THESIS

presented for the

DEGREE OF DOCTOR OF PHILOSOPHY

IN THE

UNIVERSITY OF LONDON

by

TIMOTHY GEORGE JEFFORD

1963

Department of Plant Physiology,
Imperial College of Science & Technology,
London, S.W.7.

Studies on the Physiology and Enzymology of Fructosan Utilization in the Tuber of *Helianthus tuberosus* L.

Abstract

It is shown that polymerized fructose present in tubers of Helianthus tuberosus L. is utilized in the growth of new plants; over a few weeks, tubers lost 80% of their dry weight, fructose accounting for 80% of this loss. The translocation of material from the tuber was accompanied by depolymerization of fructosan; this was consistent with the assumption that carbohydrate was transported in the form of sucrose. Depolymerization did not occur in tubers at 20°C which were not translocating material, but could be induced in dormant tubers by storage at low temperature (2-8°C).

The mechanism of depolymerization in both these circumstances was examined, and it is shown to be the same in each case; the evidence is consistent with the hypothesis that the first stage in the process is the liberation of free fructose from the fructosan.

Two enzymes which catalyse the hydrolysis of fructosans found in the artichoke tuber were separated from tuber extracts. Their specificity appeared to be confined to the hydrolysis of the β2→1 linkage between a terminal fructose and its adjacent residue, but in addition, the rate at which a particular sugar was attacked depended to a large extent on the number of residues it contained. The characteristics of one of these enzymes were investigated in detail, with particular reference to its possible role in the living plant: no properties were found which would preclude its being active in vivo; an important

feature was found to be inhibition by sucrose which could represent a possible factor in the control of fructosan breakdown.

Abbreviations and Terminology

$[\alpha]_D^{20}$	specific optical rotation. The rotation of the D-line of sodium light by a 1 decimeter length of a 100 mg./ml. solution at 20°C.
ATP	adenosine triphosphate
D.P.	degree of polymerization
D.Wt.	dry weight
F	fructose
F6P	fructose-6-phosphate
F.Wt.	fresh weight
G	glucose
Gal	galactose
K_i	inhibitor constant; equilibrium constant of the reaction $E + I \rightleftharpoons EI$
K_m	Michaelis constant; concentration of substrate at which rate of reaction is half the maximum rate.
MPA	metaphosphoric acid
mU	milliunit(s) of enzyme activity; 1 unit = the amount of enzyme which will transform 1 µmole of substrate per minute.
PEAW	n-propanol:ethyl acetate:water
R_f, $R_{sucrose}$ etc.	Chromatographic mobility : distance moved by a compound as a proportion of that moved by the solvent front (R_f) or by a reference compound, e.g. sucrose ($R_{sucrose}$)
R.S.) T.R.S.)	(total) reducing sugar

TCA trichloracetic acid

UDPG uridine diphosphateglucose

...

Helianthus tuberosus L. is referred to as the Jerusalem artichoke or artichoke.

Nomenclature of sugars

Trivial names in common use, such as inulin, sucrose and raffinose, have been used in the text. Other sugars have been given trivial names; thus O-β-D-fructofuranosyl(2→1)O-β-D-fructofuranosyl(2→←1)-α-D-glucopyranoside is 1^F-fructosylsucrose, and O-β-D-fructofuranosyl(2→1)O-β-D-fructofuranosyl(2→1)O-β-D-fructofuranosyl(2→←1)-α-D-glucopyranoside is $(1^F\text{-fructosyl})_2$sucrose. Similarly, O-β-D-fructofuranosyl(2→1)-D-fructose and O-β-D-fructofuranosyl(2→1)O-β-D-fructofuranosyl(2→1)-D-fructose are described as 1-fructosylfructose and $(1\text{-fructosyl})_2$ fructose. A single sugar has sometimes been described by more than one name, where alternatives give better indications of particular aspects of their structure; thus 1^F-fructosylsucrose is also described as inulose-1; the relationship between these names and the structure of the sugars is explained between pages 76 and 80.

Contents

Abstract	i
Abbreviations and Terminology	iii
Introduction	1
Materials and Methods	6
Experiments and Results	17
Discussion	146
Publications	189
Acknowledgements	190
Literature Cited	191

Introduction

The Jerusalem artichoke tuber contains a series of β-D-fructofuranosides, each member of which has the general formula G~F - Fn, where G~F represents a sucrosyl group and n may be any number from zero (sucrose) to about 35 (inulin). The fructose residues are all 2:1' linked, giving straight chains. A chromatogram of an extract from artichoke tubers is shown in Fig. 1; it has been shown that the spot labelled GF_2 in this figure is 1^F-fructosylsucrose (Bacon & Bell, 1953; Allen & Bacon, 1956), and it is assumed that n increases by the addition of one fructose residue per member; this is supported by the fact that when the chromatographic mobilities are plotted in the form of $\log_{10} \frac{R_f}{1-R_f}$ against the assumed D.P. up to 10, a straight line is obtained (see Fig. 21); this is consistent with their being members of a homologous series (French & Wild, 1953). Each spot on paper chromatograms seems to be represented by a single substance (Bacon, 1960), and glucose-free fructose chains could not be detected by Quillet (1957).

Bacon & Edelman (1951) state that, apart from cell wall materials, the tubers contain no other carbohydrates in significant amount; this impression has also been gained in the present work in spite of the report that Jerusalem artichoke tubers form a convenient source of stachyose (6^{Gal} - galactosylraffinose); this opinion is ascribed to Shallenberger (anon., 1962), but no account is given of the less convenient sources.

Although work on changes in the series of fructosides in the artichoke tuber during winter storage has been reported, the significance of these compounds in the economy of the plant has not been examined,



Figure 1. Chromatogram of extract from artichoke tuber. Run in n-propanol:ethyl acetate:water (6:1:3 v/v) for 2 days and sprayed with urea-MPA (see Materials & Methods).

and it was possible that, even though not metabolically inert, they were merely by-products of carbohydrate metabolism which could not be utilized in further growth. Experiments were performed to examine this question, and the results showed that most of the polymerized fructose of the tubers is lost during growth of daughter plants, and that much of it provides substance for the growing tissue. This being so, later work was directed towards elucidating the mechanism of utilization, which appeared to involve an initial depolymerization of fructosan.

It is well known that there is a gradual decrease in the average D.P. (degree of polymerization) of the fructosides in the tuber during winter storage, and that this is accompanied by a change in the optical rotation of the juice. Thus, inulin cannot be identified in the juice after about December (Belval & Chollet, 1948) while the optical rotation changes from negative to positive.

Bacon & Loxley (1952) showed by chromatography of extracts that the change in rotation is associated with an accumulation of material of low D.P. at the expense of material of high D.P. The optical rotation of extracts from tubers was therefore used in the present work to follow the effect of various factors on depolymerization.

Examination of changes which occurred in the carbohydrate series during the growth of daughter plants showed that these were compatible with the hypothesis that the hydrolytic β-D-fructofuranosidase described by Edelman & Bacon (1951) was involved in the breakdown of polymerized material. A study of this enzyme was therefore carried out; it was extracted from dormant tubers and on purification separated into two

fractions; the properties of the larger fraction were investigated, with particular emphasis on characteristics which might be relevant to its rôle in the plant.

MATERIALS AND METHODS

Materials

Artichoke tubers of unknown variety were obtained from The Botanical Supply Unit of the University of London. Various other plants were collected in the field for the extraction of fructosans.

A culture of Bacterium subtilis was kindly supplied by Dr. A.H. Dadd of this Department.

Chemical reagents were obtained commercially, bacteriological or Analar grades being used wherever possible.

Centrifugation

M.S.E. "Minor", "Magnum" and Refrigerated "High Speed 13" machines were used.

Measurement of pH

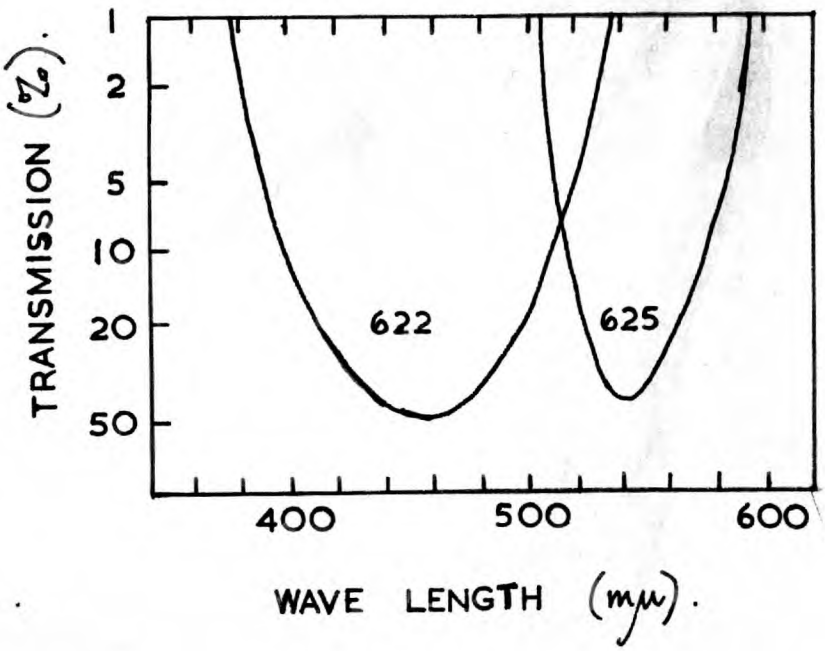
Approximate measurements of pH were made by using B.D.H. wide range, and narrow range pH indicator papers, or B.D.H. "Universal Indicator". Precise measurements were made on a pH meter* calibrated with standard buffer solutions.

Polarimetry

Optical rotation of solutions was determined in a polarimeter (Adam Hilger Ltd., No. M.S. 306/30248) which could be read to $\pm$ 0.01°. Readings were made in a controlled temperature dark room at 20°C, using a Sodium lamp as the light source, and estimations of the zero correction and of the rotation of a standard (5% w/v) sucrose solution were always included.

---

* Cambridge pH indicator, Cambridge Instrument Co. Ltd., London and Cambridge.



Absorption curves of Ilford light filters.
Data copied from brochure (F.105), published by
Evans Electroselenium Ltd., Harlow, Essex.

Colorimetry

Colorimetric determinations were carried out using an Evans Electroselenium colorimeter (EEL) and Ilford filters. Standards and blanks were included with all estimations.

Dry Weight determination

The tissue was sliced into tared metal trays and placed in an oven at 61°C for 24-30 hr. The trays were allowed to cool to room temperature in a desiccator, weighed and replaced in the oven. The treatment was repeated until constant weight was reached.

Extraction of soluble carbohydrate

The tissue was sliced into boiling 80% v/v aqueous ethanol; the boiling was continued for at least a further 5 min. It was assumed that this treatment prevented significant alteration of the carbohydrates by post mortem enzyme action. The supernatant liquid was decanted, and the slices extracted three times with boiling distilled water, and the four extracts filtered through glass wool and combined. The pH of the extracts was in the range 5.8 - 6.5 whenever tested. This procedure was considered to effect practically quantitative extraction, as a preliminary investigation showed that for every 1000 mg of combined fructose extracted in this manner, three subsequent extractions with boiling distilled water extracted only 44, 14 and 8 mg respectively. The combined extracts were made up to volume in volumetric flasks, a preliminary reduction in bulk sometimes being necessary.

Concentration and Evaporation of Solutions

Plant extracts of large volume were reduced in bulk by

evaporation using a hot plate or infra-red lamp and a current of air from a fan. The pH was checked by using B.D.H. narrow-range indicator papers in case of pH drift - adjustment of the pH was rarely necessary. Final evaporation to dryness was carried out over anhydrous $CaCl_2$ in a vacuum desiccator. Concentration of sugars separated by column chromatography (see p. 11) was achieved in a Gallenkamp rotary vacuum evaporator, the solutions being taken to dryness in the apparatus.

Determination of Sugars

Standard solutions of glucose or fructose containing 5mg/ml were made up in water saturated with benzoic acid (Folin, 1929). These were diluted with water saturated with benzoic acid to give concentrations of 50 µg/ml.

Reducing sugars were estimated colorimetrically by the Somogyi (1952)-Nelson (1944) method, using a 625 light filter. The sample for analysis containing 20-70 µg reducing hexose in 1.0 ml was mixed with 1.0 ml copper reagent and heated at 100°C for 10 min. After cooling, 1.0 ml arsenomolybdate was added, and the solution made up to 10 ml.

Total fructose, i.e. free and combined, was estimated by Cole's method (described by Bacon & Bell, 1948), in which the colour produced was measured using a 622 light filter. The sample containing 20-100 µg fructose in 3 ml was mixed with 5 ml 0.15% w/v resorcinol in absolute ethanol and 5 ml concentrated HCl containing 7.5 mg $FeCl_3$ per litre. The solution was heated for 30 min in a water bath controlled at 78 ± 1°C and then removed and cooled. The reagents were measured using polythene "rapid dispensers" ("E-MIL" Works, Pontypridd, Glam.);

it was found that the polythene vessels did not interfere with either of the reagents, and the measurement of volume was sufficiently accurate and consistent. The colour produced by free glucose in this estimation was less than 1% of that given by an equal weight of free fructose.

The glucose oxidase-peroxidase method of Huggett & Nixon (1957) was used for the estimation of free glucose in the presence of free fructose. In the first case estimations were carried out with the glucose sample (20-100 μg) in 0.2 ml together with 2.5 ml of reagent. It was later found more convenient to use a sample volume of 1.0 ml and 3.0 ml of reagent. After mixing, the digest was put in a water bath controlled at 35-37°C, and the colour which developed was measured using a 622 filter. On different occasions, incubation times of 30, 45 and 60 min were used; a preliminary investigation of the time course of the reaction showed that the EEL readings were proportional to the glucose concentration, at least up to 100 μg per sample, whatever the incubation time. The method was not satisfactory for the estimation of glucose in the presence of sucrose, because the glucose oxidase preparation (Sigma Chemical Company, St. Louis, Missouri, U.S.A.; lot G 107-55) was contaminated with invertase.

Estimation of soluble protein

The method of Nielsen (1958) was used. The sample of protein (0.2 - 4 mg. as casein) was pipetted into a centrifuge tube, made up to 1 ml, and 3 ml of 10% w/v trichloracetic acid added. The tubes were allowed to stand for 10 min. and then centrifuged for 10 min. at 3000 x g

in the bench centrifuge. The supernatant was discarded and the tubes drained. The precipitate was dissolved in 5 ml 5% w/v $Na_3PO_4.12H_2O$, or in 10 ml where necessary, and one drop of 4% w/v $CuSO_4.5H_2O$ added. The tubes were gently shaken for 30 min. on an automatic shaker (Baird & Tatlock, Chadwell Heath, Essex) and then centrifuged for 10 min. at 3000 x g. The supernatant was poured into fritted glass filter funnels of porosity grade "5", and the filtrate collected by suction. Samples were removed from the filtrate and made up to 3.0 ml with 5% $Na_3PO_4.12H_2O$; a few crystals of sodium diethyldithiocarbamate (DIECA) were added and the tubes allowed to stand for 20 min. after which 4 ml. of tertiary amyl alcohol was added, with vigorous mixing, to extract the colour. After separation, aliquots were removed from the amyl alcohol phase and the intensity of colour measured in a spectrophotometer ("Uvispek H.700/303; Hilger & Watts Ltd., London) at 440 mμ. The amount of protein was calculated by comparison either with Cu^{++} standards or standards of casein.

