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Trisaccharides of the Onion (Allium cepa)

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TRISACCHARIDES OF THE ONION (ALLIUM CEPA)

BY

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This thesis is approved as a creditable, independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

The purpose of this work was the characterization of a portion of the carbohydrate material extracted from onions. The portion under study is the third band on a paper chromatogram counting upward from sucrose as band one when the chromatogram is developed with n-butyl alcohol: acetic acid:water(6:1:2,v/v). This band is non-reducing carbohydrate material which yields on acid hydrolysis only fructose and glucose in a 2:1 ratio (16).

The determination of the conditions under which or the method by which these compounds were synthesized was not undertaken. It is known that oligosaccharides, that is, those carbohydrates containing two to ten monosaccharide units in each molecule, are sometimes formed by the hydrolysis of polysaccharides (3, 7, 17, 21, 26, 28, 29, 30, 37). This hydrolysis of polysaccharides can be brought about under conditions which are dependent upon the nature of the linkages present in the polysaccharides. The linkages vary greatly in stability to acid hydrolysis (35, 39). "Autohydrolysis" of polysaccharides can be accomplished merely by stirring the polysaccharide in boiling water for a given time (4, 5, 40). Also, hydrolysis of polysaccharides and oligosaccharides can be catalyzed by the use of enzymes (7, 20, 28, 41).

Oligosaccharides can be synthesized from disaccharides or other suitable acceptors by fructose- or glucose-transferring enzymes (2, 6, 7, 14, 19, 20, 28, 37). In fact, there is danger of isolating certain trisaccharides as artifacts in plant extracts if the enzymes present in

the plant are not inactivated quickly during the extraction (2).

Regardless of the method of synthesis or the origin of these oligosaccharides, the determination of their structure and their characterization by the preparation of derivatives still presents a challenge. The application of well-established techniques of chromatography and micro-methods of analysis makes possible the isolation of very pure oligosaccharides in amounts sufficient for the preparation of derivatives.

EXPERIMENTAL PROCEDURE

Materials

The mature onions from which the carbohydrates were extracted were of the commercial Yellow-Globe Danvers variety. At the time of purchase, some of them had green, growing shoots. These shoots were not disturbed. The whole onions were stored in a cool place before use. They were never kept longer than two weeks after the date of purchase.

Deionization of the onion extract and the invertase-sucrose preparation was accomplished by passage through Amberlite IR-120(H^+) cation exchange resin and Amberlite IR-4B(OH^-) anion exchange resin. These resins are products of Rohm and Haas Company, Philadelphia, Pennsylvania.

Whatman filter paper of grades 1, 2, and 3MM were used for chromatography. They were used either as the whole sheet (40 by 60 centimeters) or cut into pieces of various shapes and sizes for certain purposes. The Whatman Number 3MM thick filter paper was used for the separation of carbohydrates on a preparative scale.

Solvent systems for the development of chromatograms were:

1. n-butyl alcohol:acetic acid:water(4:1:5,v/v, upper phase) (36)
2. n-butyl alcohol:acetic acid:water(6:1:2,v/v) (33)
3. n-butyl alcohol:ethyl alcohol:water(40:11:19,v/v) (29)
4. n-butyl alcohol:ethylene glycol:water(2:1:1,v/v)
5. n-butyl alcohol:pyridine:water(6:4:3,v/v) (38)
6. ethyl acetate:acetic acid:formic acid:water(18:3:1:4,v/v) (29)
7. ethyl acetate:pyridine:water(8:2:1,v/v) (44)

8. ethyl acetate:pyridine:water(10:4:3,v/v) (44)

9. isopropyl alcohol:n-butyl alcohol:water(7:1:2,v/v) (45)

10. isopropyl alcohol:ethylene glycol:water(7:1:2,v/v)

11. water-saturated phenol(lower phase) (36)

The sprays for the detection of carbohydrates were prepared in the following manner:

1. Aniline-diphenylamine Phosphate (11)

4 grams diphenylamine
4 milliliters aniline
200 milliliters acetone
20 milliliters syrupy (80%) phosphoric acid

The aniline and the diphenylamine are thoroughly mixed with the acetone before the addition of the phosphoric acid. The reagent is stable 2-3 weeks when stored at 0° C. After spraying, the chromatogram is heated at 80° C. for 4 minutes to develop color at the sites of the sugars.

2. p-Anisidine Phosphate (31)

0.5 gram p-anisidine
2 milliliters syrupy phosphoric acid (specific gravity 1.75)
99% ethyl alcohol

One-half gram of p-anisidine is dissolved in 99% ethyl alcohol. Two milliliters of syrupy phosphoric acid are added and the mixture diluted to 50 milliliters in a volumetric flask. The undissolved phosphate is separated by filtration. The filtrate is a spray reagent specific for fructose. The precipitate is dissolved in the smallest possible amount of water (50 milliliters). The mixture is diluted with an equal volume of ethyl alcohol. Syrupy phosphoric acid (2 milliliters)

is then added so that its final concentration is approximately 2%. This second spray reveals all sugars except trehalose. These sprays may be either combined or used separately. After spraying, the chromatogram is heated at 95-100° C. for 3-5 minutes to develop colors. The sprays are stable indefinitely when stored in a refrigerator.

3. alpha-Naphthylamine Trichloroacetate (27)

2 grams alpha-naphthylamine
95% ethyl alcohol
5 grams trichloroacetic acid

Two grams of alpha-naphthylamine are dissolved in 95% ethyl alcohol and the mixture diluted to volume in a 100 milliliter volumetric flask.

The 5 grams of trichloroacetic acid are dissolved in water and the mixture diluted to volume in a 100 milliliter volumetric flask.

The chromatogram is first sprayed with the 2% alpha-naphthylamine in 95% ethyl alcohol solution. After air-drying, it is oversprayed with the 5% trichloroacetic acid solution. Immediately after overspraying, the chromatogram is heated at 100-105° C. for 3-5 minutes to develop colors at the sites of the sugars.

This procedure is a modification of that in the literature in which 2% alpha-naphthylamine in 1:1 v/v n-butyl alcohol-methyl alcohol containing 5% trichloroacetic acid is used instead of the separate solutions (27).

A chromatographic column was prepared according to the directions of Whistler and Durso (43). The adsorbent was a mixture of equal parts by weight of Darco-G-60 charcoal (Matheson, Coleman, and Bell, East

Rutherford, New Jersey) and Celite (L-665-A, Johns-Manville, New York). Both these substances were washed with distilled water in separate beakers until the effluent was neutral. Aqueous suspensions of the washed charcoal and Celite were poured together in a large beaker and stirred vigorously to form a slurry.

A small amount of glass wool was used to pack the bottom of the chromatographic tube. An aqueous suspension of well-washed Celite was poured over the glass wool to form a mat about 1 centimeter in depth. The slurry of the well-mixed washed charcoal and Celite was added in small portions to the tube. The mixture was allowed to drain until the liquid level was about 1-2 centimeters from the top of the column, which was never allowed to run dry. No suction was applied while filling the tube. The freshly-packed column was allowed to settle overnight by gravity. A small amount of glass wool was used again to pack the top of the column.

Extraction of Carbohydrates from Onions

The mature intact onions were not washed or peeled before weighing. The roots and shoots were left undisturbed. After weighing, the onions were cut into pieces of such size that they would fit in the Waring Blendor, quartering was usually sufficient. Boiling 95% ethyl alcohol was added so that the final alcohol concentration was between 70-80% (34). To calculate this quantity, the water content of the onions was taken to be 87% (47). A small quantity of calcium carbonate was added to keep the solution neutral during the extraction (34). The onions were

then blended until a pulpy mass resulted. This pulpy mass was separated from the alcoholic extract by filtering through a Buchner funnel with Celite as a mat on the paper. The filter containing the pulp was washed with boiling 80% ethyl alcohol and the washings were added to the extract. The pulp left on the filter was discarded.

The ethyl alcohol was removed by evaporation under reduced pressure in a 1-liter Claisen flask at a vapor temperature of less than 23° C. To expedite this evaporation, the receiver was packed in ice.

After removal of the ethyl alcohol, the resulting water solution was deionized by passage through ion-exchange resins such that the extract from 10 grams of raw onions was treated with 40 cubic centimeters of each resin. First the extract was stirred in contact with the Amberlite IR-120(H^+) cation exchange resin for 30 minutes. It was then filtered to remove resin particles. The resin was rinsed with distilled water until the effluent was colorless in order to prevent loss of carbohydrate material on the resin. The rinsings were also filtered to remove resin particles. The combined filtrates were then stirred in contact with the Amberlite IR-4B(OH^-) anion exchange resin for 30 minutes. The now neutral extract was again filtered to remove resin particles. This resin was also rinsed with distilled water until the rinsings were colorless. The rinsings were filtered to remove resin particles, and the filtered rinsings added to the filtered extract.

The neutral straw-colored water solution was concentrated by evaporation under reduced pressure under the usual conditions. The resulting small volume in the 1-liter Claisen flask was transferred with

rinsings to a 125-milliliter Claisen flask for further concentration. The concentrated extract and the rinsings with ethyl alcohol from the small Claisen flask were poured in a 50-milliliter Erlenmeyer flask fitted with a cork stopper wrapped with Saran-Wrap. The Erlenmeyer flask was then stored in a refrigerator.

Fractionation of Carbohydrates in Onions

A straight line was drawn with a graphite pencil 3 inches from and parallel to the long side of Whatman Number 3MM filter paper. Carbohydrates from the onions were applied along the line up to a point 2 inches from the edge of the paper. Sucrose was spotted on each chromatogram along this line 1 inch from the end of the streak and the edge of the paper. The end of the paper opposite the carbohydrates was serrated in order to facilitate the uniform movement of the solvent off the paper. Eight chromatograms at a time were prepared in this fashion. Two of them were hung on each tray in the chromatographic cabinet (28.5 by 19.5 by 27 inches) with the carbohydrate material on the upper end of the paper. The tray in the cabinet is provided with a bar over which the paper passes to prevent capillary siphoning. The solvent, n-butyl alcohol:acetic acid:water(6:1:2,v/v), was added without previous equilibration. After 120 hours development, the chromatograms were removed from the cabinet and hung in the fume-hood to air-dry.

