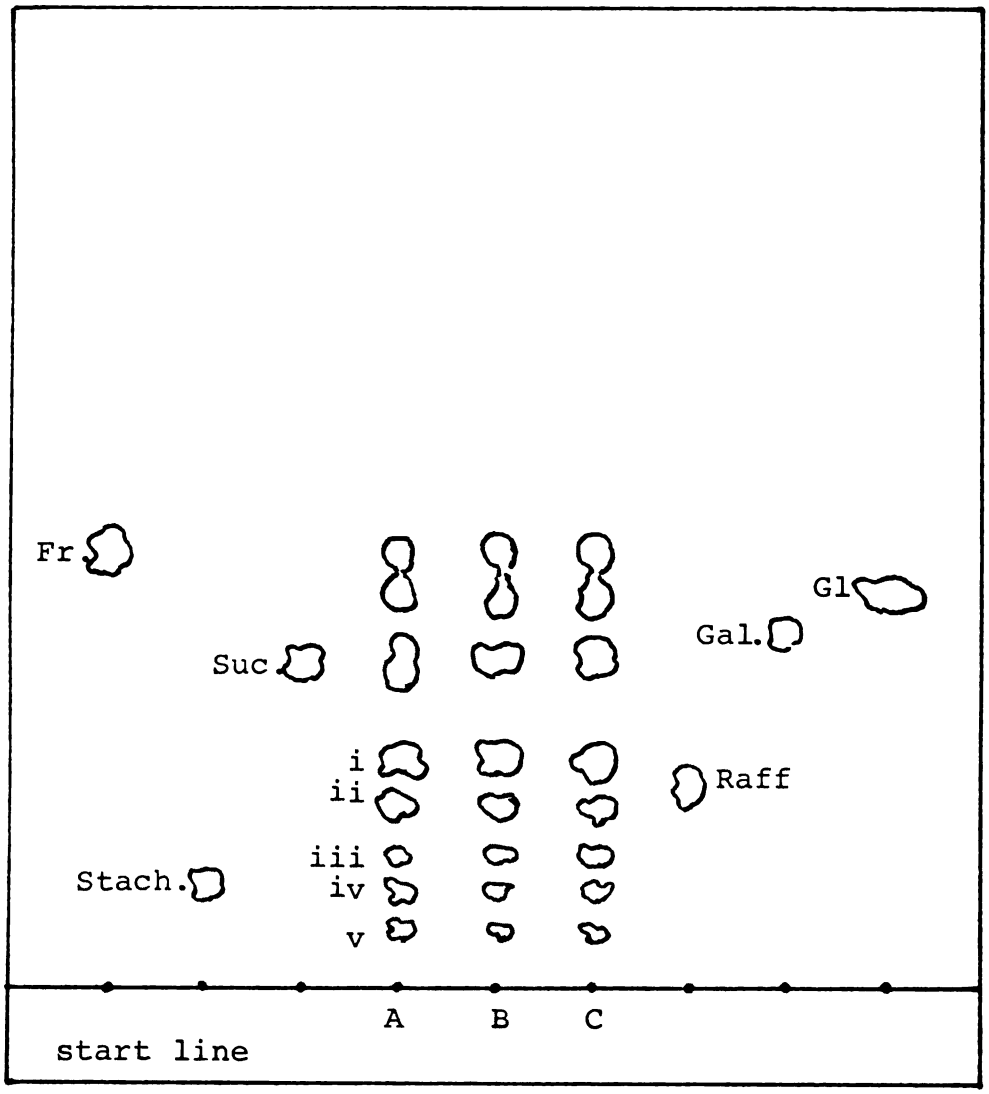


Figure 5.--Multiple thin-layer chromatography of three varieties of onions: A, Ruby; B, Spartan Banner; C, 3-Way with the solvent n-propanol : ethyl acetate : water = 6:1:3 by vol. Gl., glucose; Fr., fructose; Gal., galactose; i,ii, trisaccharides; iii,iv,v, tetrasaccharides.