Chromatography

Descending partition chromatography of sugars was carried out either on sheets of paper or, for separation of large amounts of mixed sugars, i.e. up to 1.5 g, on a hollow cylindrical column of paper (25 to 58 x 360 mm.) fitted with a pressure mantle (LKB 3502 ChroMax, LKB Produkter, Fabriksaktiebolag, Stockholm, Sweden). Whatman's No. 1 paper was used for qualitative and quantitative chromatography, 3MM for preparative separations, and No. 54 for the resolution of mixtures containing glucose, fructose, mannitol and sorbitol. The paper in the column was Grycksbo Chromatographic No. 3, quality A, wound as a continuous roll on a cylindrical rod of polythene. Chromatograms were

loaded using graduated 0.1 ml pipettes; occasionally calibrated capillaries of 10 µl total volume (Drummond Scientific Company, Philadelphia 31, PA; distributed by Shandon's Ltd., London) were used. Chromatograms were developed in glass tanks kept in a cabinet controlled at 20°C during the earlier part of the work, and later in a controlled temperature room at 25°C: development of the paper column was carried out at room temperature. The solvents used most often were mixtures of n-propanol, ethyl acetate and water in proportions of 7:1:2 or 6:1:3 by volume. The former was used principally for sugars with DP of 1-4, which were separated satisfactorily after about 48 hr development. The second mixture was used for separation of oligosaccharides of greater DP, oligosaccharides with DP of up to 10 being separated from mixtures by extending the development time to 4-5 days. Occasionally multiple development was used. The upper phase of a 4:1:5 v/v mixture of n-butanol, glacial acetic acid and water was sometimes used, in particular when a clear separation of glucose and fructose was required (72 hr. development) but it was later found that these monosaccharides were separated faster and better by the solvent of Rees & Reynolds (1958) viz. A 9:1:1 v/v mixture of methyl ethyl ketone, glacial acetic acid, and water saturated with boric acid, which also resolved mixtures containing sugars and sugar alcohols. For the resolution of mixtures of sugar phosphates, especially G6P and F6P, the solvent (pH 3.8) described by Paladini & Leloir (1952) was used.

Charcoal columns for the separation of sugars were made from a mixture of equal weights of B.D.H. activated charcoal and Celite 535. The mixture was washed with distilled water until the initially acid

washings reached a pH of about 5.5, and then dried. Columns were set up by making a weighed amount of the mixture into a slurry with distilled water, and pouring it into a glass tube plugged with glass wool. The amount of sugar applied to columns was never in excess of 1 - 1.5 g. of mixed oligosaccharides per 20 g. of C/Celite mixture (Bacon, 1954). Monosaccharides were eluted with distilled water; oligosaccharides were eluted en masse with 25% v/v ethanol.

Columns of fuller's earth were used for the separation of certain sugar mixtures which could not be resolved satisfactorily on paper or C/Celite columns. It was found advantageous to separate the fines from the fuller's earth by decantation as the sedimented earth when mixed with about $\frac{1}{4}$ of its weight of Celite 535 gave columns with a much faster rate of flow than those made from untreated fuller's earth with celite. The columns were set up by making a slurry of the mixture in absolute ethanol, and pouring it into a glass tube plugged with glass wool. Sugars were applied to the column in a small volume of ca 50% v/v ethanol, and the column eluted stepwise with ethanol containing progressively greater concentrations of water.

The eluates from columns were examined by running samples on paper chromatograms.

Detection of compounds on paper chromatograms

Sugars were located by use of benzidine-trichloracetic acid (Bacon & Edelman, 1951); dried chromatograms were sprayed with a solution containing 0.25 g benzidine in 5 ml 40% w/v trichloracetic acid, 5 ml glacial acetic acid and 90 ml absolute ethanol. After drying in a fume cupboard, the paper was heated in an oven at 100-110°C until coloured

spots developed. Glucose gave a chocolate brown and fructose a yellow colour.

Fructose in the presence of glucose was detected by use of the Urea-metaphosphoric acid spray (Wise, Dimler, Davis & Rist, 1955): 3 g of urea was dissolved in 90 ml of n-butanol saturated with water, and 6.8 ml 80% w/v metaphosphoric acid and 5 ml absolute ethanol added. Papers were sprayed, dried and heated in an oven at 100-110°C. Fructose gave blue spots against a white background, about 5 μg being detectable; glucose in the usual amounts encountered gave no reaction, but free glucose in large quantity (ca 100 μg) gave a pale khaki colour.

Oligosaccharides with a reducing ketose group were detected by the spray described by Bacon (1959), (see also Brooks, Evans, Green, Hunt, Long, Mooney & Wyman, 1958). A solution containing 20 mg 2:5-diphenyl-3-(4-styrylphenyl)tetrazolium chloride (M & B 1767, from May & Baker, Dagenham) in 10 ml absolute ethanol was mixed immediately before spraying with 10 ml 0.1N NaOH. After a few minutes at room temperature, or with slight warming, free ketoses and oligosaccharides with a ketose reducing group appeared as purple spots on a yellow background. About 5 μg of reducing fructose is detectable, but aldoses (and non-reducing ketoses) do not react unless the chromatogram is heated strongly during removal of the solvent.

Sugar alcohols and sugars were located by spraying the paper with a solution of 0.6% w/v sodium metaperiodate in distilled water. After about 5 min the papers were sprayed with a solution of 0.1 M-benzidine in 50% v/v ethanol, acetone, and 0.2N HCl (10:2:1 v/v). The positions of sugars and sugar alcohols were immediately revealed as

white areas on a dark blue background.

Sugar phosphates were detected by the method of Burrows, Grylls & Harrison (1952). The paper was dipped in a reagent containing 1 g ammonium molybdate, 8 ml of water, 3 ml concentrated HCl and 3 ml 60% w/v perchloric acid, made up to 100 ml with acetone. The paper was removed and after evaporation of the acetone heated at 100-110°C until it showed signs of charring. Exposure to ultra-violet light showed the phosphate compounds as blue spots on a grey-brown background (Bandurski & Axelrod, 1951).

Detection of radioactivity on paper chromatograms

Radioactive areas were located approximately by scanning the paper with a monitor fitted with a Geiger-Müller tube, and precisely by exposing "Kodirex No-screen" X-ray film (Kodak Ltd.) to the chromatogram. The duration of exposure was estimated from the data that a count of 10^3 per min per 1" diameter circle at the surface of the paper gave a satisfactory blackening of the developed film after being in contact for 24 hr.

When it was desired to collect substances from paper chromatograms they were located by spraying marker strips, or if radioactive, by autoradiography. In the majority of cases the materials were very soluble in water, and elution from the paper was done by setting up the area of paper as for chromatography and developing with distilled water. The solution which dripped from the tip of the pieces of paper was collected in beakers. Blank areas of paper were also eluted in cases where the eluates were to be used in quantitative estimations. When it was necessary to elute material of high molecular weight, e.g.

which had not moved from the origin during chromatographic development, the paper was pulped using a glass rod in a test tube, and extracted 3-4 times with hot distilled water. Very large areas of paper, e.g. those collected from many chromatograms, were eluted by pulping the paper in a Waring blender with distilled water, and extracting the pulp in a Büchner funnel. Eluates were evaporated to dryness before further treatment.

Assay of ^{14}C

Estimations of radioactivity were made using a thin end-window Geiger-Müller tube (Mullard MX 123) housed in a perspex castle and used in conjunction with a Panax scaler (Type D 657; Panax Equipment Ltd., Redhill, Surrey).

Solutions or suspensions of material for counting were pipetted on to 1" or $\frac{3}{4}$" diameter duralumin plates which had previously been covered with 0.1% v/v "teepol" (Shell Chemical Company) in 50% w/v aqueous ethanol, and then dried in a current of air. The dried samples were placed at a standard distance from the end-window of the tube, and counted for such a period that at least 1000 counts were registered. The activity was expressed as counts per minute (c/m) at infinite thinness, corrected for background and for lost counts due to dead time. When applicable, corrections for self-absorption were made. Where necessary, estimations made on different occasions were compared by reference to a standard source (a 1" diameter perspex disk of 1 μc/g.; The Radiochemical Centre, Amersham, Bucks).

EXPERIMENTS AND RESULTS

Physiological Experiments

Introduction

The availability of polymerized fructose for further metabolism in artichoke tubers was examined by determining whether any change in the amount of these compounds occurs when tubers sprout. As the results given below indicated that fructosan contributes to the new growth, attempts were made to elucidate the process by which they are utilized.

Change in weight and carbohydrate content of tubers during growth of daughter plants.

Tubers of similar shape were selected from a number which had been stored for two weeks in damp sand at 2°C. The tubers were weighed and divided into three series in ascending order of fresh weight, viz. 16-21 g., 22-24 g., and 27.5-36 g.; some very small and very large tubers - fresh weights 5.0-8.0 g. and 56.5-69 g. respectively - were included in the experiment to determine the effect of the size of the tuber. The tubers were numbered and planted in random order in lots of six, each lot in a 10" earthenware pot filled with John Innes No. 1 compost, and placed in a heated greenhouse at 20 ± 2°C. Samples were removed at intervals during a period of seven weeks, one tuber being taken from each of the three groups on each occasion - members of the groups of very large and very small tubers were sampled at less frequent intervals.

Tubers were washed with tap water, dried with a towel, and the shoots and roots cut off flush with the tuber surface. The shoots and the tubers were then weighed. Roots were discarded as it was not

feasible to separate them quantitatively from the compost.

Each tuber was cut in half longitudinally, to minimize variations due to any differences at various depths, or distances from the apex, and the pieces were weighed; one half was used for dry weight determination and the other for extraction of soluble carbohydrate.

Although there was no apparent change in size of any of the tubers judged qualitatively, during growth of the daughter plants, slicing of the tubers revaled a progressive sponginess of the internal tissues. This was at first most apparent in the region a few millimeters beneath the epidermis, and at the distal end of the tuber. This sponginess became more marked as growth of the new plant continued and the affected region gradually spread towards the centre, while the outer layers of the tuber became harder. After some 4–5 weeks there was a small firm central core surrounded by a deep layer of drier stringy tissue interspersed with many large gas-filled spaces, the whole contained in a rigid outer coating (Fig. 2).

The results in Table 1 show the fresh and dry weights of the tubers, the weight of fructose extracted from them, and the fresh weight of the daughter shoots.

The progressive decrease in dry weight and corresponding loss of total fructose are shown in Figure 3, as averages of the results for the three main series, and also individually for the "smallest" and "largest" series. In this figure the dry weights at the time of sampling are expressed as percentages of the original dry weight; this was calculated from the dry weight of the tubers sampled at the start of the experiments, viz. 21.4% of the original fresh weight. It is apparent

Unsprouted tuber. Sprouted for 6 weeks at 20°C.

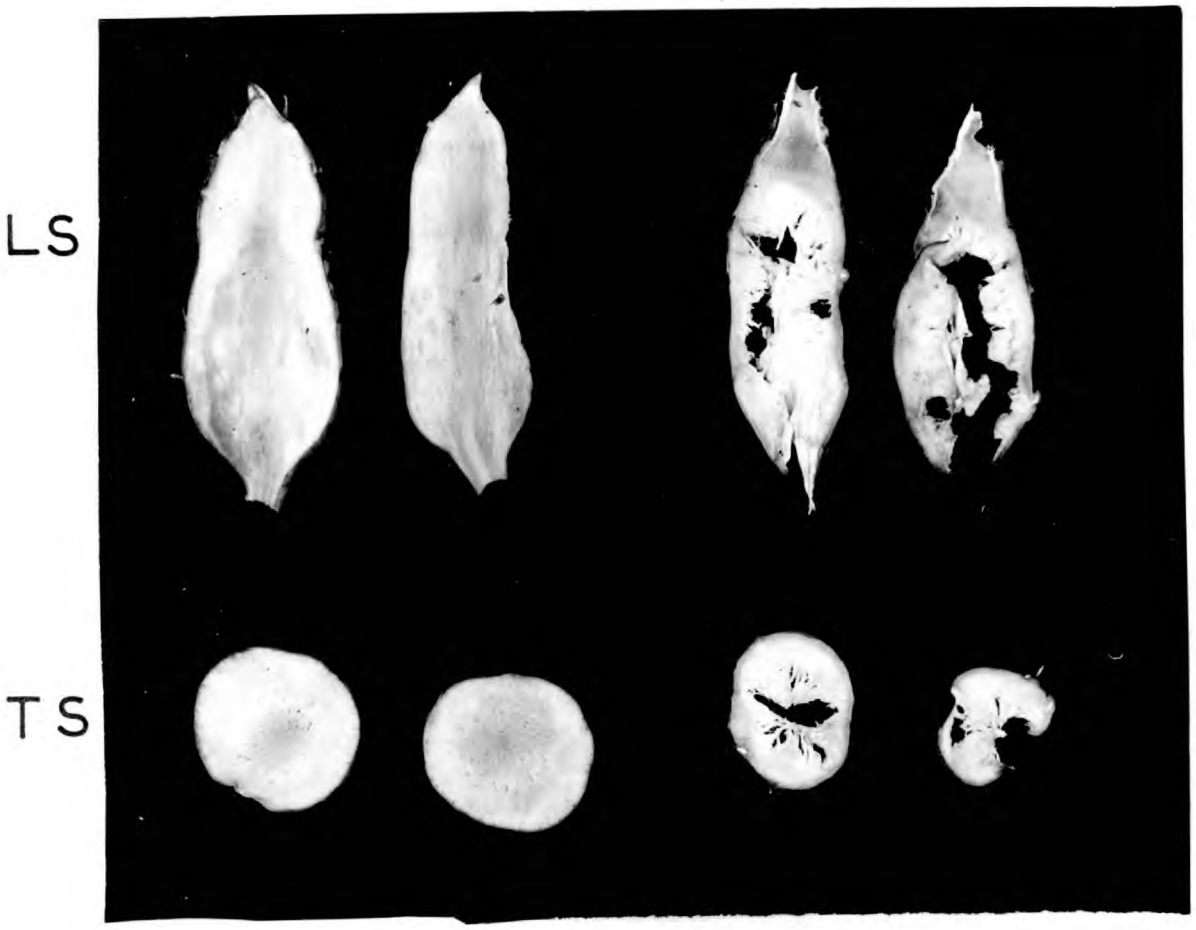


Figure 2. Appearance of sprouted tuber.

Table 1. Fresh weight, dry weight and anhydrofructose content of sprouting tubers, and fresh weight of daughter plants.

Fresh weight groups (see text) distinguished as follows:

S, 5.0–8.0 g.; A, 16–21 g.; B, 22–24 g.; C, 27.5–36 g.; L, 56.5–69 g.