After air-drying, one-inch strips were cut vertically from both ends and the center of each chromatogram. They were sprayed with p-anisidine phosphate in 50% ethyl alcohol and heated at 95° C. for 5

minutes to develop colors at the sites of the carbohydrates. The strips were then examined under ultraviolet light, because carbohydrates fluoresce after reaction with this reagent. These areas of fluorescence were marked. The marker strips were used as guides to aid in the location of carbohydrates on the unsprayed portions of the paper. The carbohydrates on the unsprayed portions were then cut out as bands and hung on the trays in a smaller glass cabinet (6 by 9 by 12 inches). Eighty per cent ethyl alcohol was added to the trays to elute the sugars present in the bands. The eluates of corresponding bands were collected and stored in flasks.

In this work, only Band III, counting upward from sucrose as Band I, was under study. Therefore only the eluate of Band III was concentrated under the usual conditions to a syrup. This syrup was taken up in the smallest possible quantity of 80% ethyl alcohol.

Determination of Homogeneity of Band III

To ascertain whether Band III could be separated on paper by a solvent other than n-butyl alcohol:acetic acid:water(6:1:2,v/v), the one used for the initial fractionation of the carbohydrates in onions, it was tested in a number of different solvents.

A straight line was drawn with a graphite pencil 3 inches from and parallel to the long side of a sheet of Whatman Number 1 filter paper. Band III was spotted alongside standard known sugars on this line with a distance of 1.5 inches between each spot. For development with each solvent, two chromatograms containing the same sugars were prepared. The side of one chromatogram opposite the sugars was serrated.

The chromatogram without the serrated edge was removed from the cabinet as soon as the solvent had reached the bottom of the paper. The one with the serrated edge was left in the cabinet for a longer time.

If the solvent consisted of only one phase, the paper containing the sugars was not equilibrated with solvent vapor prior to development. However, if the solvent consisted of two phases, equilibration of the paper prior to development was necessary. This equilibration was accomplished by placing small evaporating dishes filled with the layer of the solvent not used for development of the chromatograms in the bottom of the chromatographic cabinet.

The solvent systems used to determine the homogeneity of Band III were:

n-butyl alcohol:ethyl alcohol:water(40:11:19,v/v)

n-butyl alcohol:ethylene glycol:water(2:1:1,v/v)

n-butyl alcohol:pyridine:water(6:4:3,v/v)

ethyl acetate:acetic acid:formic acid:water(18:3:1:4,v/v)

ethyl acetate:pyridine:water(8:2:1,v/v)

ethyl acetate:pyridine:water(10:4:3,v/v)

isopropyl alcohol:n-butyl alcohol:water(7:1:2,v/v)

isopropyl alcohol:ethylene glycol:water(7:1:2,v/v)

water-saturated phenol

These solvent systems were used not only to ascertain the homogeneity of Band III, but also to provide a tentative identification of Band III. The use of a variety of solvent systems serves to eliminate from consideration those sugars which have the same R_s value as Band III

in one solvent but not in another. The R_s value is defined as the ratio of the distance moved by the sugar to that moved by the sucrose under the same conditions.

To further test the homogeneity of Band III, the Whistler-Durso column was used. The column was prepared by pouring a slurry consisting of 50 grams of a mixture of equal parts by weight of Darco-G-60 charcoal and Celite into a chromatographic tube (430 millimeters by 30 millimeters diameter) with Celite and glass wool in the bottom. Approximately 3 grams of carbohydrates extracted from the onions in the form of a 10% solution in water were added to the top of the column. Fractionation of the adsorbed sugars was effected by washing the column with water and solvents of an increasing ethyl alcohol content. The flow rate was adjusted with suction to about one milliliter every 2 minutes. Fractions of approximately 10 milliliters each were collected. One-tenth milliliter of each fraction collected was analyzed by the phenol-sulfuric acid method (18). By means of a micropipette, 0.1 milliliter of each fraction was added to 0.9 milliliter distilled water in a 5-inch test tube. One milliliter of 5% phenol (50 grams phenol twice recrystallized from water, dissolved in enough water to make one liter of solution) was then pipetted into each test tube. After the addition of the phenol, the tubes were shaken to mix the contents. Five milliliters of concentrated sulfuric acid (analytical reagent grade) were then added as quickly as possible to effect mixing without spattering. The tubes were allowed to stand at room temperature (20-25° C.) for 30 minutes to develop color. The color produced was determined in a Spectronic-20 Colorimeter at 490 millimicrons

within 6 hours against a blank containing 1 milliliter of distilled water, 1 milliliter of 5% phenol, and 5 milliliters of concentrated sulfuric acid prepared at the same time under the same conditions. It is imperative to guard against contamination of the tubes by pieces of lint and cotton fibers, as they also produce color with this procedure.

The transmittancy readings obtained by the phenol-sulfuric acid method with the Spectronic-20 Colorimeter were converted to absorbancy readings by multiplying the corresponding optical densities (24) by 2.303. These absorbancies were then plotted against the corresponding fraction number to establish the elution pattern of the column. The phenol-sulfuric acid readings also provided a means of ascertaining whether or not enough solvent of a given ethyl alcohol concentration had been used to elute the column.

Those column eluates having a high absorbancy were analyzed by paper chromatography with sucrose, glucose, and fructose as standards on Whatman Number 1 filter paper. The solvent used for the development of the chromatograms was n-butyl alcohol:acetic acid:water(6:1:2,v/v).

Hydrolysis of Band III

The method chosen to determine the increase in reducing power on graded acid hydrolysis of Band III was Nelson's colorimetric modification of Somogyi's method (32, 42).

The low-alkalinity copper reagent was prepared in the following manner (42):

12 g. Rochelle salt
24 g. anhydrous sodium carbonate
16 g. sodium hydrogen carbonate
144 g. anhydrous sodium sulfate

The above solid substances were dissolved in distilled water and diluted to 800 milliliters.

4 g. cupric sulfate pentahydrate
36 g. anhydrous sodium sulfate

These two solid substances were dissolved in distilled water and diluted to 200 milliliters.

Immediately before use 4 volumes of the first solution were well-mixed with 1 volume of the second solution.

The arsenomolybdate reagent was prepared in the following manner (32):

25 g. ammonium molybdate
21 ml. concentrated sulfuric acid
3 g. disodium hydrogen arsenate heptahydrate

To 25 grams of ammonium molybdate dissolved in 450 milliliters of distilled water are added 21 milliliters of concentrated sulfuric acid. After mixing, 3 grams of disodium hydrogen arsenate heptahydrate dissolved in 25 milliliters of distilled water is added. The mixed solution is incubated 24 hours at 37° C. and stored in a glass-stoppered brown bottle.

One milliliter of the concentrated eluate of Band III in ethyl alcohol was pipetted into a mixture of 2 milliliters of distilled water and 1 milliliter of 0.01 N HCl in a 5-inch test tube. The test tube was

well-stoppered with a cork wrapped with Saran-Wrap. After shaking the test tube to insure homogeneity of its contents, it was placed in an oven set at 80° C. One-tenth milliliter aliquots were withdrawn after the lapse of 5, 10, 15, 30, 45 minutes and 1 hour for the first hour and every 15 minutes thereafter. The aliquots were pipetted into 5-inch test tubes containing 1.9 milliliters distilled water. The hydrolysis was carried out for 6 hours. At the end of this time, the remaining material in the test tube was neutralized with solid sodium carbonate until effervescence ceased (38). It was then stored in a refrigerator.

Two milliliters of low-alkalinity copper reagent were added to the aliquots and a test tube containing 2 milliliters of distilled water. The test tubes were shaken to effect mixing of the contents. The mouths of the test tubes were covered with marbles before the tubes were placed in a boiling water bath to retard evaporation of the solution and a subsequent change in volume. After 10 minutes in the water bath, the tubes were withdrawn and allowed to cool to room temperature. The slight cuprous oxide precipitate in the bottom of the tubes was resuspended by placing each tube on a Vortex mixer. Two milliliters of Nelson's arsenomolybdate reagent were added and the tubes were again mixed. After 40 minutes had lapsed, the amount of color produced was determined against a blank containing 2 milliliters of distilled water, 2 milliliters of low-alkalinity copper reagent and 2 milliliters of Nelson's arsenomolybdate reagent at 520 millimicrons on the Spectronic-20 Colorimeter.

Band III was hydrolyzed under the same conditions as before, but for only 70 minutes. One milliliter aliquots were withdrawn every 10

minutes for 70 minutes after an initial time of 20 minutes, and immediately neutralized with solid sodium carbonate (38). These aliquots were examined by paper chromatography.

Preparation of Reference Compounds

On the basis of the fact that Band III had an R_s value of 0.62 in isopropyl alcohol:n-butyl alcohol:water(7:1:2,v/v), and the fact that the components of Band III separated on the Whistler-Durso column, the identity of Band III was tentatively established as a mixture of 1- and neo-kestose (45).

The kestoses that have been characterized are 6-kestose(O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(6→2)-β-D-fructofuranoside), 1-kestose(O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside), and neo-kestose(O-β-D-fructofuranosyl-(2→6)-O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) (1, 8, 10, 23).

In an effort to prove further this identity, the kestoses were prepared by the action of the enzyme invertase on sucrose under acid conditions (22, 45).