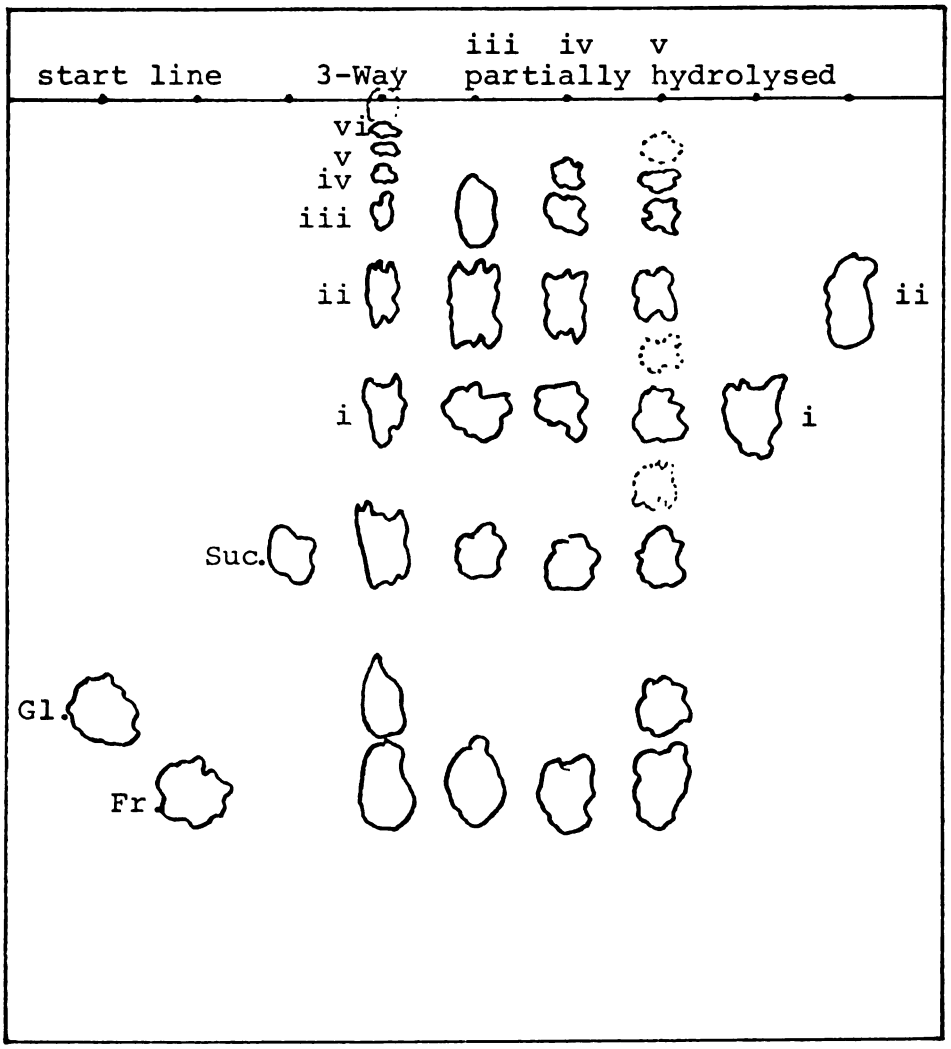
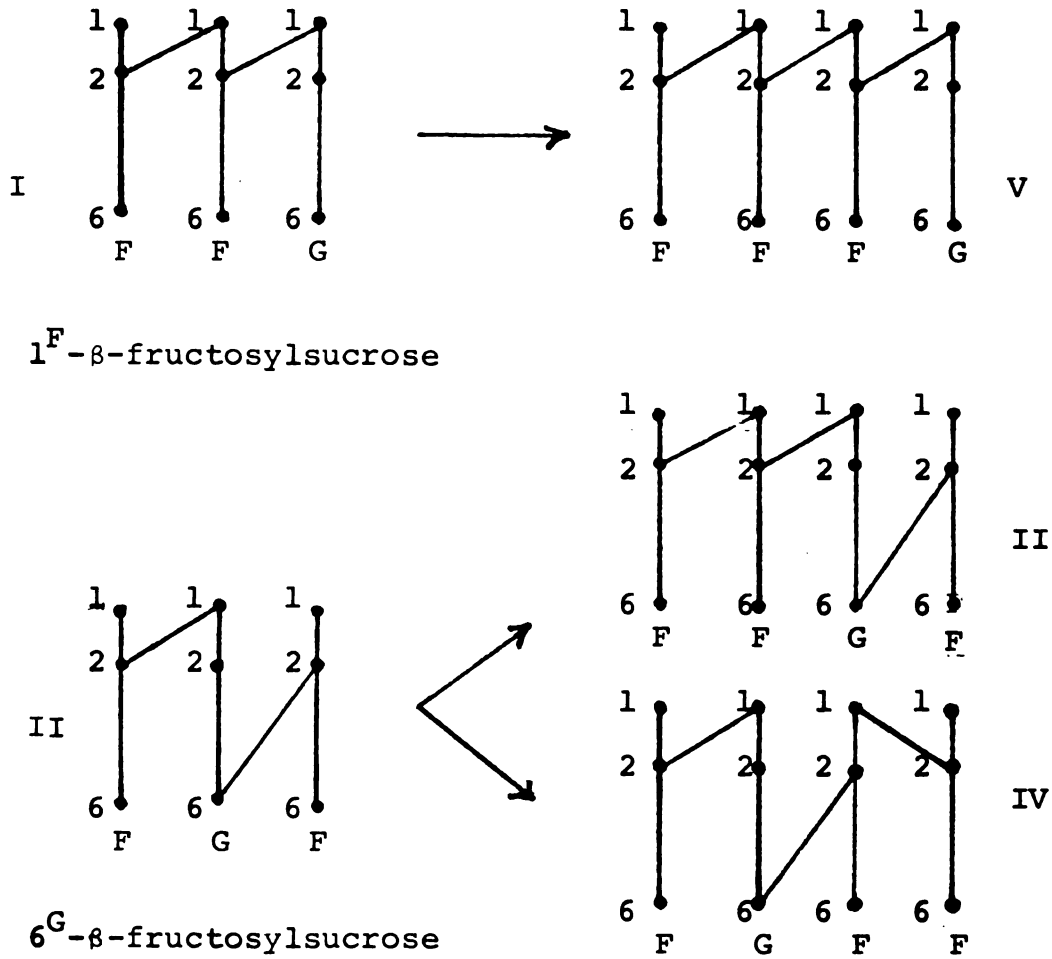


Figure 6.--Multiple descending paper chromatography of partial hydrolysis of tetrasaccharides iii,iv and v of the onion with the solvent n-butanol : ethanol : water = 5:3:2 by vol. Gl., glucose; Fr., fructose; Suc., sucrose; i,ii, trisaccharides; iii,iv,v, tetrasaccharides; vi, pentasaccharide. The unhydrolysed sugars of 3-Way are shown for a comparison.



1^{F}-β-fructosylsucrose

6^{G}-β-fructosylsucrose

Figure 7.--Three tetrasaccharides can be formed from 1^{F}-β-fructosylsucrose and 6^{G}-β-fructosylsucrose by the substitution of the C-1 primary alcoholic group with a fructose residue of the sucrose molecule (transfructosidation); F, fructose; G, glucose.

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