Weeks from planting	Series	Original F. Wt. (g.)	Current F.Wt. (g.)	D.Wt. (g.)	F.Wt. shoot (g.)	Total Anhydrofructose (g.)
	S	6.0	5.05	1.28	–	
	A	21.0	18.5	4.60	–	2.94
0	B	22.0	19.3	4.95	–	4.12
	C	32.0	27.7	6.57	–	4.43
	L	57.5	54.1	11.9	–	
	A	19.0	18.8	3.80	1.21	2.70
2	B	22.0	21.9	4.08	2.14	2.58
	C	27.5	27.0	5.71	1.12	4.49
	S	5.5	4.29	0.44	4.2	0.33
	A	20.5	17.9	1.83	20.0	0.77
4	B	22.0	20.5	2.81	18.1	1.66
	C	36.0	31.4	4.78	17.5	2.63
	L	62.5	60.8	11.1	18.9	6.12
	A	20.0	16.8	1.21	34.1	0.41
5	B	22.5	18.1	2.37	18.3	1.42
	C	32.0	25.8	2.78	41.2	1.40
	S	8.0	4.46	0.29	14.5	0.08
	A	20.0	10.3	0.77	45.5	1.06
6	B	22.0	16.0	0.99	46.0	0.27
	C	29.5	21.9	1.60	47.5	0.56
	L	56.5	43.9	4.79	58.0	2.42
	A	18.5	6.49	0.67	51.9	0.11
7	B	24.0	8.41	0.99	43.5	0.19
	C	33.5	15.9	1.70	71.2	0.41

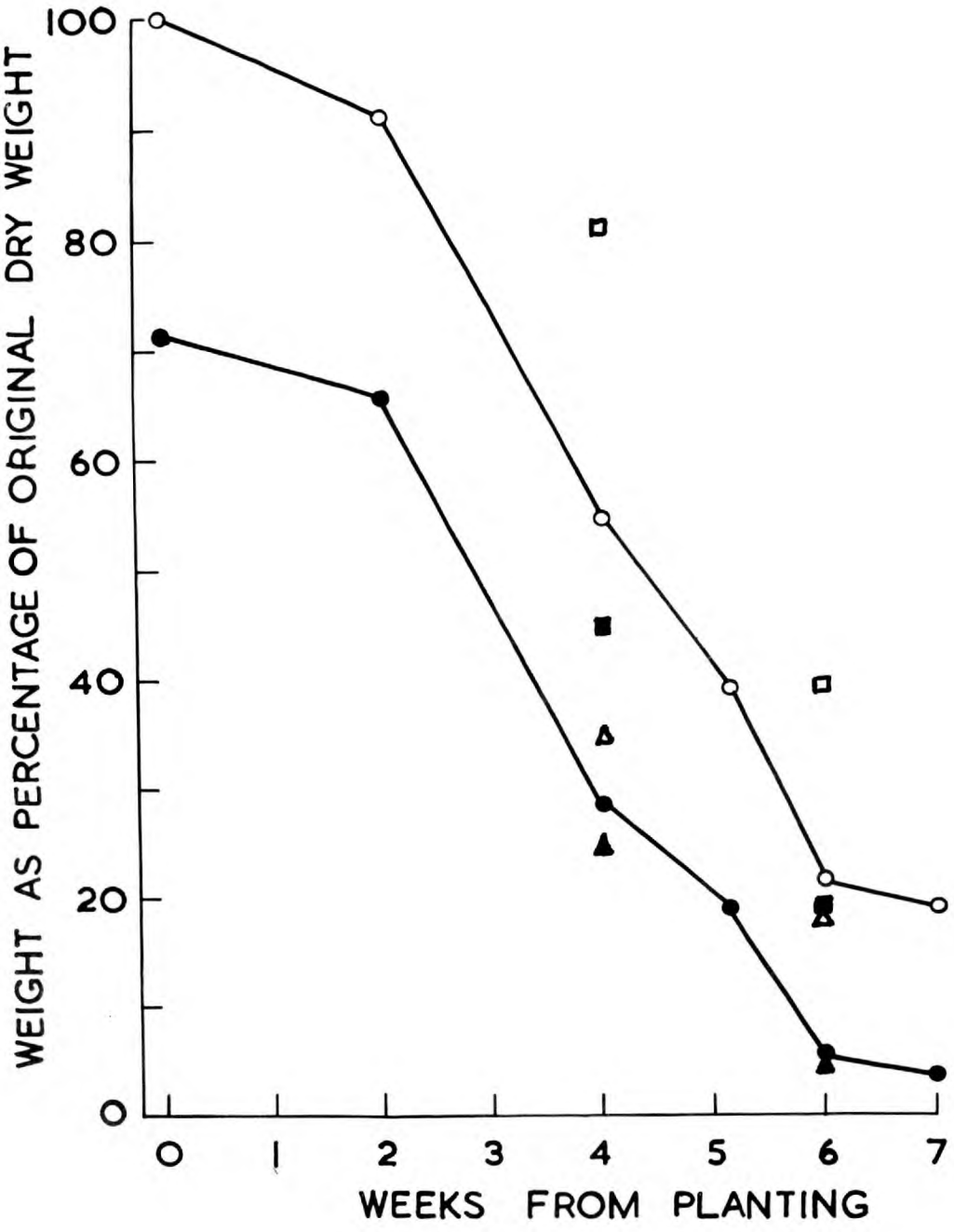


Figure 3. Changes in dry weight O, and anhydrofructose content ● of tubers. Each value is an average of three observations.

■■ large tubers ▲▲ small tubers.

from Table 1 and Fig. 3 that differences in tuber size had no significant effect on the pattern of change.

The individual fructosides in the extracts of the alternate halves of the tubers were estimated and the changes in these are shown in Table 2, in which only the fructose content of the fructosides is given. Values for the glucose content of each of the compounds of D.P. 2 - 5 can be calculated from the formula $G\sim F-(F)_n$, as "n" can be given known values - this cannot be done for the polysaccharide fraction. The most obvious change is the decrease in the fraction containing the polymer viz. 5-n, and the increase in free fructose which occurs at the time when loss of combined fructose from the polymer and from the tuber as a whole is proceeding at the maximal rate; these data are consistent with the hypothesis that the process of polymer breakdown is initiated by the hydrolytic enzyme described by Edelman & Bacon (1951).

The previous experiment indicated that there was a loss of dry weight and total fructose from sprouting tubers, and a concomitant increase in weight of the shoots, which grew rapidly during the experimental period, reaching a height of several feet by the seventh week. As the plants were grown in the light, and the roots could not be recovered quantitatively from the soil, it was not possible to discover to what extent material lost from the tuber was translocated to the daughter plants. It was necessary in drawing up such a balance sheet to prevent photosynthesis, and also to measure the dry weight of the roots. Weighed tubers, which had been stored in damp sand at 2°C for about 15 weeks, were grown in continuous darkness at 20°C. They were planted singly in 6" pots containing fine washed sand to facilitate

Table 2. Changes in individual carbohydrates present in tubers during the growth of daughter plants.

Weeks after planting	Series	D.Wt.	Free Glucose	Fructose (as mg. free F per gm. D.Wt.) DP 1	DP 2	DP 3	DP 4	DP 5	DP 6-n
	S	1.28	1.6	6.4	31.9	28.6	36.4	42.8	559.
	A	4.60	-	3.2	34.7	25.1	40.6	40.6	551.1
0	B	4.95	-	5.3	40.9	32.9	46.2	50.2	702.2
	C	6.57	-	3.9	48.5	43.7	54.4	52.4	574.3
	L	11.9	-	-	54.1	43.0	53.1	53.1	516.
	A	3.80	2.5	11.0	36.0	27.5	40.5	47.0	629.5
2	B	4.08	2.2	9.7	41.6	30.8	54.1	57.8	566.5
	C	5.71	7.2	11.5	27.9	26.4	35.6	34.1	738.0
	S	0.44	4.4	259.	70.0	35.0	43.8	31.2	399.
	A	1.83	-	240.	76.4	20.2	12.4	11.2	106.
4	B	2.81	3.3	214.	68.0	32.0	19.5	20.3	302.
	C	4.78	4.5	162.	58.6	24.1	21.8	18.0	326.
	L	11.1	1.5	110.	50.8	30.5	31.1	32.2	358.
	A	1.21	3.9	182.	72.1	26.2	14.8	14.8	68.9
5	B	2.37	0.6	183.	80.0	36.2	27.6	25.7	316.
	C	2.78	4.5	202.	92.0	36.8	23.0	26.4	181.
	S	0.29	2.0	163.	45.6	19.4	16.9	17.5	49.8
	A	0.77	2.3	144.	37.3	14.0	9.3	7.0	21.5
6	B	0.99	7.3	199.	52.0	16.2	9.3	9.0	20.6
	C	1.60	6.3	194.	76.3	26.5	17.0	16.4	57.6
	L	4.79	5.1	222.	53.5	39.4	26.6	26.6	195.
	A	0.67	17.0	60.1	42.5	8.3	7.5	8.1	56.0
7	B	0.99	23.4	82.9	22.0	9.8	9.8	9.8	79.6
	C	1.70	5.9	108.	68.9	20.6	13.0	16.0	34.9

recovery of the roots. Samples were lifted at suitable intervals, and the roots washed free of sand. Shoots and roots were cut off flush with the tuber surface and fresh and dry weights determined. The results are given in Table 3. From these data the original dry weights of tubers were calculated from the original fresh and dry weights of the initial (0 weeks) samples, and in Fig. 4 the current dry weights of the tubers and of the shoots and roots are plotted as percentages of their corresponding original calculated tuber dry weights.

Table 3. Fresh and dry weight of tubers and daughter plants grown in darkness.

Weeks after planting	Original Fresh Wt. (g.)	Current D.Wt. shoot + root (g.)	Current D.Wt. tuber (g.)	Total current D.Wt. (g.)
0	33.5	0.205	6.56	6.76
	37.5	0.150	7.54	7.69
2	35.5	0.850	6.08	6.93
	33.5	0.855	5.56	6.42
4	46.5	2.40	6.18	8.57
	34.5	2.66	2.82	5.47
6	41.0	3.91	2.31	6.22
	33.5	2.49	1.29	3.78

It is clear that the change in the dry weight of the tubers is similar to that depicted in Fig. 3, and that a substantial amount of material is transported from the tuber to the daughter plant. Respiration would account for that proportion of the dry weight lost entirely (and represented by the dotted lines in Fig. 4) but most of this was respired by the daughter plant following translocation from the tuber, as can be deduced from the results of the next experiment.

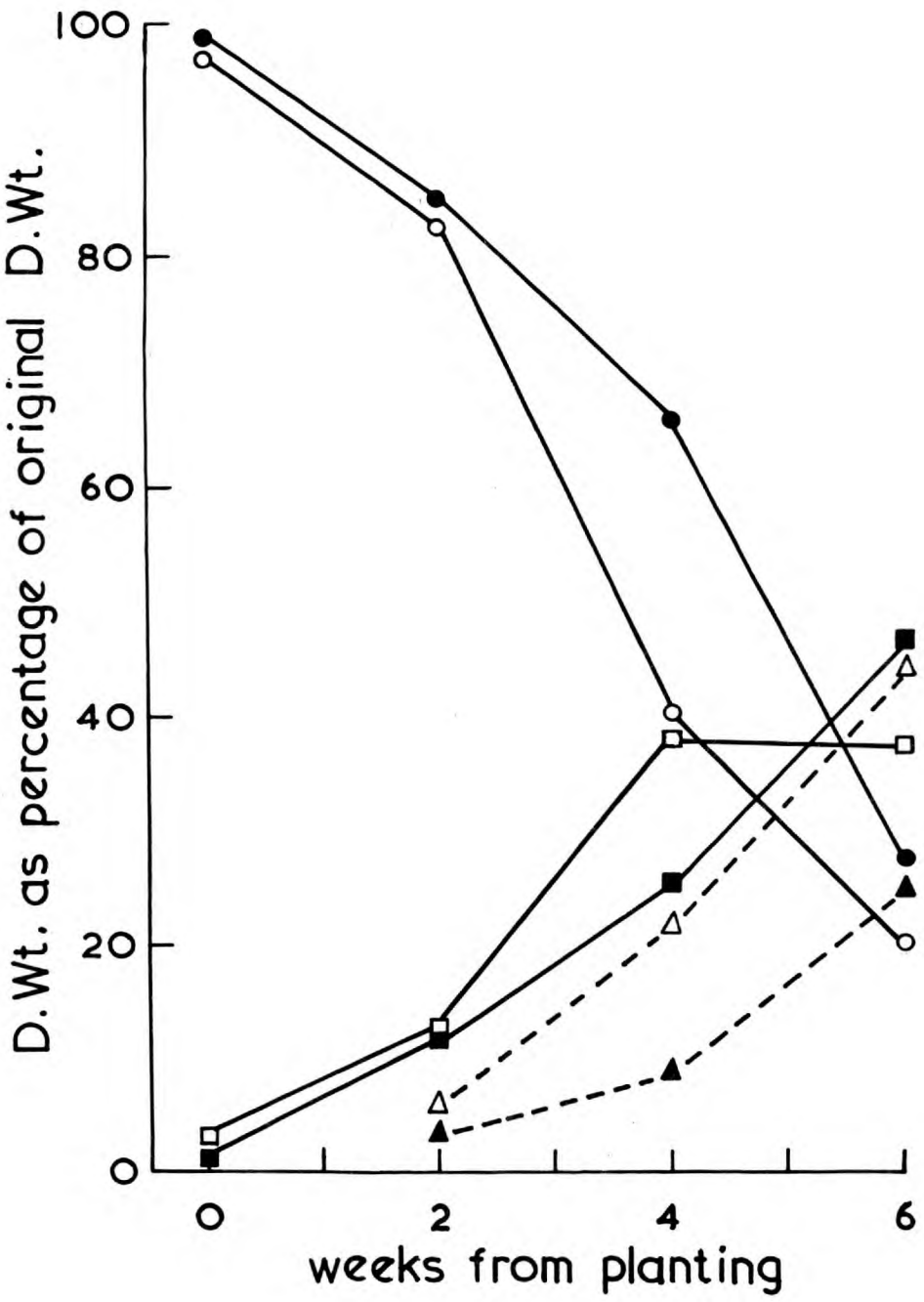


Figure 4. Fate of material lost from tubers sprouted in darkness.

● Tuber. ■ shoot plus root. ▲ loss by respiration, calculated by difference. ○ □ △ values for duplicate experiment.

Further investigation of depolymerization - effect of removing growing or potentially growing tissue.

The data on the distribution of fructose among the various sugar fractions (Table 2) were consistent with the hypothesis that the process of polymer breakdown is initiated by the hydrolytic enzymes described by Edelman & Bacon (1951). As a working hypothesis it was assumed on current opinion in plant physiology that sucrose is the sole translocatory carbohydrate. This would imply that the free fructose released is converted to sucrose before leaving the tuber. An attempt to implicate the hydrolytic system more directly by inducing an accumulation of free fructose or of sucrose was made by removing the actively growing shoots from sprouting tubers. This treatment would also show if respiratory activity in the tuber contributed significantly to its loss of dry weight (cf. previous experiment). Twelve weighed tubers were planted in compost and allowed to grow in the open on a roof in South Kensington. When the daughter plants were about a foot high, at which stage carbohydrate was judged to be depolymerizing and disappearing from the tubers at the maximal rate (see Fig. 3, Table 2), six of the tubers were dug up and all roots, shoots and dormant buds were cut off; the wounds on the tuber surface were immediately covered with vaseline containing Tetracycline-HCl (kindly donated by Cyanamid Ltd., London) to discourage invasion by microörganisms, and the tubers replaced in their original positions in the pots. No decay occurred for the duration of the experiment. At intervals pairs of tubers of similar weight, one treated and one not, were sampled:- the tubers were sliced

longitudinally after removal of any shoots or buds, and the halves weighed - one half was used for dry weight determination and the other for extraction of soluble carbohydrate. Slicing the tubers revealed the development of gas-filled spaces as described on p.19 in the control tubers but such lacunae did not develop in the treated tubers which remained turgid; in the latter a red pigmentation appeared on the cut surfaces, where it seemed to be confined to the ends of the vascular bundles. The results of the dry weight determinations are shown in Table 4, and are plotted in a form comparable to Figs. 3 and 4 in Fig. 5.

Table 4. Fresh and dry weight of tubers after removal of growing tissue.