A buffer solution with a pH of 4.6 was prepared by mixing together 98 milliliters of 0.2M sodium acetate and 102 milliliters of 0.2M acetic acid (25). Two and one-half milliliters of this solution were diluted with 54 milliliters of water. The pH of this dilute solution was determined on a Beckman Model 72 pH meter standardized with three solutions: 0.05M potassium acid phthalate, 0.05M sodium tetraborate decahydrate, and a phosphate buffer 0.025M in respect to

both disodium hydrogen phosphate and potassium dihydrogen phosphate (12).

The commercial Invertase Analytical preparation (Difco Laboratories, Detroit 1, Michigan) was rehydrated by the addition of 10 milliliters of distilled water. One milliliter of Invertase Analytical will cause complete inversion of 10 milliliters of a 100% sucrose solution in 1 hour at room temperature. The rehydrated enzyme preparation was not dialyzed before use.

Sixty grams of sucrose were added to the dilute solution buffered at a pH of 4.6. As soon as the sucrose had dissolved, 6 milliliters of the rehydrated Invertase Analytical preparation were added with stirring at room temperature. Every 5 minutes thereafter, 0.5 milliliter aliquots were withdrawn, and the enzyme in each aliquot was inactivated by the addition of 0.1 milliliter of 0.01M mercuric chloride (13). At the end of 45 minutes, the invertase in the main reaction flask was inactivated by the addition of 6.5 milliliters of 0.01M mercuric chloride (13). Fifteen minutes later, a slight cloudiness resulted. The solution was then ion-exchanged with Amberlite IR-120 and Amberlite IR-4B ion-exchange resins.

Three milliliters of the ion-exchanged solution were then heated in a water bath with 1 milliliter of 0.25% ninhydrin solution in 95% ethyl alcohol. At the same time 1 milliliter of the rehydrated enzyme preparation diluted with 2 milliliters of distilled water, and a blank consisting of 3 milliliters of distilled water were both tested with ninhydrin.

The neutral deproteinized solution was then concentrated to a small volume (96 milliliters) under the usual conditions.

Both the concentrated purified preparation and the aliquots were examined by paper chromatography.

Fractionation of Reference Compounds

Ten and one-half milliliters containing 6 grams of carbohydrates resulting from the action of invertase on sucrose were added to the top of a Whistler-Durso column. The column was prepared by pouring a well-washed slurry of 100 grams of an equal parts by weight mixture of charcoal and Celite into a chromatographic tube (270 by 45 millimeters in diameter).

The column was eluted with water and solvents with an increasing concentration of ethyl alcohol. The flow rate of the column was about 60 milliliters per hour. Fractions of 5 milliliters each were collected automatically on a Vanguard fraction collector (Vanguard Instrument Company, LaGrange, Illinois). Again 0.1 milliliter aliquots of the column eluates were analyzed by the phenol-sulfuric acid method. The readings obtained were plotted against the corresponding fraction number.

Preparation of Acetates of Reference Compounds and Onion Carbohydrates

The acetates were prepared according to the method of Pazur and Gordon (38).

The carbohydrate material was taken to dryness in a 125-milliliter Claisen flask. The dry material was removed from the Claisen flask by adding small portions of cold pyridine to the flask and transferring

each portion to a sample bottle. For every 3 milliliters of pyridine, 2 milliliters of cold acetic anhydride were added. The sample bottle was stoppered with a sheet of polyethylene under the screw cap. The sample bottle was well shaken before it was placed in the refrigerator. At the end of one week, the contents of the bottle were poured into 200 milliliters of ice water to destroy the excess acetic anhydride. The acetylated derivative was then extracted from the water with diethyl ether. The ether was allowed to evaporate at room temperature in a fume-hood. The crystalline derivative was then treated with decolorizing charcoal and recrystallized from boiling 95% ethyl alcohol. These crystals, still contaminated by a yellow syrup, were then dissolved in water-saturated methyl ethyl ketone. The solvent was allowed to evaporate at room temperature. The residue was taken up in chloroform, and the chloroform allowed to evaporate at room temperature. Methyl alcohol was added to the flask containing the residue from which the chloroform had evaporated. Upon the addition of the methyl alcohol, small white crystals precipitated. These small crystals were separated from the mother liquor, recrystallized from hot water, and air-dried.

RESULTS

Results of Fractionation of Carbohydrates in Onions

The initial fractionation of the carbohydrates extracted from onions on Whatman Number 3MM paper by development with n-butyl alcohol: acetic acid:water(6:1:2,v/v) for 120 hours resulted in the appearance of nine bands. The first band, labeled Band I, traveled with the standard sucrose. At the end of this time, the monosaccharides are eluted from the paper. A chromatogram of the carbohydrates extracted from onions developed under these conditions is depicted in Figure 1.

Fractionation of Band III

The homogeneity of Band III was tested on paper by the use of a number of different solvents for development of the chromatograms. Known sugars were spotted alongside Band III on these chromatograms both to establish the possible identity of Band III and to obtain information about the resolving power of the solvent. The R_s values of Band III and the known sugars obtained on development with the various solvents are given in Table I.

The homogeneity of Band III was further tested by means of a Whistler-Durso column. The column on which the carbohydrates extracted from the onions were adsorbed was eluted with 500 milliliters water, 150 milliliters 2.5% ethyl alcohol, 1.5 liters 5% ethyl alcohol, and 1.5 liters 10% ethyl alcohol. Fractions of approximately 10 milliliters each were collected. One-tenth milliliter of each fraction was analyzed by the phenol-sulfuric acid method. However, 1 milliliter each of

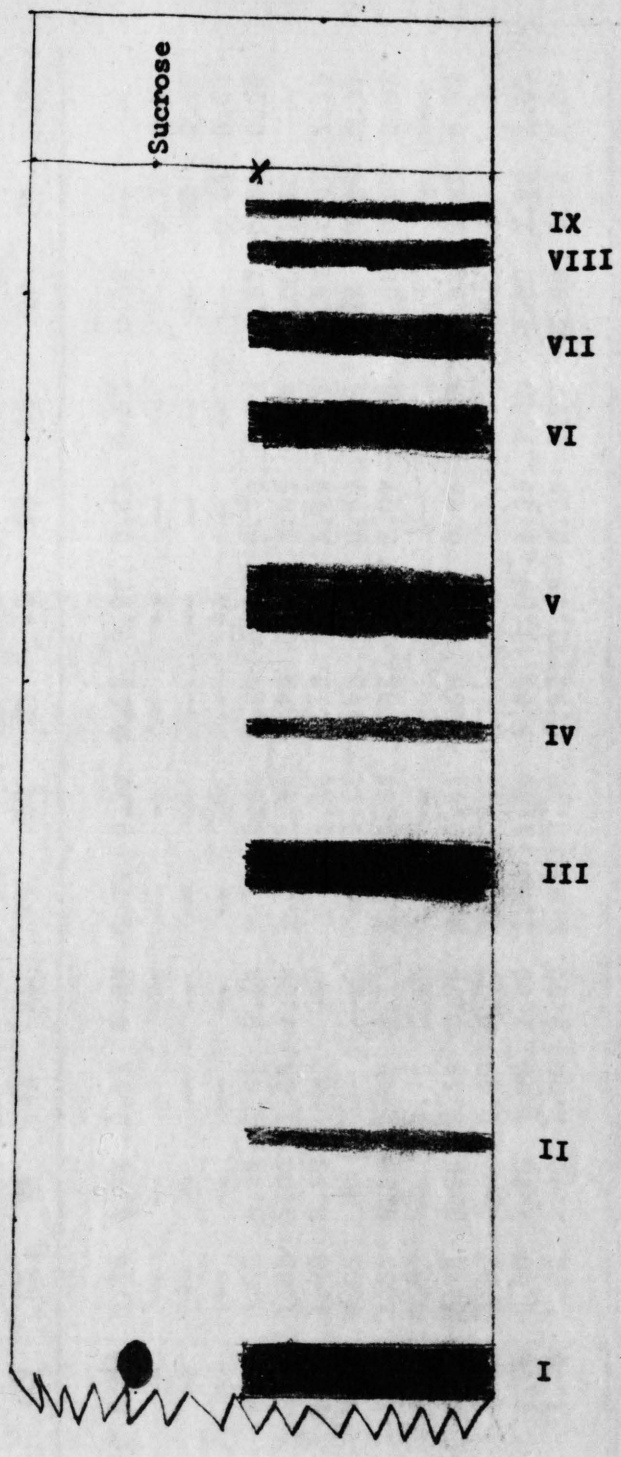

Figure 1. Chromatogram of onion carbohydrates with sucrose as standard (n-butyl alcohol:acetic acid:water[6:1:2,v/v] 120 hours, Whatman No. 3MM, p-anisidine phosphate in 50% ethyl alcohol)

Table I. R_s Values of Band III and Standard Sugars

Solvent	A		B	C	D		E		F	G		H	I	
Development time (hours)	14	85	26	72	11	24	12	27	12	23	48	23	24	59
Carbohydrates														
Band III	0.61	0.54	0.98	0.55	0.84	0.74	0.72	0.67	0.98	0.62	0.63	0.99	--	--
III_a	--	--	--	--	--	--	--	--	--	--	--	--	0.72	0.71
III_b	--	--	--	--	--	--	--	--	--	--	--	--	ND	0.77
III_c	--	--	--	--	--	--	--	--	--	--	--	--	0.86	0.84
Cellobiose	0.63	0.65	0.92	0.69	0.84	0.78	0.84	0.81	0.96	0.70	0.72	0.87	0.80	0.79
Fructose	2.13	1.95	1.03	1.69	1.30	1.37	1.56	1.72	1.11	1.45	1.34	1.06	1.30	--
Galactose	1.64	1.48	0.98	--	--	--	1.16	1.18	0.99	1.19	1.15	0.97	1.07	1.15
Impurity*	0.50	0.45	ND	--	--	--	0.55	0.45	ND	0.50	0.55	ND	0.74	0.77
Glucose	1.84	1.62	0.99	1.45	1.13	1.18	1.33	1.41	1.06	1.27	1.26	0.99	0.94	0.99
Maltose	0.74	0.72	--	0.76	0.85	0.84	--	--	--	--	--	--	--	--
Melezitose	0.61	0.46	0.96	0.55	0.76	0.70	0.64	0.59	0.92	0.56	0.63	0.93	0.81	0.78
Raffinose	0.45	0.29	--	0.29	0.73	0.64	--	--	--	--	--	--	--	--
Sucrose	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Turanose	1.00	1.02	1.00	1.00	0.93	0.94	0.98	0.97	1.03	0.94	1.02	0.99	1.12	1.04

ND - not detected. * Impurity noted in CP-Galactose.

A - n-butyl alcohol:ethyl alcohol:water(40:11:19,v/v), Whatman Number 1 paper.

B - n-butyl alcohol:ethylene glycol:water(2:1:1,v/v), Whatman Number 1 paper.

C - n-butyl alcohol:pyridine:water(6:4:3,v/v), Whatman Number 2 paper.

D - ethyl acetate:acetic acid:formic acid:water(18:3:1:4,v/v), Whatman Number 1 paper.

E - ethyl acetate:pyridine:water(8:2:1,v/v), Whatman Number 1 paper.

F - ethyl acetate:pyridine:water(10:4:3,v/v), Whatman Number 1 paper.

G - isopropyl alcohol:n-butyl alcohol:water(7:1:2,v/v), Whatman Number 1 paper.

H - isopropyl alcohol:ethylene glycol:water(7:1:2,v/v), Whatman Number 1 paper.

I - water-saturated phenol, Whatman Number 1 paper equilibrated 17 hours with phenol-saturated water.

fractions 70-115 eluted with 5% ethyl alcohol was analyzed, because these fractions were very dilute. The plot of these readings of the 5% and 10% ethyl alcohol eluates against the corresponding fraction number is shown in Figure 2.

The phenol-sulfuric acid readings showed no definite peak in either the water fractions or the 2.5% ethyl alcohol fractions. On chromatographic analysis of these fractions on Whatman Number 1 paper irrigated with n-butyl alcohol:acetic acid:water(6:1:2,v/v) for 24 hours, they were found to contain a mixture composed of only the monosaccharides, glucose and fructose. These fractions were then discarded.

Fractions 1 through 60 eluted with 5% ethyl alcohol were analyzed by paper chromatography under the same conditions. The first small peak detected by the phenol-sulfuric acid readings extending from fractions 1 through 7 was too weak to be detected by paper chromatography. Sucrose was found to be present in fractions 20-50. Fractions 70 through 115 were not analyzed by paper chromatography because they were so dilute that they required concentration before analysis by this method.

The two peaks eluted with 10% ethyl alcohol were analyzed by paper chromatography to decide which one corresponded to Band III. Fractions 130 through 160 were each spotted 20 times 1 inch apart on 2 sheets of Whatman Number 1 paper. Glucose, fructose, sucrose, and Band III (isolated from paper) were included as standards on each chromatogram. These 2 chromatograms were developed with n-butyl alcohol:acetic acid: water(6:1:2,v/v) for 72 hours. After air-drying, they were sprayed with p-anisidine phosphate in 50% ethyl alcohol and heated at 95° C. to develop

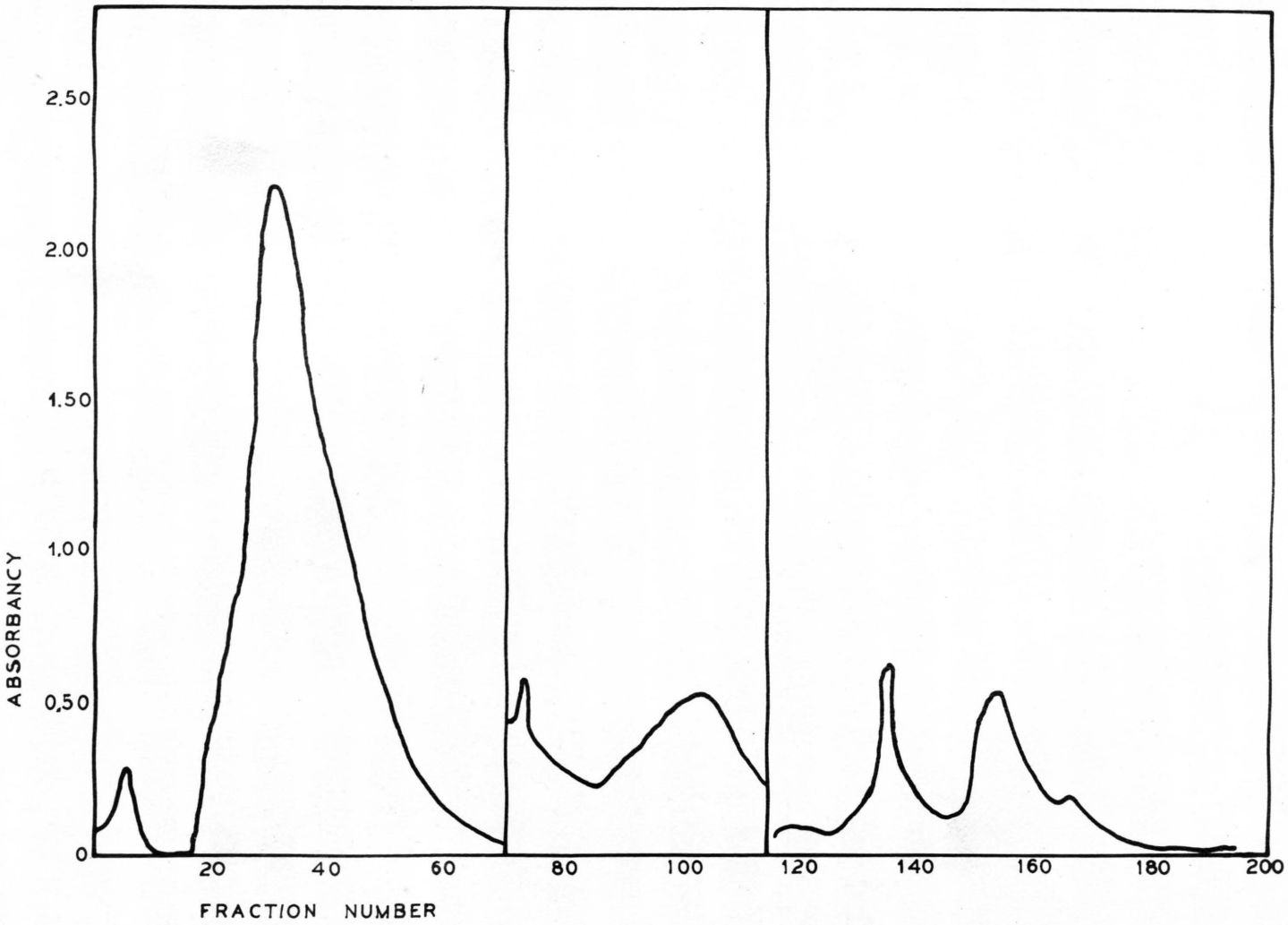

Figure 2. Onion carbohydrates. Phenol-sulfuric acid readings of 5% and 10% ethyl alcohol eluates from Whistler-Durso column

colors at the sites of the sugars. The appearance of these 2 chromatograms is shown in Figures 3 and 4. These chromatograms showed that the major component of both peaks traveled with the same R_s value as Band III in this solvent. The first peak extending from fractions 132 through 139 seemed to contain only one substance. However, the second peak extending from fractions 151 through 160 was contaminated with some material which has a lower R_s value than Band III.

In order to establish any correlation that may exist between the two peaks detected in the 10% ethyl alcohol column eluates by the phenol-sulfuric acid method, and the three spots which result on development of Band III (isolated from paper) with water-saturated phenol, the column eluates containing the components of Band III were again analyzed by paper chromatography with water-saturated phenol as solvent. Fractions 130 through 160 were each spotted 1 inch apart on 2 sheets of Whatman Number 1 paper. Sucrose and Band III (isolated from paper) were included as standards on each chromatogram. These two chromatograms were equilibrated with phenol-saturated water for 16 hours before development with the water-saturated phenol for 58 hours. After development with this solvent, it is important that the chromatograms air-dry for at least 36 hours as the phenol interferes with the color produced by the spray. The sugars were again detected by p-anisidine phosphate in 50% ethyl alcohol. The appearance of these two chromatograms is shown in Figures 5 and 6.