A: growing tissue removed after 13 days

B: controls

Time after planting (days)	Treatment	Original F.Wt.	Current F.Wt.	Current D.Wt.	Original D.Wt. (calc.)
0		44.0	39.5	5.69	6.99
		39.5	37.0	6.43	6.27
14	A	35.0	27.2	3.44	5.57
	B	35.5	28.6	3.94	5.64
16	A	32.5	27.6	3.47	5.17
	B	32.0	23.9	3.12	5.08
19	A	45.0	39.9	5.43	7.14
	B	46.5	29.7	3.00	7.39
24	A	39.5	31.9	3.95	6.27
	B	36.0	17.3	1.38	5.72
29	A	44.5	37.4	4.94	7.07
	B	41.5	15.5	1.56	6.38

These results show that there is little loss of dry matter from the tuber when the newly grown tissue is removed. The distribution of fructose

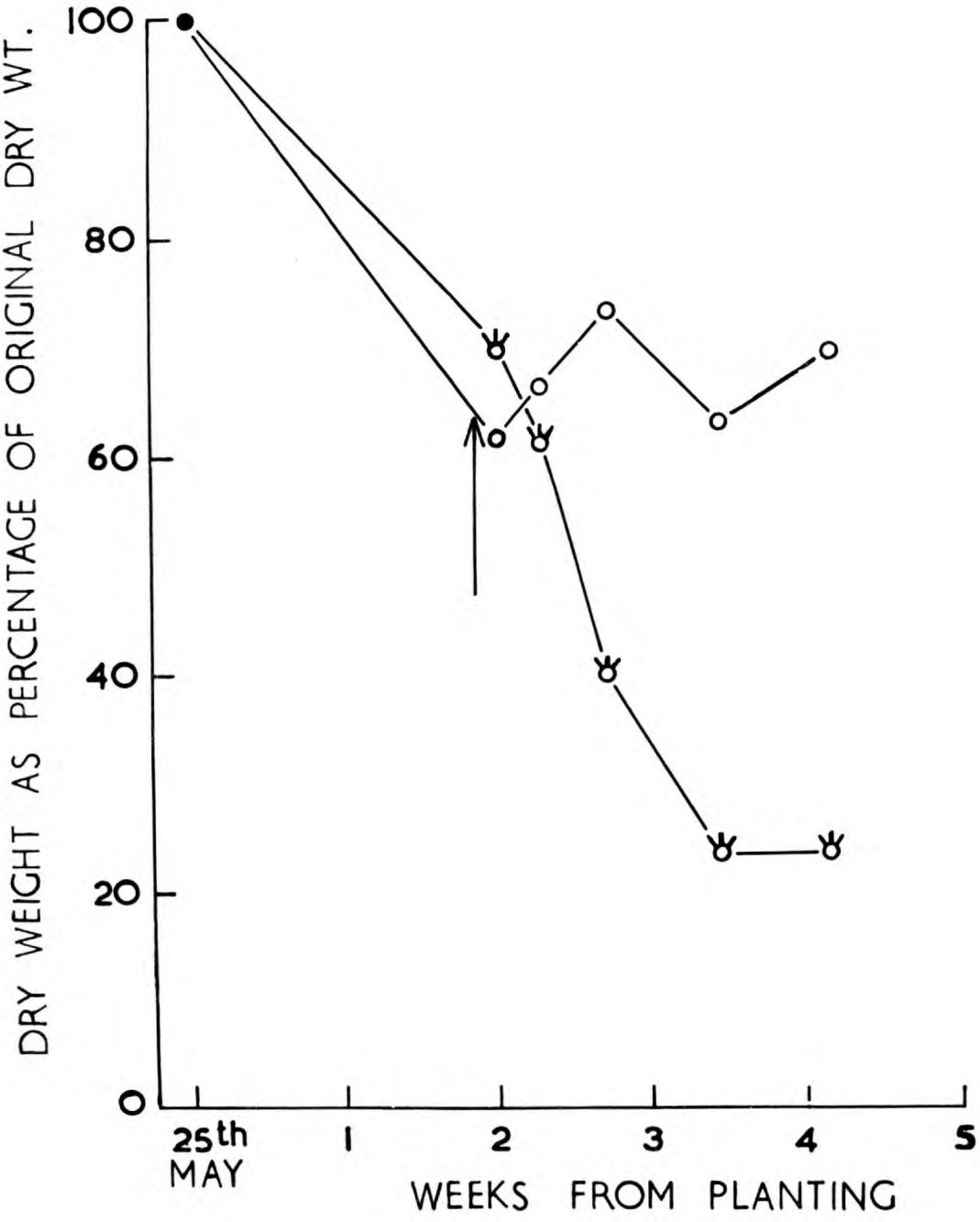


Figure 5. Dry weight of tubers at intervals after removal of growing tissue.

● original dry weight. ○ growing tissue removed at time indicated by arrow. ☿ control.

among various fractions separated by paper chromatography is shown in Table 5. The results indicate that there was no further change in the carbohydrate complex once the shoots had been removed, and suggest that the shoots exercise some control over the breakdown of the fructose polymers, per se or by the removal of sucrose from the tuber.

Table 5. Changes in individual carbohydrates present in tubers during growth of daughter plants, and after removal of growing tissue.

A: growing tissue removed after 13 days;

B: controls

Time after planting (days)	Treatment	D.Wt.	mg. free glucose	mg. fructose/gm. Current D.Wt. DP 1	2	3	4	5-n
0		5.69	6	45	58	51	65	398
		6.43	7	25	58	43	53	439
14	A	3.44	2	199	111	30	22	221
	B	3.94	1	134	53	23	24	288
16	A	3.47	0	168	65	16	26	360
	B	3.12	3	172	65	26	24	417
19	A	5.43	12	100	66	47	52	348
	B	3.00	3	290	78	21	21	181
24	A	3.95	25	93	63	38	41	406
	B	1.38	0	233	54	12	8	69
29	A	4.94	17	76	49	34	34	309
	B	1.56	10	126	64	28	21	85

Depolymerization, however, is known to occur in dormant artichoke tubers as demonstrated by the well-known over-winter change in the optical rotation of the tuber juice; the drift in the positive direction of the optical rotation has been correlated with conversion of fructosans of high molecular weight to sugars of lower D.P. (Bacon &

Loxley, 1952). If this process is mediated by the same system as that occuring during sprouting, it is possible that the dormant buds exercise control over it. An experiment to test this by removal of the buds was carried out: Tubers were weighed, divided into three batches, and treated as follows:

1. all buds cut off
2. parts of cortex, but not buds, cut off
3. untreated.

All cut surfaces were covered with a layer of vaseline, and the tubers placed in "Alkathene" bowls filled with damp sand at 2°C in the cold room. After four weeks storage the buds and cut surfaces contained a quantity of red pigment which in the latter case was apparently confined to the vascular tissue. The three batches were sampled at intervals; the tubers were halved longitudinally and the halves weighed; one half was used for dry weight determination and the other for the extraction of soluble carbohydrates. The extracts of carbohydrates, when brought to a uniform volume of 50 ml, were too deeply coloured for use in the polarimeter. The solutions were decolorized by the addition of a little charcoal (about 0.5 g.), mixed well, and the charcoal separated by centrifugation. It was found that little carbohydrate was lost during this procedure; data obtained for some representative samples before and after treatment with charcoal are given in Table 6.

After the extracts had been decolorized a sample was hydrolysed with 0.1 N oxalic acid, and the total fructose and total reducing

Table 6. Effect of charcoal treatment on carbohydrate content of extracts

Weight of tuber extracted (gm.)	Total reducing substances (mg./ml. extract)	
	Before charcoal treatment	After charcoal treatment
18.3	10.9	11.7
12.7	8.55	8.75
13.7	7.83	7.83
11.6	6.15	5.93
14.2	9.28	9.20
16.8	11.2	11.1

substances estimated. The results are shown in Table 7; the values for the dry weights of the tubers are plotted (together with the change in dry weight of tubers sprouting at 20°C (Table 1)) in Fig. 6 and show that although all series at 2°C may have lost some dry material, there was no significant difference in the loss from tubers with buds as compared to those without buds, despite individual variability; this indication that the buds made no growth during the experimental period was supported by their appearance. The tissues of all the tubers remained healthy and turgid.

The specific rotation of the extracted juice was calculated from the optical rotation of the decolorized extract and the value obtained in the total reducing substance estimation after total hydrolysis of the carbohydrates. The results of these calculations are given in Fig. 7, which shows that there was a large and rapid change in the rotation of the juice, which is apparently unaffected by the presence or

Table 7. Dry weight and soluble carbohydrate content, and optical rotation of extracts from tubers stored at 2°C.

A, controls; B, all buds removed; C, parts of cortex removed.

Period of storage (weeks)	Treatment	Original dry wt. (calc.)	Current dry weight	Fructose mg./g. current D.Wt.	TRS mg./g. current D.Wt.	TRS mg./ 100 ml.	Optical rotation
							α/dm.
		4.81	5.24	621	657	4.55	-0.56
0		3.74	3.34	628	645	2.22	-0.34
		5.53	5.57	626	659	3.20	-0.46
	A	5.71	5.08	379	500	2.23	+0.36
		4.64	4.60	493	497	2.48	+0.32
	B	3.30	3.20	432	539	1.51	-0.24
4		4.01	3.58	395	484	1.63	-0.00
	C	4.72	3.90	428	537	1.75	+0.36
		3.74	3.23	415	547	1.67	+0.25
							α/4dm.
	A	5.08	5.01	476	560	0.480	+0.37
		6.78	5.84	446	567	0.543	+0.56
	B	6.15	4.48	377	494	0.404	+0.66
8		4.37	4.51	437	495	0.418	+0.22
	C	5.71	5.45	456	539	0.468	+0.34
		4.28	3.77	474	481	0.295	+0.27
	A	3.92	3.43	446	620	0.357	+0.35
13	B	6.15	5.26	449	536	0.467	+0.40
	C	5.44	5.09	451	556	0.468	+0.46
	A	5.35	4.42	384	516	0.390	+0.42
16	B	4.64	3.87	442	568	0.345	+0.36
	C	4.99	4.69	520	589	0.441	+0.28

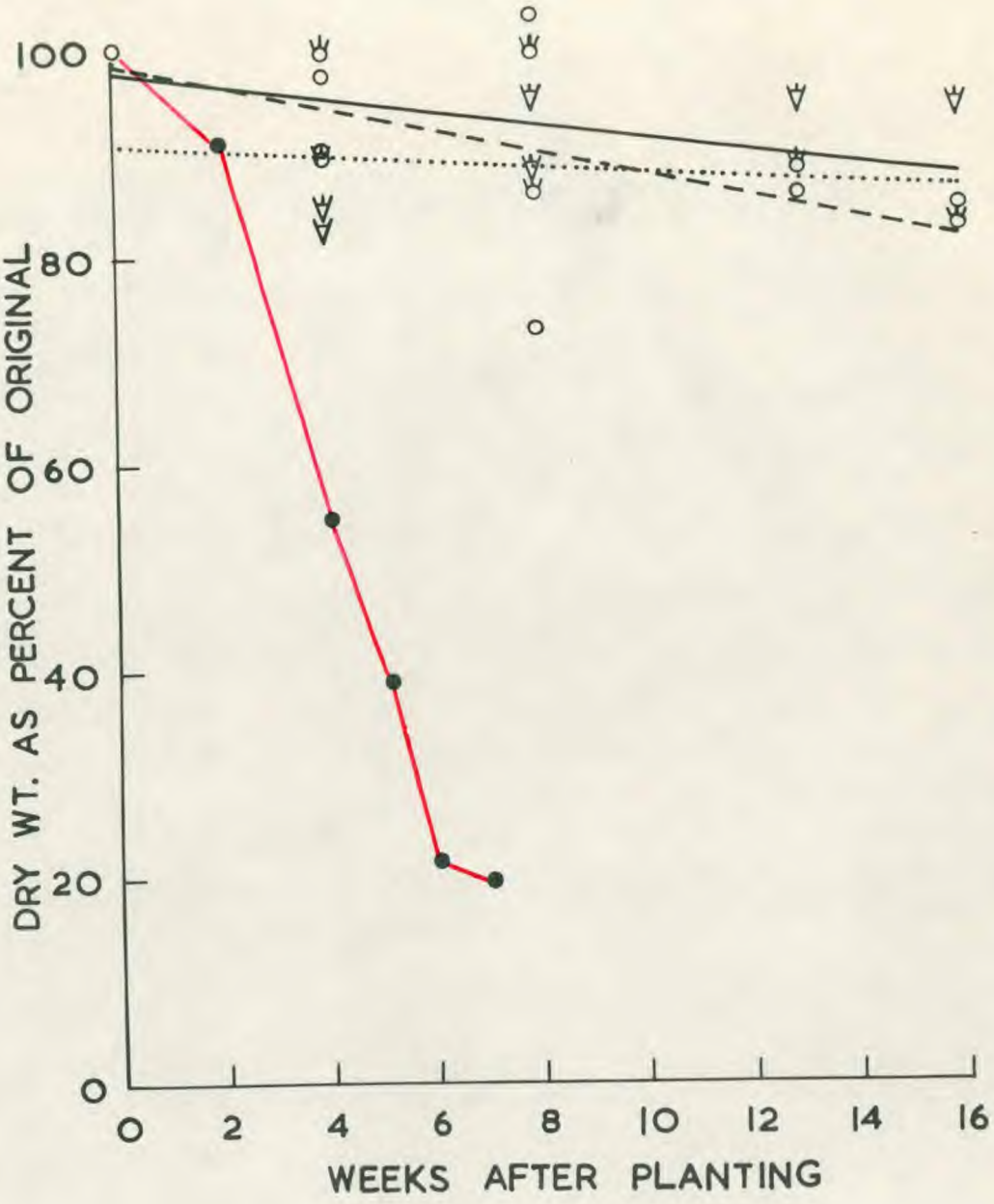


Figure 6. Dry weight of tubers stored at 2°C.

controls, O—O all buds cut off, parts of cortex cut off. The slopes of the lines were calculated.

data from Fig. 3 (tubers at 20°C) given for comparison.