The first peak eluted with 10% ethyl alcohol composed of fractions 132 through 139 corresponded to the spot termed III_a. Fractions 140

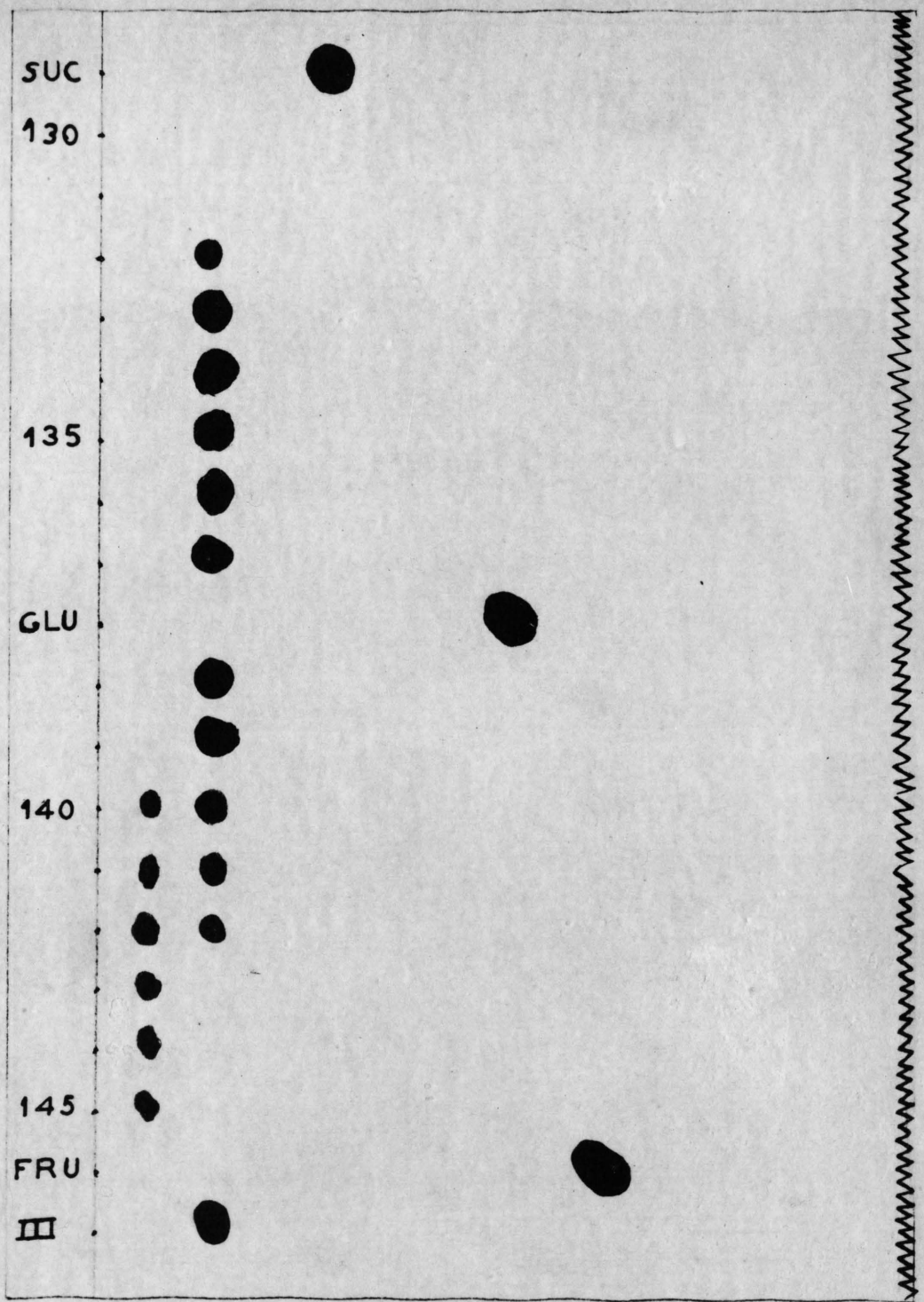

Figure 3. Chromatogram of fractions 130-145 eluted with 10% ethyl alcohol including sucrose (suc), glucose (glu), and fructose (fru) as standards (n-butyl alcohol:acetic acid:water [6:1:2,v/v], 72 hours, p-anisidine phosphate in 50% ethyl alcohol, Whatman Number 1)

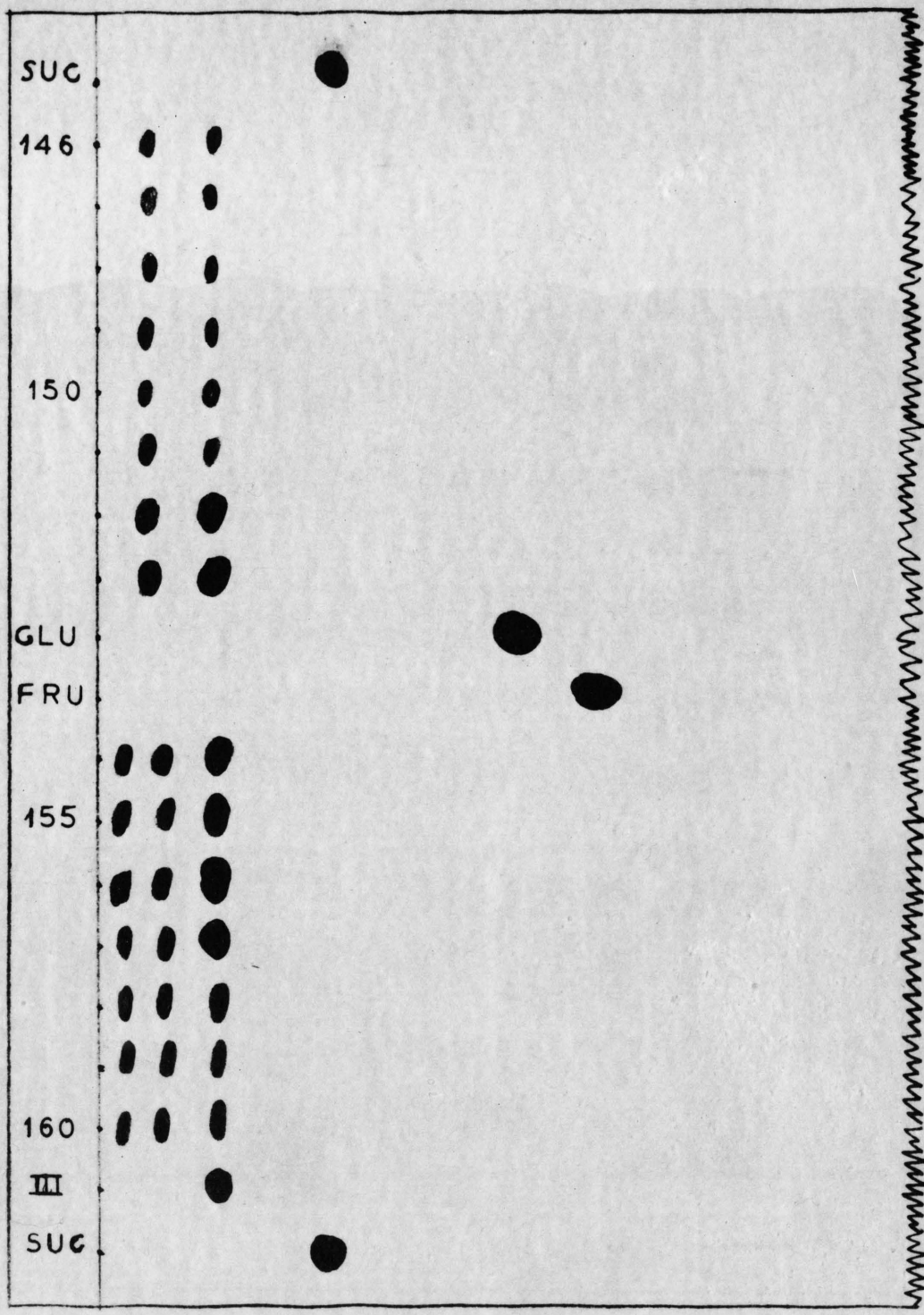

Figure 4. Chromatogram of fractions 146-160 eluted with 10% ethyl alcohol including sucrose (suc), glucose (glu), and fructose (fru) as standards (n-butyl alcohol:acetic acid:water [6:1:2,v/v], 72 hours, p-anisidine phosphate in 50% ethyl alcohol, Whatman Number 1)

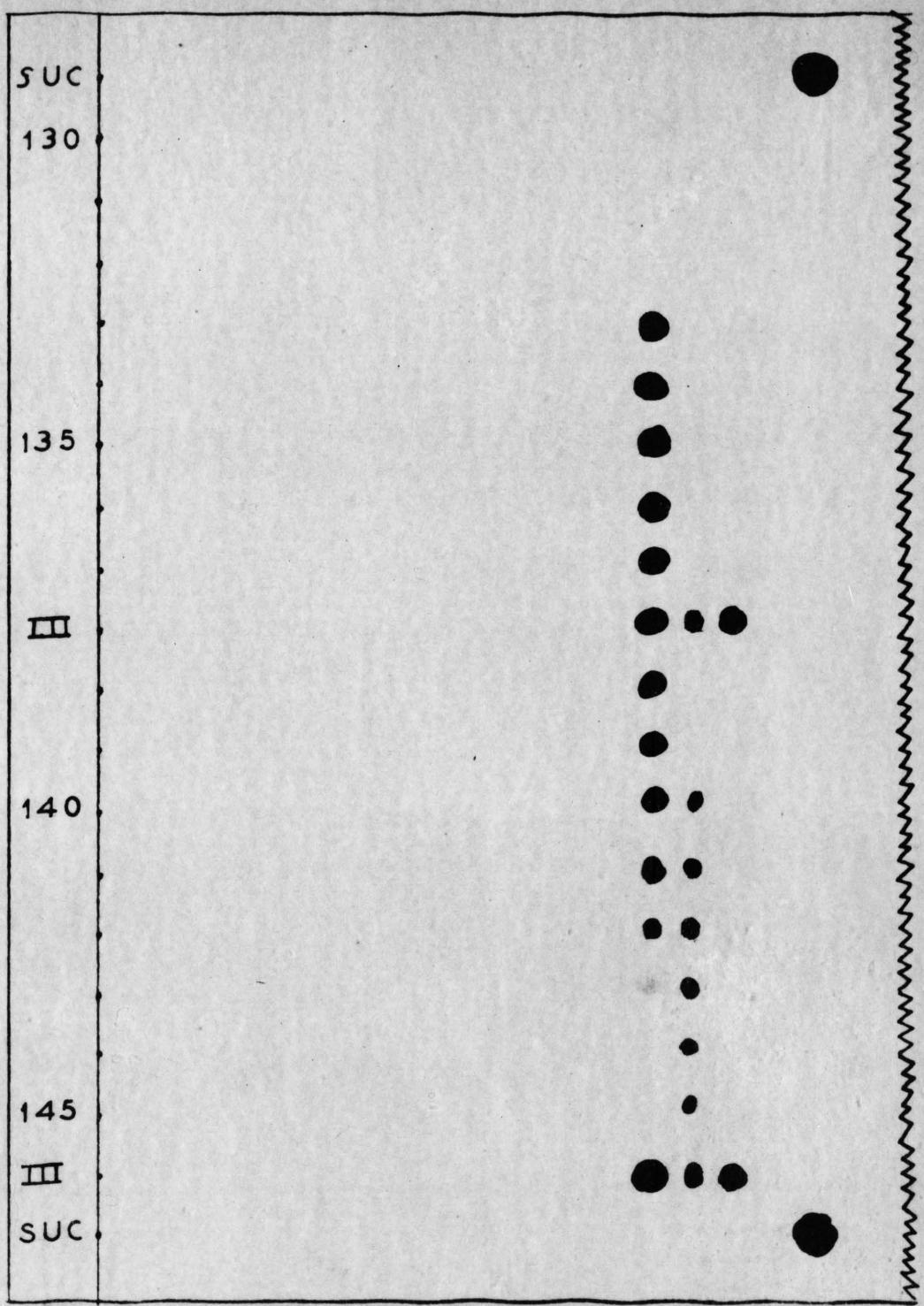

Figure 5. Chromatogram of fractions 130-145 eluted with 10% ethyl alcohol including sucrose (suc), glucose (glu), and fructose (fru) as standards (water-saturated phenol, 58 hours, p-anisidine phosphate in 50% ethyl alcohol, Whatman Number 1)

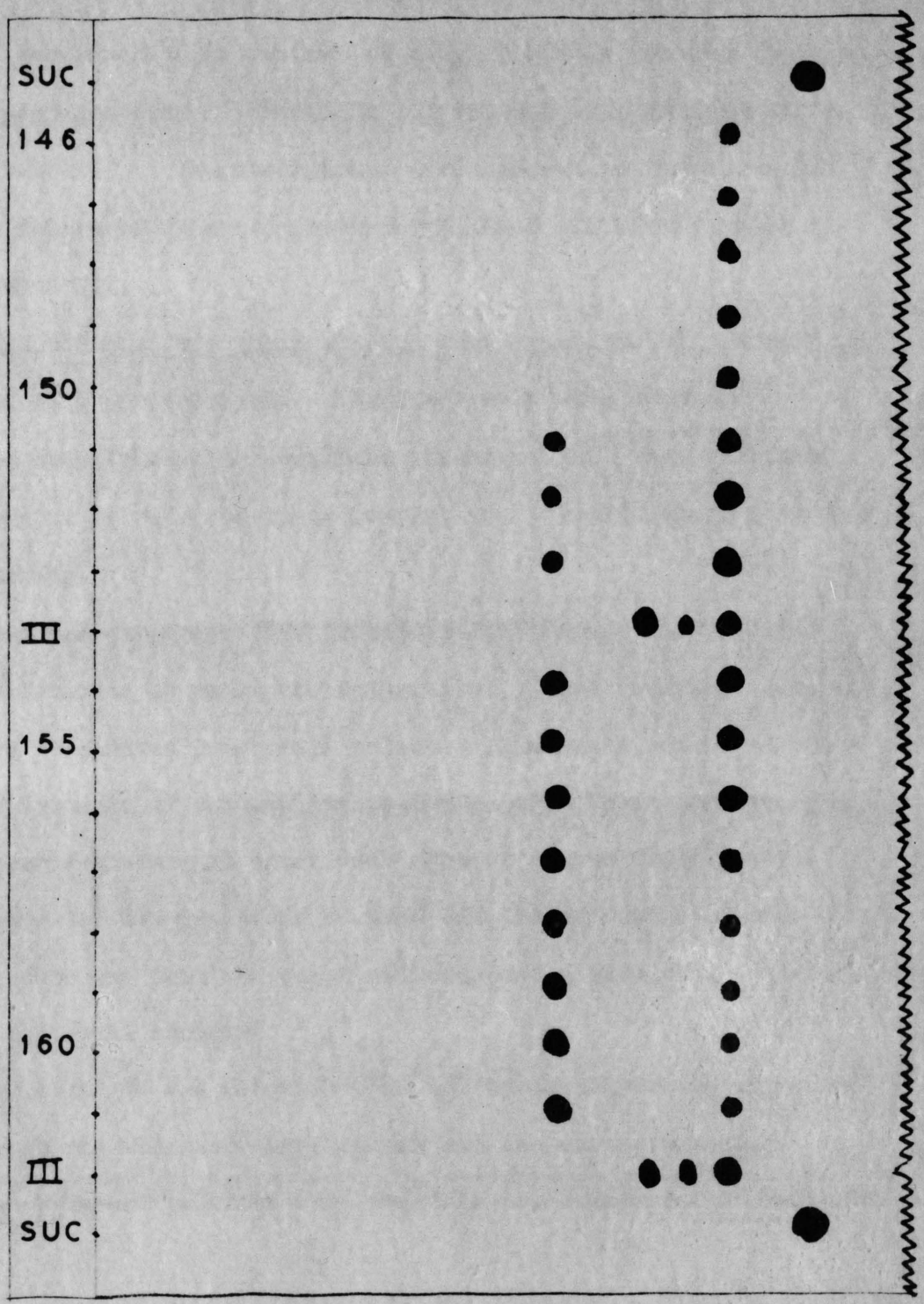

Figure 6. Chromatogram of fractions 146-160 eluted with 10% ethyl alcohol including sucrose (suc), glucose (glu), and fructose (fru) as standards (water-saturated phenol, 58 hours, p-anisidine phosphate in 50% ethyl alcohol, Whatman Number 1)

through 145 contained trace amounts of III_b, a barely discernible spot. The second peak composed of fractions 151 through 160 corresponded to the spot termed III_c. The contaminant also present in fractions 151 through 160 separated from the component of Band III with a lower R_s value than Band III.

It was obvious from these results that fractions 132 through 139 consisted of only one substance. Therefore they were combined.

Those fractions which contained traces of III_b were discarded since the amount of this substance present was insufficient for further characterization.

It was also apparent that fractions 151 through 160 required further purification by paper chromatography. These fractions were then combined and evaporated to a small volume. This small volume was then streaked on 4 sheets of Whatman Number 3MM paper. These chromatograms were then developed for 120 hours with n-butyl alcohol:acetic acid: water(6:1:2,v/v). The component of Band III, identified by marker strips, was then cut from the paper as bands and eluted with 80% ethyl alcohol in the small cabinet.

The results of the fractionation of the carbohydrates extracted from onions on the Whistler-Durso column and the chromatographic examination of these fractions when possible are summarized in Table II.

Table II. Onion Carbohydrates. Composition of Fractions from Whistler-Durso column

Fraction number	Eluant	Components
---	Water	glucose and fructose
---	2.5% ethyl alcohol	glucose and fructose (trace amounts)
1-7	5% ethyl alcohol	not detected
20-50	5% ethyl alcohol	sucrose
70-115	5% ethyl alcohol	not analyzed by paper chromatography
132-139	10% ethyl alcohol	III_a
140-145	10% ethyl alcohol	mixture of III_a and III_b
151-160	10% ethyl alcohol	III_c and other material with lower R_s value than any component of Band III

Acid-Sensitivity of Band III

The graded acid hydrolysis of Band III was followed by the accompanying increase in reducing power with Nelson's colorimetric modification of Somogyi's method. The results of this procedure are plotted in Figure 7.

These results were checked by repeating the hydrolysis under the same conditions for a shorter time. The course of the hydrolysis was followed by paper chromatography. Aliquots labeled to indicate the time of hydrolysis in minutes were spotted 15 times on Whatman Number 1 paper. Sucrose, glucose, fructose, turanose, maltose and cellobiose were