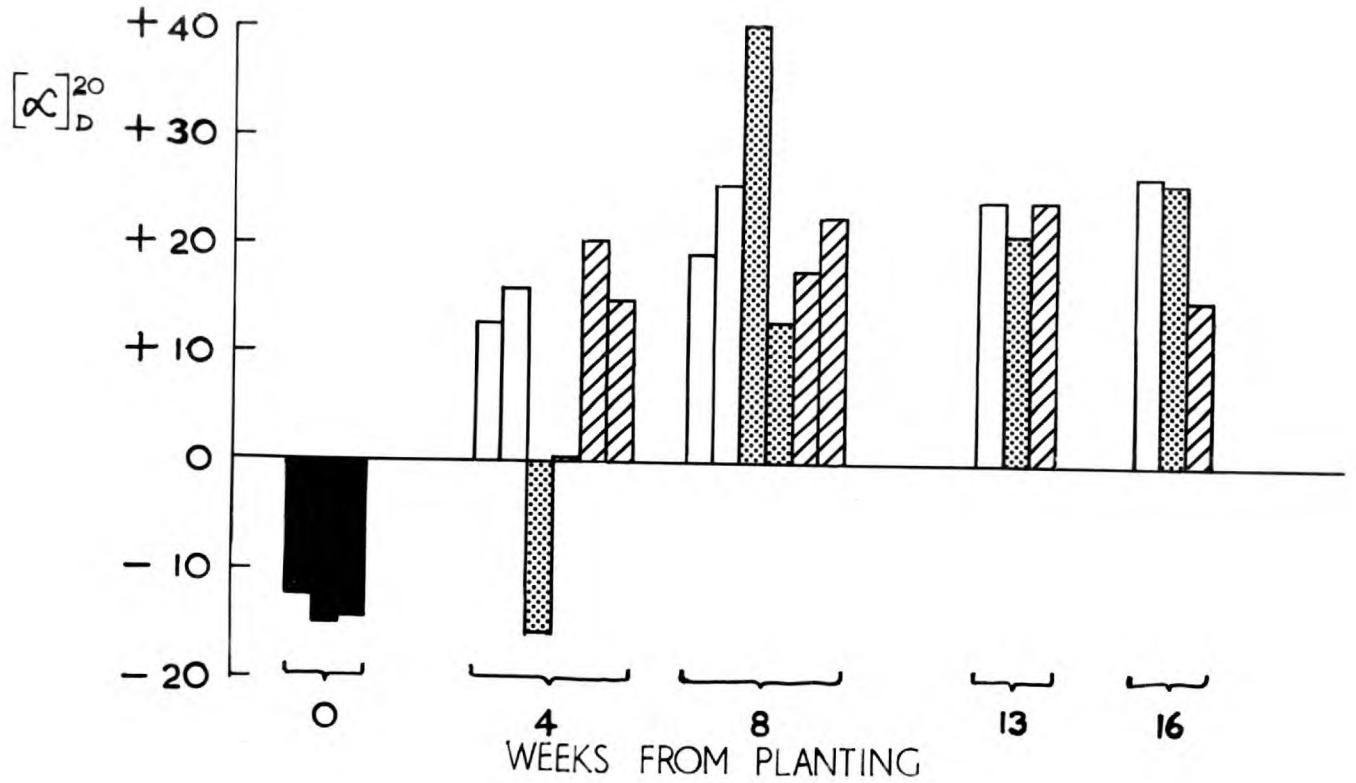


Figure 7. Optical rotation of soluble carbohydrate from tubers stored at 2°C.

Open columns – controls; stippled columns — all buds cut off; hatched columns — parts of cortex cut off.

absence of buds; the observed differences between the controls and the treated tubers are taken to be due to variation between individual tubers and not to effects of the different treatments.

At this stage of the experiment (16 weeks) several tubers remained unsampled; these were used in an attempt to find if the observed change in rotation could be reversed by transferring them from their storage temperature of 2°C to higher temperatures. Before this transfer was made buds were removed from all tubers which still bore them, to prevent sprouting, and the cut surfaces covered with vaseline. The tubers were divided into four groups which were stored in constant temperature rooms or incubators kept at 2°, 16°, 25°, and 30°C respectively. Samples were removed from each group at intervals, and treated as in the first part of the experiment. Towards the end of the experimental period examination of the surfaces which had been cut showed signs of cell proliferation in those tubers stored at temperatures of 15°C and above. Although in some cases this had resulted in the growth of a considerable bulk of tissue, the new growth remained as a callus, and there was no indication of any development of organised structures. The results of the experiment are shown in Table 8 and the derived specific rotations are plotted in Fig. 8; although the latter do not allow any unequivocal conclusion to be drawn from the experiment, large scale reversal of optical rotation to the negative was not brought about by this high temperature treatment although a slight trend in this direction may be discerned, and it would therefore appear that any resynthesis of polymer must have been on a small scale.

Table 8. Dry weight and soluble carbohydrate content, and optical rotation of extracts of tubers stored at different temperatures after 14 weeks storage at 2°C.

Period of storage (weeks)	Temp. of storage (°C)	Original dry wt. (calc.)	Current dry wt.	Fructose (mg./g. original dry wt.)	T.R.S. (mg./g. original dry wt.)	$[\alpha]_D^{20}$
2	4	5.62	4.39	363	516	+24.7
	15	7.31	5.47	421	578	+16.6
	22	3.83	3.22	447	633	+14.6
	30	5.88	5.00	375	523	+15.9
4	4	4.55	3.81	484	587	+19.4
	15	5.44	4.56	431	544	+19.6
	22	3.39	3.03	489	586	+12.1
	30	5.53	4.93	415	533	+12.9
6	4	3.48	3.08	393	482	+18.2
	15	4.72	4.46	491	600	+10.8
	22	7.13	6.18	464	595	+ 7.5
	30	6.06	5.32	482	612	+12.3
8	4	6.06	4.71	340	456	+38.0
	15	4.19	3.75	517	644	+ 4.8
	22	5.97	4.71	367	438	+12.0
10	4	4.72	2.78	388	538	+32.8
	15	4.19	2.86	547	640	+16.9
		5.17	4.45	489	575	+22.6

The initial rapid change in specific roation during storage at 2°C was re-examined using tubers which had been grown in pots on a roof in South Kensington. Tubers were harvested from the pots, washed in tap water and dried with a towel. Twenty-one of these tubers were selected and treated as in the previous experiment, namely

1. all buds cut off
2. parts of cortex, but not buds, cut off
3. untreated.

The cut surfaces were daubed with vaseline and the tubers stored in damp

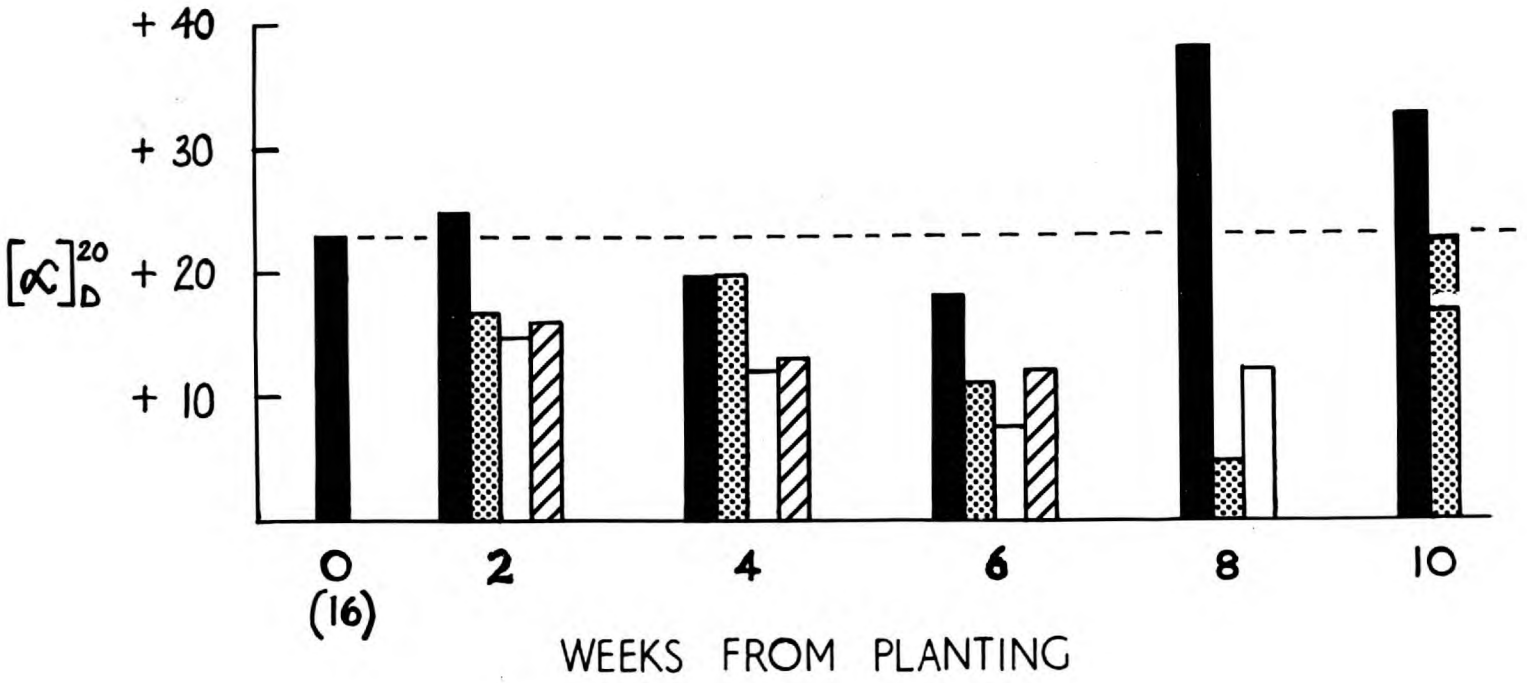


Figure 8. Optical rotation of soluble carbohydrate from tubers stored at different temperatures after 16 weeks at 2°C. All the tubers were disbudded.

Solid columns — 2°C; stippled columns — 16°C; open columns — 22°C; hatched columns — 30°C.

sand in "Alkathene" bowls at 2°C in the cold room. Samples were taken from each series at intervals, which were shorter than in the previous experiment; no dry weight determinations were made, but the fresh weight of the tubers at the time of sampling was recorded. There was no visible sign of sprouting over the experimental period in any group. The extracts of soluble carbohydrates were diluted to 250 ml instead of being concentrated to 50 ml; this saved time as the optical rotation could still be read with sufficient accuracy, owing to the magnitude of the changes. Some of the batch of tubers from which the experimental material had been selected were left in damp sand at room temperature. Although their fresh weights at the time of the start of the experiment had not been recorded, some of these tubers were sampled at the same time as two of the later samples kept at low temperature: these tubers had not been kept with this purpose in mind and the control of temperature and of watering was lax. The results obtained from these intact tubers are nevertheless included with those of the experiment as originally planned, in Table 9. The values for the specific rotation of the extracted carbohydrate are given in Fig. 9 together with the data of Bacon & Loxley (1952) for a similar period: the tubers used by these authors were dug from the ground as required and were all untreated. The results of the experiment show that under the conditions of storage used, the presence or absence of buds is not a significant factor in the control of the change in rotation. The results also show that the change in optical rotation during winter storage is accelerated and magnified at 2°C, and indicate that the change is depressed at 20 °C.

Table 9. Fresh weight and soluble carbohydrate content, and optical rotation of extracts of tubers stored at 2°C.

A, controls; B, all buds removed; C, parts of cortex removed; A*, untreated tubers stored at room temperature.

Period of storage (weeks)	Treatment	Original fresh wt. (g.)	Current fresh wt. (g.)	Fructose mg./ml.	T.R.S. mg./ml.	$[\alpha]_D^{20}$
		18.7	18.7	11.6	11.7	-20.0
0		14.7	14.7	7.34	7.83	-14.4
		13.1	13.1	8.19	8.75	-18.5
	A	14.5	14.9	8.14	9.20	-17.1
1	B	12.3	12.2	5.90	5.93	-22.8
	C	18.6	18.4	10.6	11.1	-15.4
	A	12.1	12.9	7.15	7.75	-11.0
2	B	15.4	15.8	8.75	9.57	-11.2
	C	12.1	12.7	7.10	7.50	-10.7
	A	18.1	19.0	9.52	10.2	- 5.4
3	B	15.3	15.9	8.56	9.37	- 7.2
	C	10.6	11.0	6.20	6.90	- 6.2
	A	14.0	15.5	6.47	7.50	+ 7.0
5	B	15.0	16.0	7.83	8.88	+ 3.1
	C	15.3	16.1	5.31	6.06	+ 7.0
	A	12.7	14.1	6.10	7.26	+ 5.9
	B	10.2	11.4	4.54	5.32	+ 7.0
	C	14.1	15.0	6.28	7.58	+ 4.9
6	A*	-	9.4	5.83	5.98	-17.1
	A*	-	16.0	9.26	9.42	-17.3
	A*	-	8.9	4.68	4.86	-18.5
	A	11.3	11.9	5.59	6.52	+13.0
	B	14.4	15.8	6.72	8.58	+16.3
	C	14.6	15.8	6.13	7.48	+23.8
10	A*	-	8.0	3.26	3.58	-13.0
	A*	-	6.7	3.35	3.61	-19.4
	A*	-	12.8	8.19	8.98	-13.9

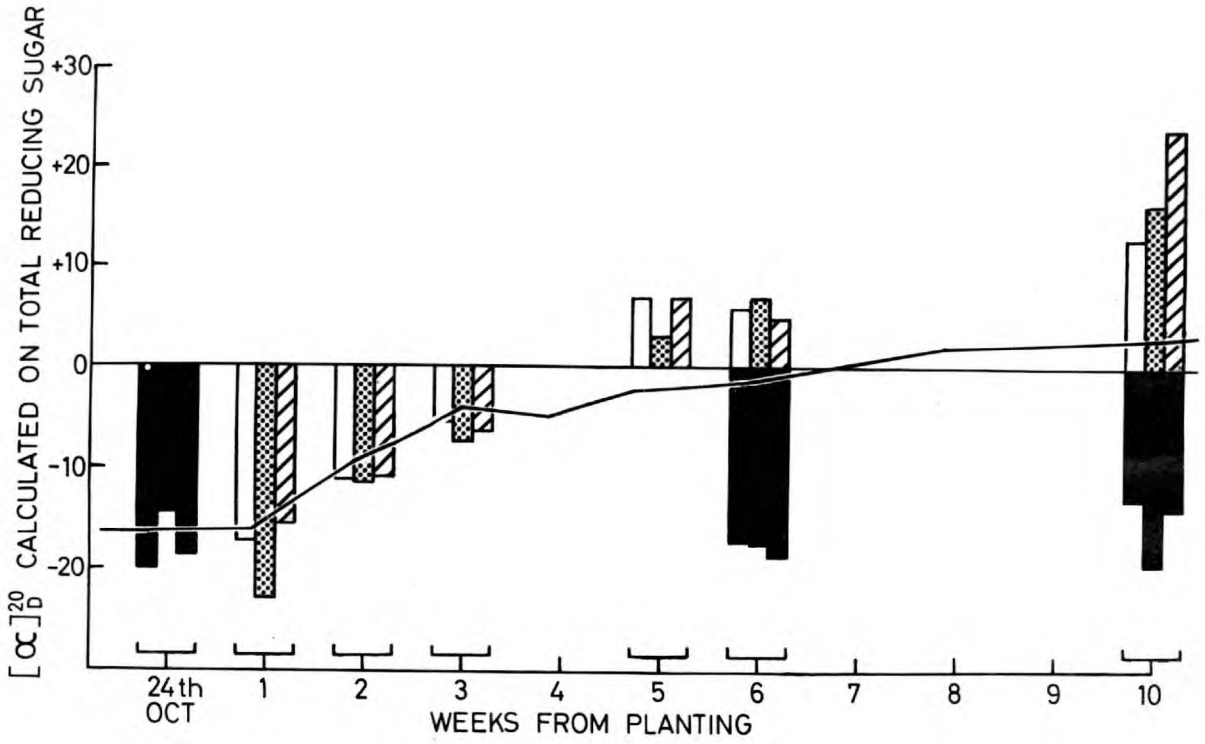


Figure 9. Effect of storage temperature of tubers on optical rotation of the soluble carbohydrate.

Solid columns - untreated tubers stored at room temperature (15-20°C); other columns - tubers stored at 2°C: open columns - untreated, stippled - all buds cut off, hatched - parts of cortex cut off. The data of Bacon & Loxley (1952) are given for comparison (continuous line).

The Effect of temperature on depolymerization

A further experiment was made to determine the optical rotation of the juice extracted from tubers stored at different temperatures. Tubers supplied freshly dug in September by the Botanical Supply Unit, were washed, disbudded, numbered and weighed. Three series were established by random selection and stored at 2°, 8°, and 20°C respectively, the tubers being kept in damp sand in Alkathene bowls. Each series was sampled at intervals and the fresh and dry weights and optical rotation measured; the fructose and reducing substances were estimated after total hydrolysis of the carbohydrates. After seven weeks the specific rotation of the sample from 2°C was +21.6°, compared with the initial value of -13.5°: at this stage a number of tubers was transferred from 2° to 20°C, and vice versa, making five series in all which were sampled at further intervals. The tubers stored at 20°C for the duration of the experiment showed signs of callus formation as noted in a previous experiment, and in two cases daughter tubers had developed, probably from existing buds which had escaped excision rather than from newly formed adventitious buds. In these, and in the cases where the callus was of some magnitude, a "sucking-out" similar to that occuring during sprouting could be observed on slicing the tuber, indicating transport of material from these tissues to the proliferating cells as to a growing shoot. The results of the experiment are shown in Table 10 and Fig. 10. The treatments at 2° from the start, or at 2° after 7 weeks at 20°C confirm that storage at 20°C depresses the rate of change in optical rotation. The effect of storage at 8°C as compared to

Table 10. Dry weight and soluble carbohydrate content, and optical rotation of extracts from disbudded tubers stored at different temperatures.

Period of storage (weeks)	Temp. of storage (°C)	Original dry wt. (g.) (calc.)	Current dry wt. (g.)	Fructose (mg./g. dry wt.)	T.R.S. (mg./g. dry wt.)	$[\alpha]_D^{20}$
	-	5.58	5.98	540	603	- 6.7
0	-	22.9	23.6	643	705	-18.8
	-	12.5	10.1	540	597	-15.0
	2	8.23	8.52	573	612	-16.4
2	8	6.10	4.89	447	517	+ 1.0
	20	7.83	7.79	699	738	-17.0
	2	8.87	8.15	545	644	+ 9.1
4	8	7.83	6.61	489	558	+ 8.8
	20	15.7	14.7	612	618	-17.4
	2	12.3	12.0	464	559	+21.6
7	8	8.45	8.98	517	649	+ 8.8
	20	9.77	9.49	532	569	-11.4
	2	8.12	7.05	400	557	+31.2
	2*	10.0	9.93	476	573	+14.4
10	8	12.3	11.4	469	619	+23.2
	20	17.5	14.7	520	581	- 3.1
	20/	8.78	8.85	494	608	+12.8
	2	10.1	9.52	478	598	+19.5
	2*	9.85	8.02	427	528	+21.6
14	8	16.2	12.3	465	597	+24.5
	20	7.30	5.71	480	579	+10.4
	20/	18.0	16.0	484	600	+16.6

* transferred from 20°C after 7 weeks

/ " " 2°C " " "

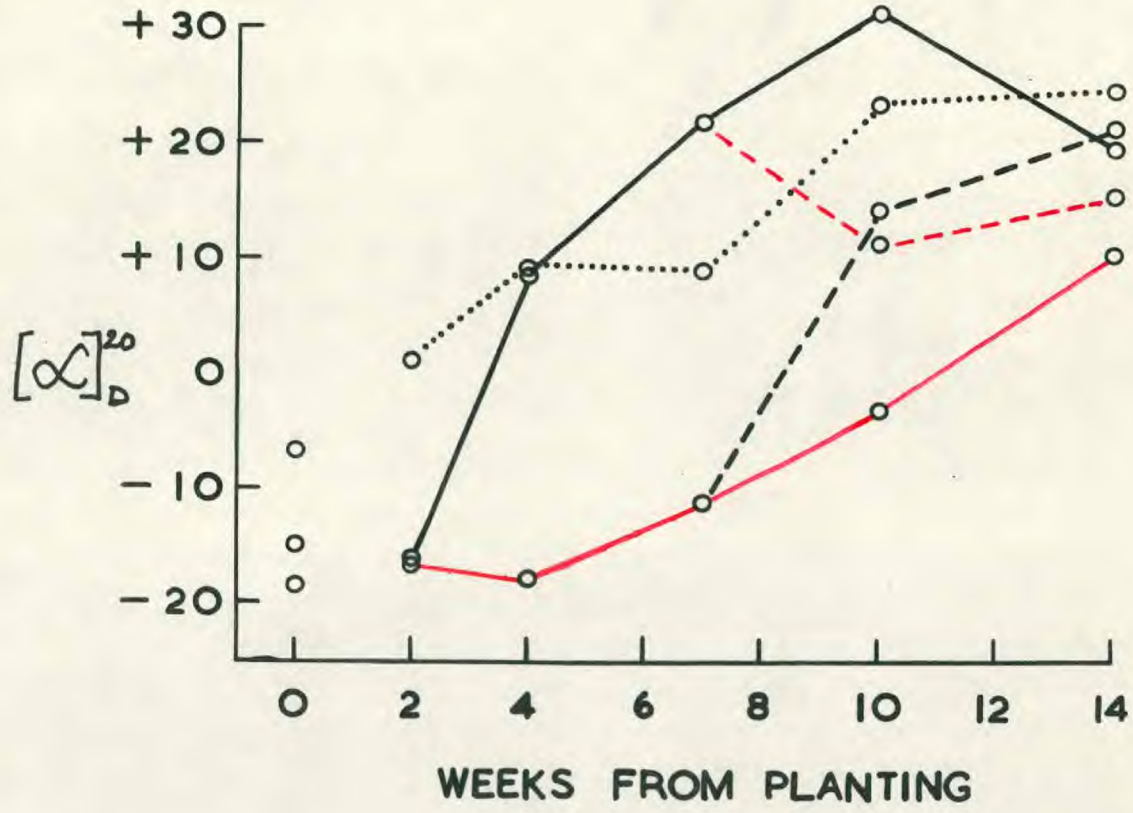


Figure 10. Effect of storage temperature of tubers on optical rotation of the soluble carbohydrate. All tubers were disbudded.

—— 2°C; ····· 8°C; —— 20°C;
— — — tubers transferred to 2°C after 7 weeks at 20°C
— — — tubers transferred to 20°C after 7 weeks at 2°C

that at 20°, shows the former to be essentially a "cold" treatment. It appears from the rotation of extracts from tubers transferred from 2° to 20°C that the change is to some extent reversible (cf. Fig. 8).

All tubers used in the germination experiments described previously had been stored in the cold for various periods, all long enough on the basis of the above results for the change in optical rotation to occur. The next experiment was designed to show if the redistribution and isomerization of fructose residues which this change reflects is a prerequisite for sprouting. Tubers of the same origin as those used in the previous experiment were washed, weighed and numbered, and stored in the dark at either 2° or 20°C in damp sand. The groups were sampled at intervals; the fresh and dry weights of each sample and of the buds and new growth were determined. An extract of the soluble carbohydrates was made from the tuber and decolorized before measuring the optical rotation. A sample of the extract was hydrolysed with 0.1 N oxalic acid, and the fructose content and total reducing substances estimated.

After seven weeks, a set of tubers was transferred from storage at 2°C to 20°C, and samples taken from all three groups over a further seven weeks. At the end of the 14 weeks no significant increase in the size or dry weight of the buds of tubers stored at 2°C was detectable; in the other groups however there was a considerable increase in the dry weight of the buds taken as a whole, although not all the buds on each tuber grew. The morphological form of the new growth differed profoundly in these two groups: that which was stored at 2°C for seven weeks and then transferred to 20°C produced negatively geotropic sprouts

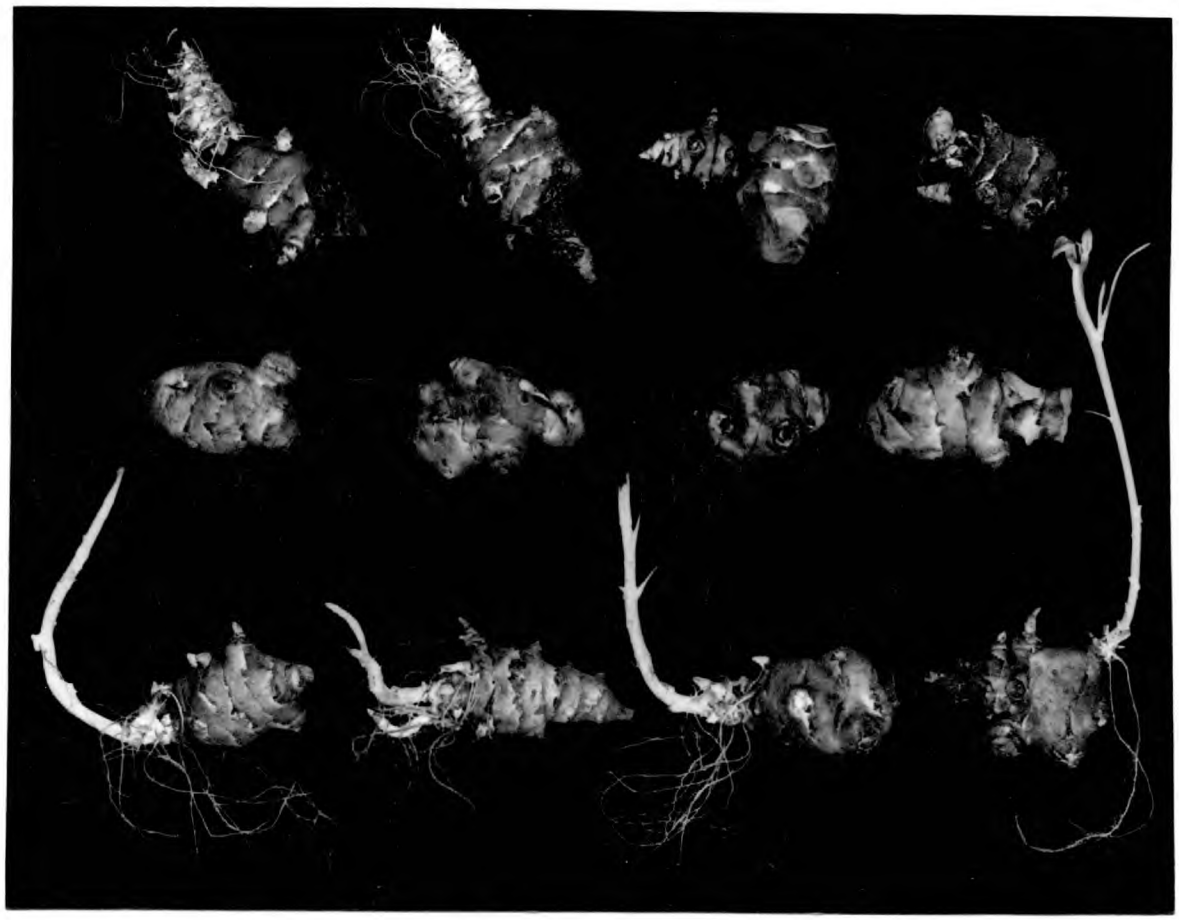


Figure 11. Effect of storage temperature on the subsequent sprouting of tubers. All tubers were kept in damp sand in the dark.

Top row: 15 weeks at 20°C
Middle row: 15 weeks at 2°C
Bottom row: 7 weeks at 2° followed by 8 weeks at 20°C.

which showed symptoms typical of etiolated stems, while tubers stored at 20°C for the whole of the 14 weeks produced swollen diageotropic organs, i.e. daughter tubers. These differences are illustrated in a photograph, (Fig. 11). The results of the analyses of the samples are shown in Table 11, and the derived changes in specific rotation in Fig. 12.

Table 11. Dry weight and soluble carbohydrate content, and optical rotation of extracts from tubers stored at different temperatures.

Period of storage (weeks)	Temp. of storage (°C)	Original dry wt. (calc.) (g.)	Current dry wt. (g.) buds + roots	Current dry wt. (g.) tuber	Carbohydrate content g./mg. D.Wt. fructose	Carbohydrate content g./mg. D.Wt. T.R.S.	$[\alpha]_D^{20}$
	–	5.58	0.42	5.98	540	603	- 6.7
0	–	22.9	1.15	23.6	643	705	-18.8
	–	12.5	0.61	10.1	540	597	-15.0
	2	15.9	0.22	15.3	584	644	- 3.6
2	20	16.4	0.55	13.2	569	612	- 9.8
	2	10.9	0.35	10.0	507	600	+20.9
4	20	15.4	0.71	13.7	573	586	-19.1
	2	20.9	0.78	21.2	375	451	+28.5
7	20	13.4	1.68	11.4	518	572	- 4.9
	2	13.7	0.39	12.5	421	545	+28.6
10	20*	18.2	0.87	15.1	543	668	+ 2.8
	20	18.5	2.78	13.2	550	657	+ 1.9
	2	11.2	0.37	9.89	439	582	+23.8
15	20*	16.1	3.00	9.53	476	559	- 7.1
	20	17.6	4.33	10.9	553	653	+ 5.8

* tubers transferred to 20° after 7 weeks at 2°C.

Bacon & Loxley (1952) point out that as all the fructosides contain a glucose residue, the depolymerization implies a rising proportion of combined glucose to fructose as the process continues, and they conclude

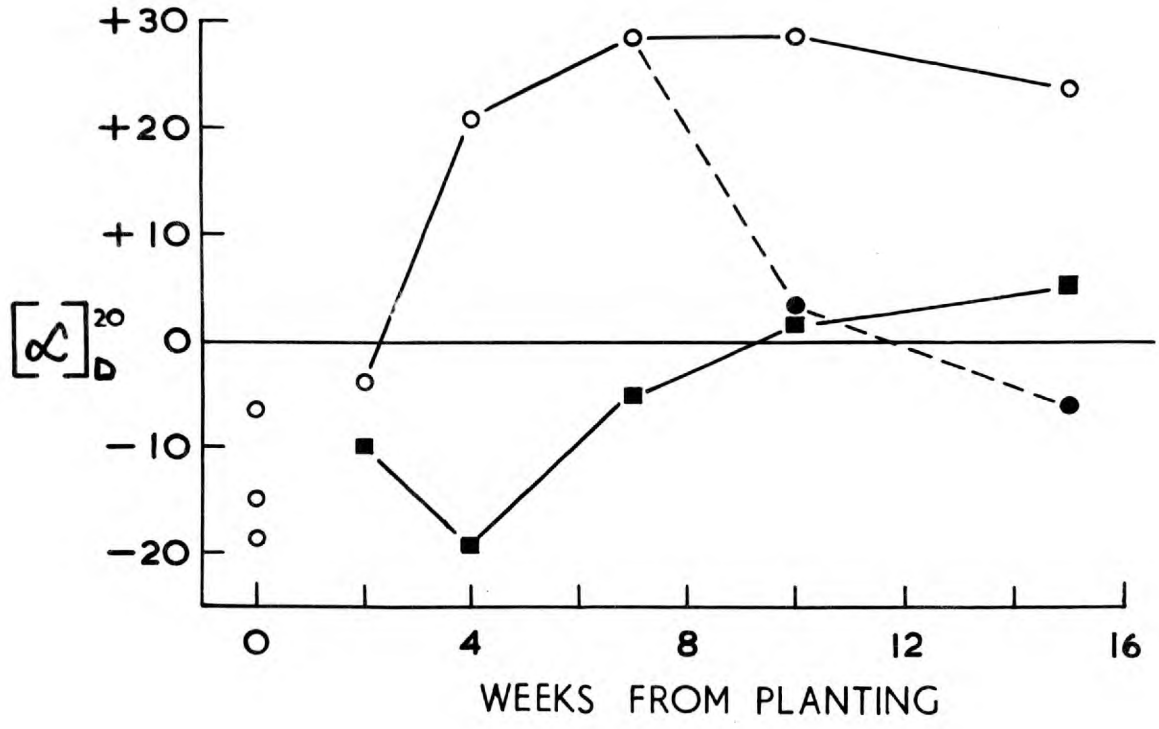


Figure 12. Effect of storage temperature of tubers on optical rotati of the soluble carbohydrate.