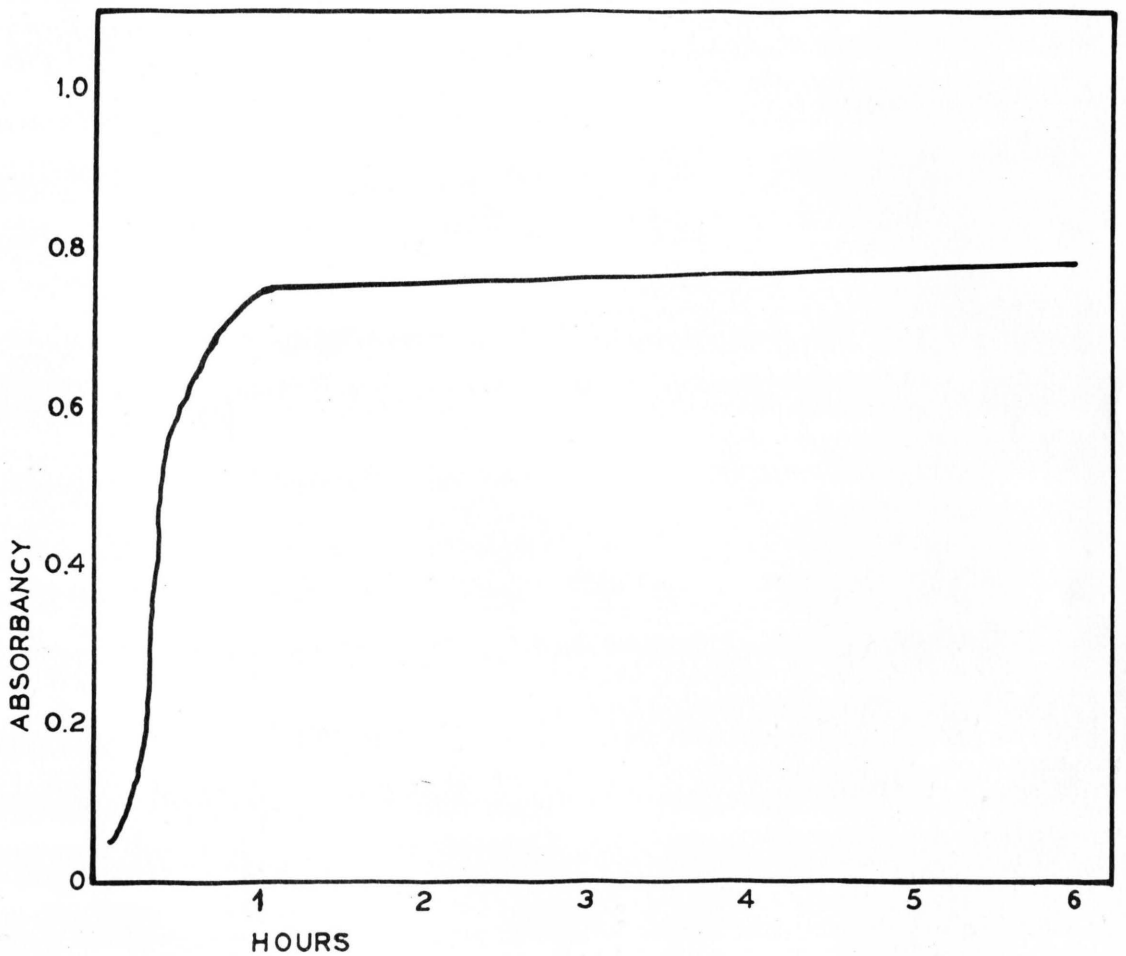

Figure 7. Colorimetric determination of graded acid hydrolysis of Band III

included as standards. This chromatogram, developed 84 hours with n-butyl alcohol:acetic acid:water(6:1:2,v/v), is depicted in Figure 8.

According to the results of the colorimetric procedure, hydrolysis of Band III was complete in 45 minutes, as the reducing power had then become constant. The chromatographic examination of the aliquots withdrawn when the hydrolysis was repeated supported this finding, since no material other than glucose and fructose could be detected after a hydrolysis time of 40 minutes.

Fractionation of Reference Compounds

The details of the fractionation on a carbon column of the carbohydrates resulting from the action of invertase on sucrose were presented in B. H. Howard's paper (28). As usual, the monosaccharides, glucose and fructose were eluted with water. Sucrose, the other disaccharides, and one trisaccharide, the 6-kestose, were found in the 5% ethyl alcohol fractions. One-kestose and a material composed of 3 fructose and 1 glucose units were present in the 7.5% ethyl alcohol eluates. The neo-kestose remained on the column until elution with 10% ethyl alcohol.

However, the elution pattern of the column in the fractionation of the carbohydrates extracted from onions denoted that the components of Band III, tentatively identified as neo- and 1-kestose, could be separated by elution with 10% ethyl alcohol.

The column on which the carbohydrates resulting from the action of invertase on sucrose were adsorbed was eluted with 1.5 liters of

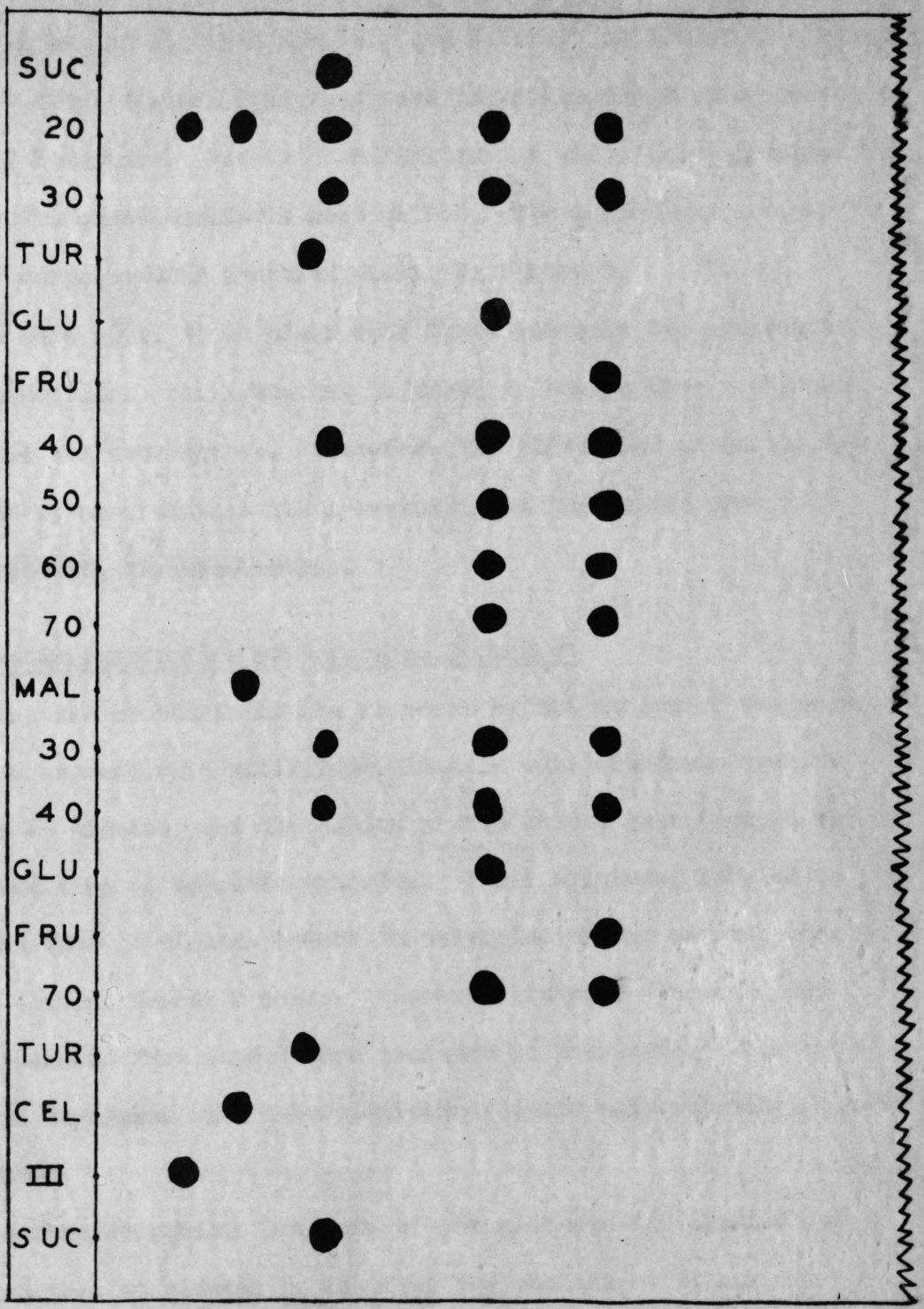

Figure 8. Chromatogram of the hydrolysates of Band III with sucrose (suc), glucose (glu), fructose (fru), turanose (tur), maltose (mal) and cellobiose (cel) as standards (n-butyl alcohol: acetic acid:water[6:1:2,v/v], 84 hours, p-anisidine phosphate in 50% ethyl alcohol)

water, 3.5 liters of 5% ethyl alcohol, and 3 liters of 10% ethyl alcohol. Only the 10% ethyl alcohol fractions were not discarded as they contained the neo- and 1-kestose. One-tenth milliliter of every third fraction was analyzed by the phenol-sulfuric acid method. These readings are plotted against the corresponding fraction number in Figure 9.

From this plot, it is clear that there are only two substances under consideration. The 1-kestose is known to emerge from a charcoal column before the neo-kestose. Therefore the first peak extending from fractions 30-90 must contain the 1-kestose, and the second peak from fractions 240-315, the neo-kestose.

Chromatographic Examination of Reference Compounds

During the synthesis of the kestoses by the action of the enzyme invertase on sucrose, 0.5 milliliter aliquots were withdrawn every 5 minutes for 45 minutes, and the action of the enzyme then stopped by the immediate addition of mercuric chloride. These aliquots, labeled to indicate the time in minutes before inactivation of the enzyme, were spotted on Whatman Number 2 paper. Sucrose, glucose, fructose, and Band III (isolated from paper) were included as standards. This chromatogram was developed with n-butyl alcohol:acetic acid:water(6:1:2,v/v) for 169 hours.

This chromatographic analysis of the aliquots was carried out to determine whether 45 minutes is too long for the enzyme to act on sucrose. Invertase completely hydrolyzes sucrose to its components, fructose and glucose. However, in the course of the hydrolysis, higher