○—○ 2°C; ■—■ 20°C; ●--● transferred to 20° after 7 weeks at 2°C.

from indirect evidence that this occurs by conversion of fructose to glucose structures and not by preferential disappearance of fructose residues. The hydrolysed extracts of some of the tubers from each of the two previous experiments were used to determine the absolute glucose content of the soluble carbohydrate mixture. The depolymerization was also measured by analysis of the fructose oligo- and polysaccharides. Separation of polymers of high and low DP was made arbitrarily by grouping compounds into those of DP 3-6 and those of DP 6. The total glucose content of the extracts was estimated after total hydrolysis by 0.1 N oxalic acid, by the glucose oxidase-peroxidase method. The results, given in Table 12, show that there is an absolute increase in the total glucose content of the soluble carbohydrate extracts of the tubers. The depolymerization is also illustrated qualitatively in Fig. 13: the chromatograms were made up so that each spot put on the paper contained the same amount of total fructose, namely 250 µg. Quantitative results are shown in Fig. 14, which is constructed from some of the data given in Table 12.

As mentioned above, a few tubers of the dis-budded group developed daughter tubers. One such specimen was divided and the parent and daughter tubers extracted and treated in the same way as above. The results are shown in Table 13. The specific rotation of the parent tuber indicated, and chromatography of a sample showed (Fig. 15), that extensive depolymerization had occurred by this stage, even at 20°C.; the appearance and dry weight measurement showed that a large amount of total fructose had disappeared from the tuber; much of this had been transferred to the daughter tuber however where it had been

Table 12. Total glucose and total fructose content of soluble carbohydrate extracts from tubers stored at different temperatures, and the distribution of fructose between various fractions.

Period of storage (weeks)	Storage temp. (°C)	$[\alpha]_D^{20}$ of T.R.S.	Total glucose content mg./g. of original dry weight	Total fructose content mg./g. of original dry weight	Distribution of fructose residues (%) DP 1	DP 2	DP 3–6	DP > 6
		- 6.7	106	540	0.2	4.5	17.3	77.9
		-18.8	69.3	643	0.3	3.4	16.4	79.9
		-15.0	88.4	540	-	-	-	-
	2*	+21.6	159	451	0.7	17.5	49.1	32.6
	2	+28.5	136	379	0.9	17.8	48.8	32.4
7	8*	+ 8.8	159	549	-	-	-	-
	20*	-11.4	105	515	3.4	6.1	21.7	68.8
	20	- 4.9	102	441	1.3	8.1	26.3	64.3
14	2*	+19.5	156	451				
15	2	+23.8	167	386				
14	8*	+24.5	145	353				
14	20*	+10.4	125	375				
15	20	+ 5.7	102	339				

* all buds removed at start of experiment

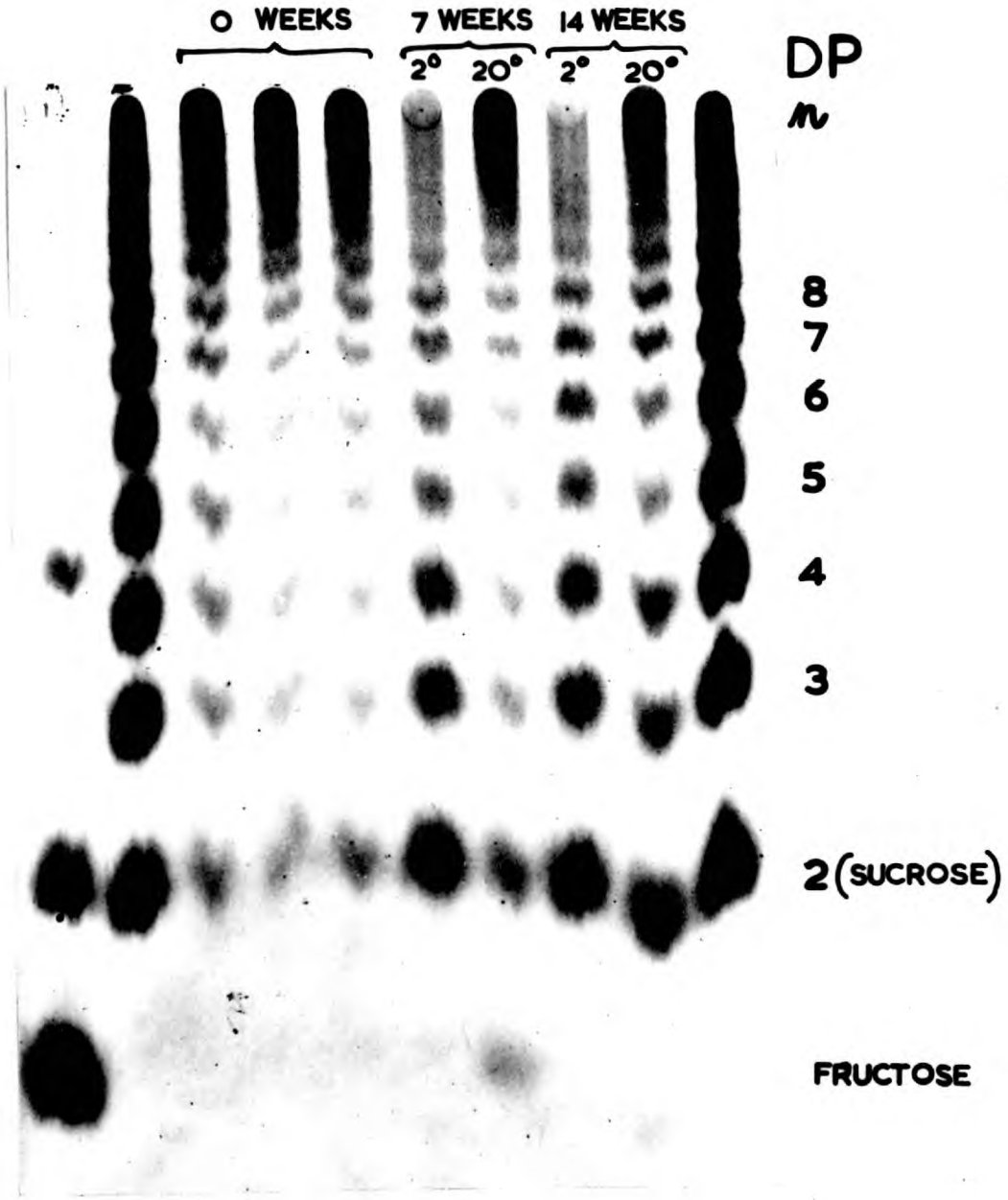


Figure 13. Distribution of fructose among various fractions in soluble carbohydrate extracts from tubers stored at 2° and 20°C.

Each spot contained about 250 µg. of total fructose; chromatogram developed in PEAW 6:1:3 for 2 days and sprayed with urea-HPA reagent.

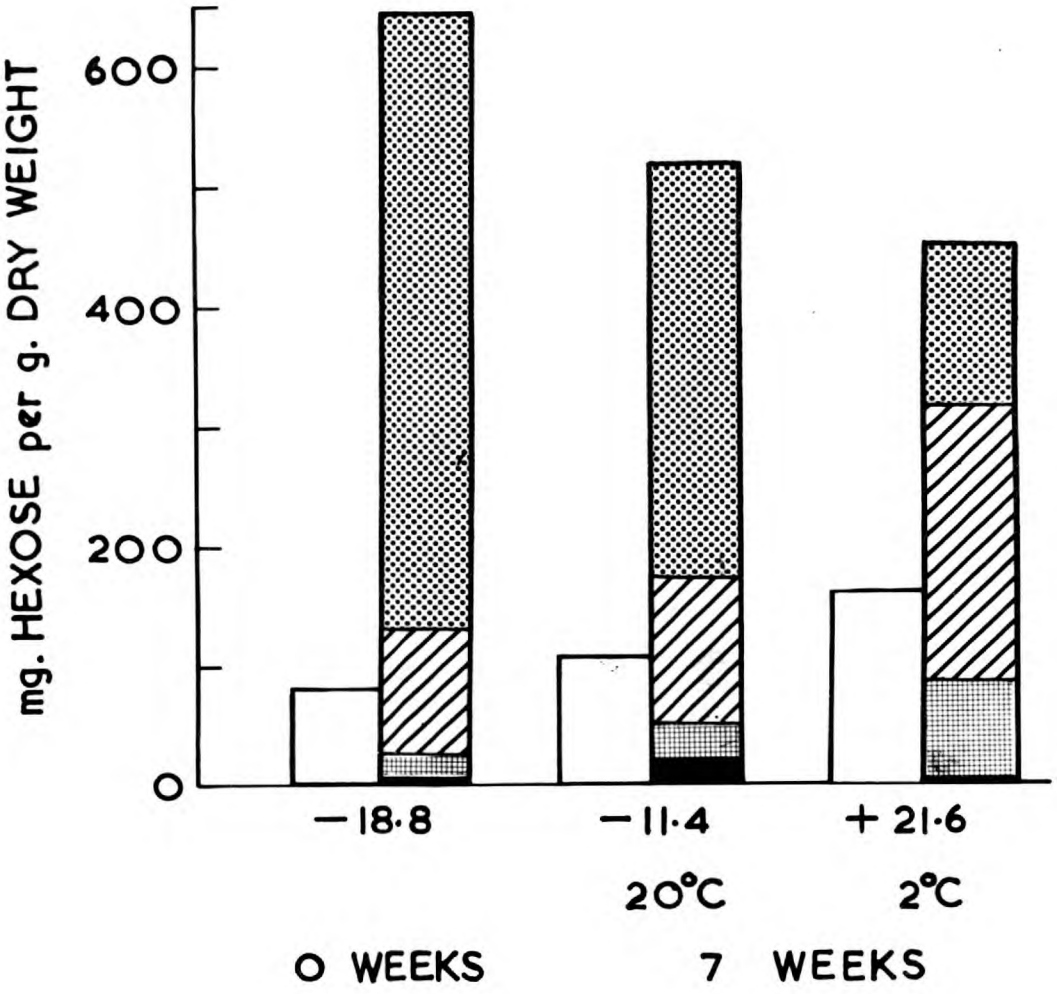


Figure 14. Effect of temperature on depolymerization of fructosan in disbudded tubers stored at 2° and 20°C.

Open columns - total glucose content; other columns - fructose content of various fractions: solid columns - DP 1 (free fructose), squared - DP 2 (sucrose), hatched - DP 3-6, stippled - DP ≥ 7. The figures at the base of the columns give the $[\alpha]_D$ of the total reducing sugar.

converted largely to material of high DP.

Table 13. Optical rotation and carbohydrate content of soluble carbohydrate extracts from parent and daughter tubers.

	Dry weight as % of original dry weight of parent tuber	Fructose mg./g. dry wt.	T.R.S. mg./g. dry wt.	$[\alpha]_D^{20}$
Parent tuber	39.0	442 (172)*	505 (197)*	- 5.9
Daughter tuber	29.3	522	528	-13.9

* mg./g. of original dry weight.

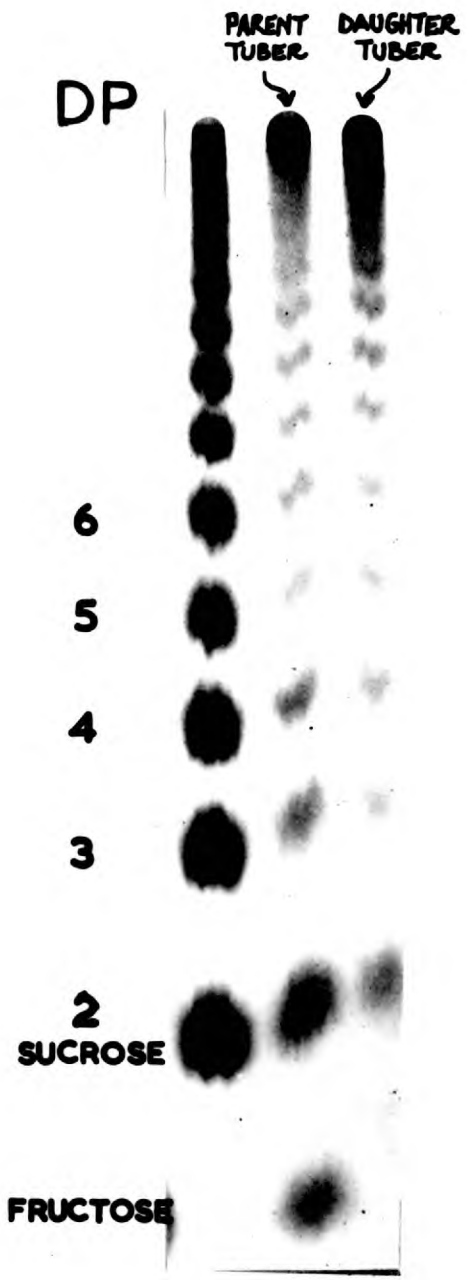


Figure 15. Distribution of fructose among various fractions in extracts from parent and daughter tubers stored at 20°C. Each spot contained about 250 µg. of total fructose; chromatogram developed in PEAW 6:1:3 for two days, and sprayed with urea-MPA reagent.

Hydrolysis of Fructosans by extracts from artichoke tubers.

Introduction

The results of the physiological experiments described above are consistent with the hypothesis that the hydrolytic enzyme described by Edelman & Bacon (1951) initiates the utilization of polymerized fructose, both in germination and in the depolymerization occuring during cold storage. Bacon & Loxley (1952) also postulated the involvement of this enzyme in the process leading to change in the optical rotation of extracts from stored tubers. The enzyme hydrolyses the bond of the terminal fructose of inulin-type sugars:-

$$G\sim F - F_n + H_2O \longrightarrow G\sim F - F_{n-1} + F$$

the final products of a succession of such reactions being sucrose and free fructose. The enzyme is thus a β-fructofuranosidase (see Report of the Commission on Enzymes, 1961) but differs from all other enzymes of this type in that it does not hydrolyse sucrose and is thus not an invertase; the enzyme is also inactive against raffinose. There is an enzyme present in the artichoke tuber which will hydrolyse sucrose and raffinose but not melezitose and is hence also a β-fructofuranosidase. It is intended in this section to use the name "invertase" for this enzyme and the name "hydrolase" for β-fructofuranosidase activity which will not hydrolyse sucrose.

Recaldin (1961) found that the activity of a hydrolase preparation from artichoke tubers was inhibited by sucrose; this characteristic could form a basis for the control of polymer breakdown, for instance when growing shoots are removed from sprouting tubers (q.v.).

The hydrolase was extracted from tubers and examined to gain information about its action upon different members of the oligosaccharide series present in the artichoke tuber, the nature of the inhibition by sucrose, and its substrate specificity, in order to gain some insight into its mode of action.

Chromatography of the extract on DEAE-cellulose resulted in at least two distinct active fractions of hydrolase: both of these were separate from the invertase activity.

Experimental

An acetone powder was prepared from artichoke tubers for study of the hydrolase. Tubers were washed in tap water, dried with a towel and 361 g. shredded through a stainless steel grater. The mush was strained through four thicknesses of muslin, yielding 210 ml. of juice which was centrifuged at 3000 x g for 10 min. at 0°C. The solid debris was discarded and the supernatant liquid dialysed overnight against tap water in the cold room - Recaldin (1961) states that this denatures most transfructosylase activity, while leaving the hydrolase activity substantially unimpaired. The dialysed solution was centrifuged at 7000 x g for 10 min. at 0°C. The supernatant solution was poured into ten times its volume of acetone at -15°C; after standing for 20 min. in a deep freeze at -15°C the clear supernatant aqueous acetone was decanted, and the precipitate separated from the remainder by centrifugation at 4000 x g for 10 min. at -10°C. The precipitate was washed by resuspending it in dry acetone at -15°C, recentrifuged, and the acetone discarded. The precipitate (except for about one-sixth of the total which

was lost) was dried under suction, giving 7.1 g. of pale brown powder.