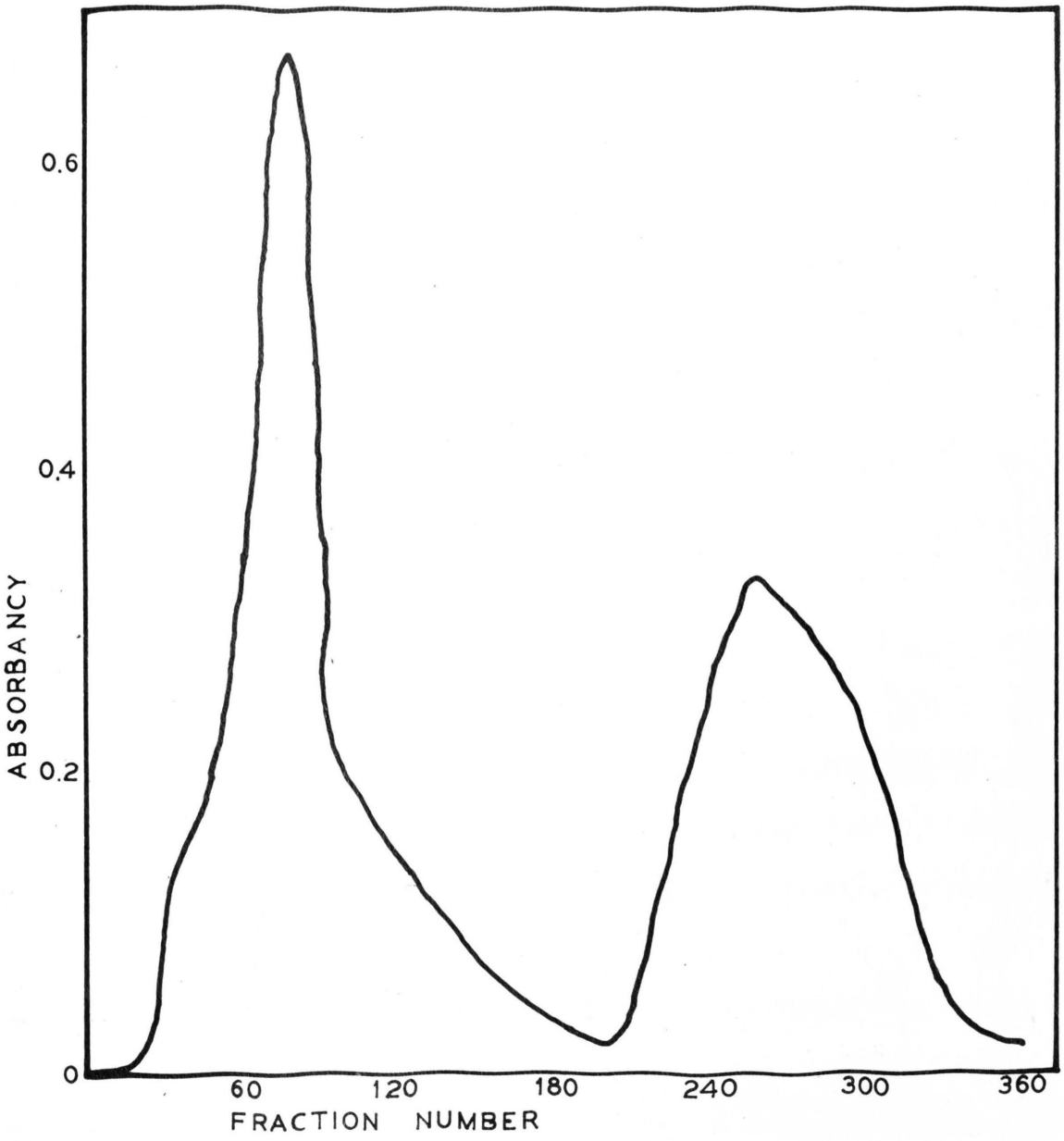

Figure 9. Invertase-sucrose carbohydrates. Phenol-sulfuric acid readings of 10% ethyl alcohol eluates of Whistler-Durso column

molecular weight products are formed (6, 9, 15, 46). These high molecular weight products are destroyed on complete hydrolysis of the sucrose (9). In the event that only glucose and fructose were present at the end of 45 minutes, the time for the maximum yield of these high molecular weight products would be known.

This chromatogram showed that there were three spots with lower R_s values than sucrose present at the end of 45 minutes, and that one of these spots corresponded to Band III.

The contents of the main reaction flask were purified by ion-exchange resins and then concentrated. This concentrated purified preparation was spotted in two places on a line 3 inches from and parallel to the long side of the sheet of Whatman Number 2 paper. One spot on this line was 1 inch from the edge of the paper. The other spot on the opposite end of the line was 3 inches from the edge of the paper. This chromatogram was equilibrated with the lower phase of n-butyl alcohol:acetic acid:water(4:1:5,v/v) for 30 minutes before development with the upper phases of this solvent for 144 hours. After air-drying, a strip 2 inches wide containing the first spot and the substances which had separated from it was cut vertically from the paper and sprayed with p-anisidine phosphate in 50% ethyl alcohol. The R_s values of the spots were calculated and compared to those in the literature (2, 20). The R_s values and the probable identity of these compounds are given in Table III.

The sheet from which the strip was cut was rotated through an angle of 90° so that the substances which had separated from the other

Table III. R_s Values and Probable Identities of Compounds Formed by the Action of Invertase on Sucrose

Spot	R_s	Probable identity
I	1.87	D-fructose
II	1.48	D-glucose
III	1.24	2- or 4-β-D-fructofuranosyl D-glucose
IV	1.00	sucrose
V	0.76	6-O-β-D-fructofuranosyl-D-glucose
VI	0.56	O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(6→2)-β-D-fructofuranoside
VII	0.44	O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside and O-β-D-fructofuranosyl-(2→6)-O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside
VIII	0.21	material containing glucose and fructose in a ratio of 1 to 3

spot on the first development would lie on a line parallel to and 3 inches from the short side. The paper was folded along this line, replaced in the cabinet and developed a second time with n-butyl alcohol: acetic acid:water(6:1:2,v/v). This two-dimensional chromatogram showed that the separation achieved with the first solvent was lost with the second.

Chromatographic Examination of Neo- and 1-Kestose

Fractions 30 through 90 were combined and concentrated to a small volume under the usual conditions. Fractions 240 through 315 were handled in the same manner. These two fractions were then each spotted on strips of Whatman Number 1 filter paper 1.5 inches wide and 12.5 inches long. The spots were applied 1 inch from the short side of the strips. These strips were put in the Gordon-Misco cabinet and developed

with isopropyl alcohol:n-butyl alcohol:water(7:1:2,v/v) for 4 hours. After air-drying and spraying, these chromatograms showed that both these substances were homogeneous.

The neo- and the l-kestose were spotted on Whatman Number 1 paper, together with sucrose and Band III. This chromatogram was equilibrated with the phenol-saturated water for 17 hours and 30 minutes before the development with water-saturated phenol for 58 hours. The spots were detected with p-anisidine phosphate in 50% ethyl alcohol. The R_s values of the reference compounds and of the components of Band III are given in Table IV.

Table IV. R_s Values of Band III and Reference Compounds

Substance	R_s
Band III_a	0.78
Band III_c	0.96
l-kestose	0.75
neo-kestose	0.93
sucrose	1.00

Melting Points of the Acetates

Melting points of the acetates of the neo- and l-kestose were taken on a Fisher-Johns melting point apparatus, because the quantity of the derivatives was insufficient for a melting point determination using a capillary tube and the Thiele apparatus. The melting points of the acetates are given in Table V. The appearance of the neo-kestose acetate changes at 38° C., and the sample slowly decreases in size until the

melting point at 56-58° C.

Table V. Melting Points of Acetates

Compound	Melting point (° C.)
l-kestose acetate (from onions)	41-43
l-kestose acetate (reference compound)	41-42.5
Mixed l-kestose acetate from onions and reference compound	42-43.5
Neo-kestose acetate (from onions)	56-58
Neo-kestose acetate (reference compound)	56-58
Mixed neo-kestose acetate from onions and reference compound	56-58

DISCUSSION

Paper chromatography on Whatman Number 3MM filter paper with n-butyl alcohol:acetic acid:water(6:1:2,v/v) as irrigant failed to resolve Band III into its components.

Separation of the components of Band III was effected by a charcoal-Celite column described by Whistler and Durso. The 1-kestose emerged from the column in a high state of purity. The neo-kestose was contaminated with some material distinct from Band III; consequently, it had to be purified by paper chromatography before the preparation of a derivative.

The fractionation on a Whistler-Durso column of the reference compounds prepared by the action of invertase on sucrose yielded the neo- and 1-kestose in quantities sufficient for chromatographic examination and preparation of the acetates.

Although both these substances are non-reducing, the method used for the preparation of the acetates of the reducing sugars was chosen because these compounds are acid sensitive. It is not known whether these compounds are fully acetylated, because the amounts of the acetates were insufficient for a determination of total acetyl groups present.

Contrary to the reports in the literature that neo- and 1-kestose do not separate on paper by development with any known chromatographic solvent, they do separate in water-saturated phenol, with R_s values of 0.93 and 0.75, respectively. Unfortunately, this solvent is not

suitable for product isolation on a preparative scale because it has a corrosive action on paper. The R_s values obtained with water-saturated phenol are dependent on the temperature. If the temperature falls below 20-25° C., the spots have a tendency to become elongated and distorted.

The nature of the third substance which travels between the neo- and 1-kestose in water-saturated phenol is unknown. Since the neo- and 1-kestose were not known to separate in any known chromatographic solvent, the existence of compounds other than these two substances has been an open question.

By means of paper and column chromatography, Bacon claimed to have isolated neo- and 1-kestose from onions extracted with boiling water, followed by the addition of an equal volume of ethyl alcohol, and removal of any precipitate by filtration. The extract was evaporated to dryness under reduced pressure and dissolved in water. The water extract was fractionated on a Whistler-Durso column. He found that the pattern of elution of the oligosaccharides from charcoal-Celite was complex. In his paper it was reported that the 1-kestose emerged from the Whistler-Durso column in a high state of purity, whereas the neo-kestose was contaminated by additional material. He stated that the behavior of the 1- and neo-kestose isolated from onions was chromatographically indistinguishable from that of the known reference compounds. However, no R_s or R_f values with any solvent were given. He said that the infrared spectra of these materials were examined and compared with those of authentic specimens. No spectra of any compounds were shown in

the paper. No characterization or physical constants of these compounds were reported (7).

SUMMARY

Neo- and 1-kestose were isolated from mature onions of the Yellow-Globe Danvers variety.

These substances were tentatively identified by the fact that they had an R_s value of 0.62 in isopropyl alcohol:n-butyl alcohol: water(7:1:2,v/v).

The known compounds, neo- and 1-kestose, were prepared by the action of invertase on sucrose under acid conditions. They were separated on a Whistler-Durso column.

The reference compounds and the compounds of Band III from onions were examined by paper chromatography in water-saturated phenol. The R_s values of the neo-kestose (IIIc) and 1-kestose (IIIa) are 0.93 and 0.75, respectively.

The acetates of the components of Band III and the reference compounds were prepared according to the procedure of Pazur and Gordon. Their mixed melting points were as follows: 1-kestose acetate, 42-43.5° C., and neo-kestose acetate, 56-58° C.

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