The ketose content of the powder was estimated: 12.4 mg. of powder was found to contain 9.98 mg. of total ketose, expressed as free fructose. The weight of combined fructose, i.e. anhydrofructose, was thus 8.98 mg., which accounts for 72.4% of the weight of the powder.

The powder was assayed for hydrolase activity upon its endogenous fructosan. Digests were made up so that the final concentration of ketose was either 2% or 5% w/v (expressed as free fructose) by suspending the appropriate weights of acetone powder in 0.1 M-acetate buffer pH 5. The digests were covered with a layer of toluene and incubated at room temperature (about 17°C). At intervals, 100 µl. samples of the digests were delivered into 1.9 ml. of distilled water which had been heated during the previous minute in a boiling water bath; the tubes were covered with a glass marble and replaced in the water bath for a further three minutes to destroy the enzyme activity. After cooling, the reducing power of the samples was estimated by the total reducing sugar method. The results are given in Table 14, and show that reducing power was produced in the digests, indicating the presence of a hydrolase. It is also obvious that the rate of production of reducing power was very different in the two digests: if the substrate were saturating the enzyme at both levels, the rates of hydrolysis should have been proportional to the amounts of enzyme present, that is by a factor of 2.5. This was not so, indicating a substantial effect of change in substrate concentration from 20 to 50 mg./ml, as is shown more clearly in Table 15.

Table 14. Liberation of reducing sugar from endogenous fructosan in digests of acetone powder in 0.1 M-acetate buffer pH 5, incubated at about 17°C.

mg. acetone powder/ml.	Endogenous substrate as combined fructose (μM)	Reducing sugar as fructose (μM/ml)		
		1 min.	6 hr.	24 hr.
25	111	0.89	2.33	6.34
62.5	278	1.78	10.8	26.6

Table 15. Effect of concentration of endogenous fructosan on rate of production of reducing sugar.

(Values calculated from data in Table 14)

Substrate concentration mg./ml.	Rate of increase in reducing sugar as μM fructose/ml./hr./mg. acetone powder	
	6 hr.	24 hr.
20	0.0096	0.0091
50	0.024	0.017

The effect of sucrose on the hydrolysis of the native substrate was investigated, quantitatively by reducing sugar estimation and qualitatively by paper chromatography. Three concentrations of sucrose were used together with a control digest without sucrose. Samples were taken immediately after the addition of the reconstituted suspension of acetone powder to the sucrose solutions and again after five hours. The results of the reducing sugar estimations, given in Table 16, show that the increase in reducing sugar is drastically inhibited by the presence of sucrose. The chromatograms when sprayed with benzidine-TCA showed that glucose was liberated in the digests in which sucrose was present; in these digests a spot assumed to be 1^F-fructosylsucrose also

appeared. The formation of these sugars suggests that both invertase and transfructosylase* activities were present in the acetone powder. As the figures given in Table 16 are based on the total reducing sugar estimations, the inhibitory effect of sucrose on fructosan hydrolysis was greater than these values indicate since reducing sugar produced by the action of invertase on sucrose and perhaps other oligosaccharides was not differentiated from reducing sugar liberated by the action of the hydrolase.

Table 16. Effect of sucrose on hydrolase activity.

Digests contained 70 mg./ml. of acetone powder in 0.1 M-acetate buffer pH 5, and were incubated at 30°C.

Sucrose concentration	Reducing sugar, as fructose μM/ml			Inhibition (%)
	Initial	5 hr.	Increase	
0	2.68	15.7	13.0	-
0.01 M	2.44	10.3	7.86	39.6
0.05 M	2.94	9.63	6.67	48.8
0.10 M	3.06	9.00	5.95	54.3

During the incubation periods of the previous experiments, it was noticed that much of the acetone powder had settled out - this undissolved material was not examined, and could have included both protein and substrate. The relative solubilities of the ketose and the active protein were investigated by the removal and extraction of the insoluble material. Acetone powder (500 mg.) was suspended in 8.0 ml. of 0.1 M-acetate buffer pH 5, and 1.0 ml. removed; the remainder was centrifuged at 3000 x g for five minutes. The supernatant solution

* cf. Edelman & Bacon, 1951 a.

(4.7 ml.) was decanted and replaced by an equal volume of acetate buffer; the solid was resuspended and 1.0 ml. removed. This process of extraction was repeated a further four times and total ketose estimations were made on each of the extracts. A second lot of acetone powder was treated in the same manner, except that the extraction was done with 0.1 M-acetate buffer containing 6% w/v inulin. The 1.0 ml. samples were incubated for 4 hr. and sampled for reducing sugar before and after the incubation period. The results in Table 17 show that the active protein was less soluble than the native substrate. It is probable that the comparison of hydrolase activity in the different samples is not an exact one since it is based on the assumption that the rate of hydrolysis of the endogenous substrate and of inulin did not differ greatly, although the fructosans present in the acetone powder seem by their solubility to be of considerably shorter chain length than commercial inulin (but refer to later results).

Table 17. Relative solubility of hydrolase activity and endogenous fructosan of acetone powder in 0.1 M-acetate buffer pH 5.

For details of experiment see text. Enzyme digests were incubated at 30°C.

Number of washings	Total fructose content (mg./ml.)	Hydrolase activity as reducing sugar released (μM/ml./ 4hr.)	Total fructose content as percentage of original	Hydrolase activity as percentage of original
0	65	8.96	100	100
1	18	4.36	28	49
2	6.2	3.17	10	35
3	2.6	2.46	4.0	28
4	1.1	1.31	1.7	15
5	0.7	0.90	1.1	10

The inhibitory effect of sucrose was re-examined using 5% w/v inulin as substrate and acetone powder preparation washed twice in acetate buffer to remove the bulk (90%) of the endogenous substrate. The digests were incubated in a water bath at 29°C and sampled 1 minute after the addition of the enzyme solution and again after 4 hr. The results are given in Table 18; as before sucrose hydrolysis was not differentiated from that of inulin. The relative contributions of the inulin and the sucrose to the increase in total reducing sugar was

Table 18. Effect of sucrose on hydrolysis of inulin.

Each digest contained 0.01 M-inulin on 0.1 M-acetate buffer pH 5, and the amount of enzyme remaining after extracting 125 mg. of acetone powder twice with 0.1 M-acetate buffer pH 5 (see Table 17). Digests were incubated at 30°C.

Sucrose concentration	Reducing sugar as fructose (μM/ml)			Increase as percent. of control
	Initial	4 hr.	Increase	
0	4.18	8.00	3.82	(100)
0.001 M	4.92	8.98	4.06	106
0.0033 M	4.96	9.17	4.21	111
0.01 M	5.22	7.27	2.05	66.2
0.033 M	5.26	7.12	1.86	48.5

examined at 0.01 M-sucrose by making up a series of incubation mixtures as follows; enzyme without substrate, enzyme with inulin, enzyme with sucrose, and enzyme with both inulin and sucrose. The suspension of acetone powder was centrifuged, and the digests incubated at 30°C. The results are given in Table 19 which shows that there was considerable hydrolysis of sucrose. Chromatograms of samples taken from the digests after 26 hr. incubation were run in order to separate the glucose and

fructose, which were then eluted and estimated separately. The results of the experiment, corrected for paper blanks, (Fig. 16) show that the extent of sucrose hydrolysis was the same whether inulin was present or not.

Table 19. Effect of hydrolase preparation on inulin, sucrose, and a mixture of inulin and sucrose.

Each digest contained the amount of enzyme remaining after extracting 125 mg. of acetone powder twice with 0.1 M-acetate buffer pH 5. Inulin and sucrose were supplied at concentrations of 0.01 M in 0.1 M-acetate buffer pH 5. Digests were incubated at 30°C.

Substrate	Reducing sugar as fructose (μM/ml)		
	1 min.	4 hr.	26 hr.
None	1.46	2.23	5.28
Inulin	5.00	9.95	25.4
Sucrose	1.76	3.33	10.1
Inulin + sucrose	5.07	8.72	23.1

Preparation of Enzyme free of Substrate.

A preparation of hydrolase was made from tubers which had been stored in the cold room for 11 weeks; from the results in Section I it can be assumed that extensive depolymerization had occurred over this period, the high molecular weight compounds such as inulin being converted to more dialysable compounds such as sucrose and other lower oligosaccharides. In order to minimize invertase activity buds were first removed as Recaldin (1960) states that invertase is more concentrated in the buds of sprouting tubers. The tubers were then washed, and shredded through a stainless steel grater: the juice was extracted

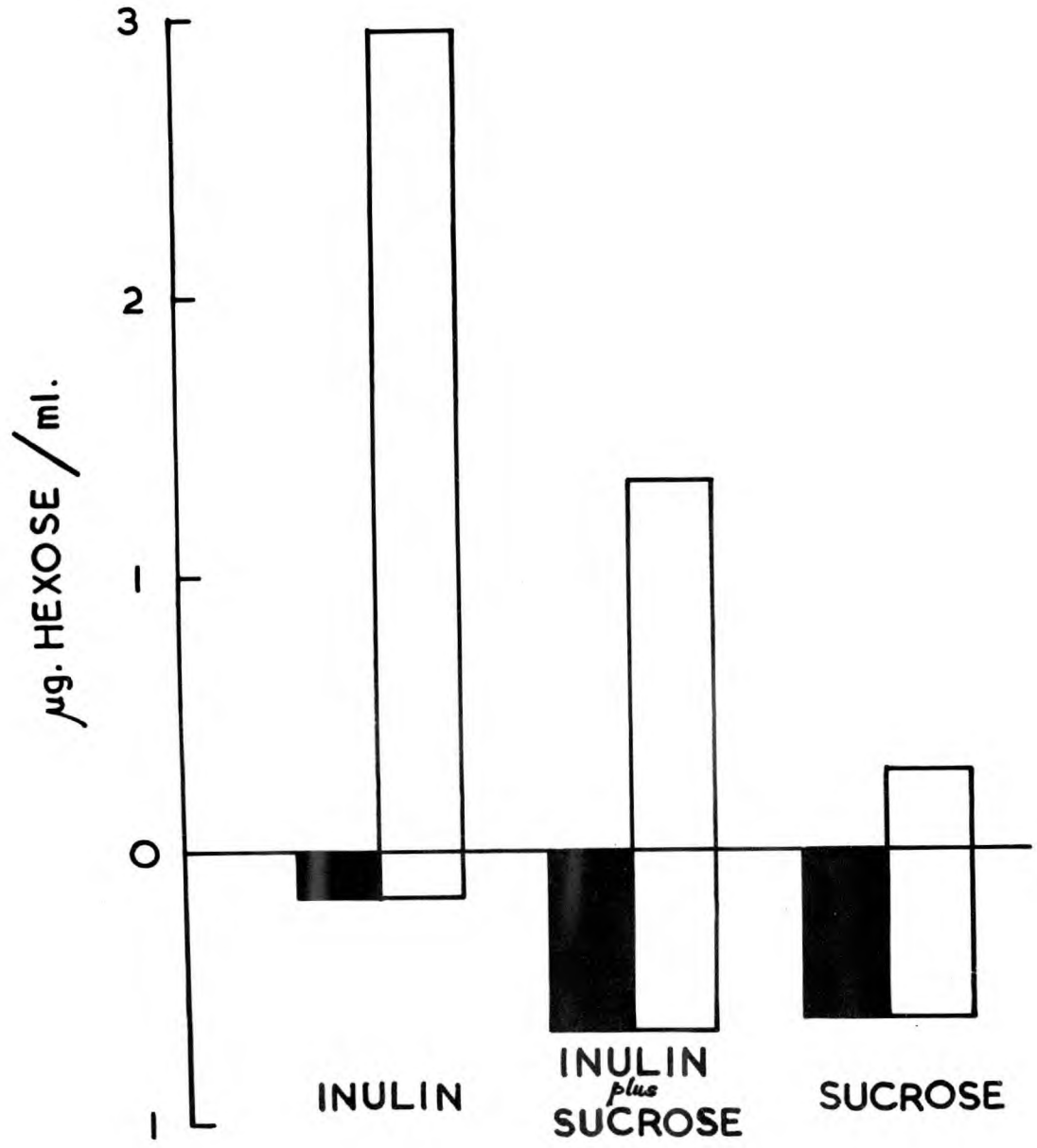


Figure 16. Action of hydrolase preparation in digests containing inulin, sucrose, and inulin plus sucrose. For details of experiment see text. The columns represent the amounts of reducing sugar recovered from chromatograms of samples from the digests; solid columns - glucose, open columns - fructose.

from the grated material by squeezing through four thicknesses of muslin, the yield being 295 ml. (pH about 6) from 497 g. of tubers. The juice was centrifuged at 3000 x g for 10 min. at 0°C and the precipitate discarded. The supernatant was dialysed overnight against 0.005 M-phosphate buffer pH 6.5. The calculated amount of ammonium sulphate was added gradually to the dialysed juice until the solution reached 70% saturation with respect to this salt; during this process the solution was maintained at about pH 7.5 by the addition of N-NaOH (external indicator: bromothymol blue). The suspension was allowed to stand for 20 min. in the cold room and then centrifuged at 8000 x g for 10 min. at 0°C. The supernatant solution was discarded and the precipitate washed by resuspending it twice in 70% saturated ammonium sulphate solution at pH 7. The washed precipitate was dissolved in 0.001 M-phosphate buffer pH 6.5 and the solution, which contained a small precipitate, was dialysed overnight against the same buffer. The dialysed solution was poured into ten times its volume of dry acetone at -15°C and allowed to stand for 20 min. at the same temperature. After decanting the bulk of the aqueous acetone, the precipitate was separated from the remainder by centrifuging at 8000 x g for 10 min. at -10°C, suspended in dry acetone at -15°C, recentrifuged, and dried under suction: the yield of dark brown, granular material was 860 mg., corresponding to 1.73 mg./g. of fresh tuber.

The ketose content of the powder was determined by suspending 27.8 mg. in 3.0 ml. of 0.1 M-acetate buffer pH 5; samples were taken for the total fructose estimation but when the acid reagent was added a faint yellow colour was produced - the colour intensity was therefore

read before as well as after the customary heating. The anhydrofructose content of the dry powder after correction for the initial colour was found to be only 0.3% which, when compared with the value of 72.4% for the unpurified preparation (see p.57), demonstrates that virtual elimination of endogenous substrate had been achieved.

The activity of the preparation against 5% w/v inulin was determined. A number of digests was made up containing differing quantities of the acetone powder: as this was added partly in suspension, it was not possible to measure the quantities accurately. The digests were incubated at 28°C, and samples removed immediately after the addition of the enzyme, after 4 and after 20 hr. The increase in total reducing substances was estimated. The results given in Table 20 show the enzyme was active. The rate of hydrolysis was virtually constant at least up to 16% breakdown of the inulin present, and within the limits of accuracy of dispensing the enzyme preparation, was independent of enzyme concentration.

This powder was used to investigate the influence of substrate concentration on the rate of hydrolysis of inulin, over the range 1.25 - 50 mg./ml. The digests were made up in 0.1 M-acetate buffer pH 5 and incubated at 30°C. They were sampled at intervals and the amount of reducing substances estimated. The results (Fig. 17) show that there is an increase in the rate of hydrolysis with increase in substrate concentration over the whole of the range used in this experiment. When the data are expressed in the form of a Lineweaver-Burk plot, they fall on the curve shown in Fig. 18 - such a line is inconsistent with any of

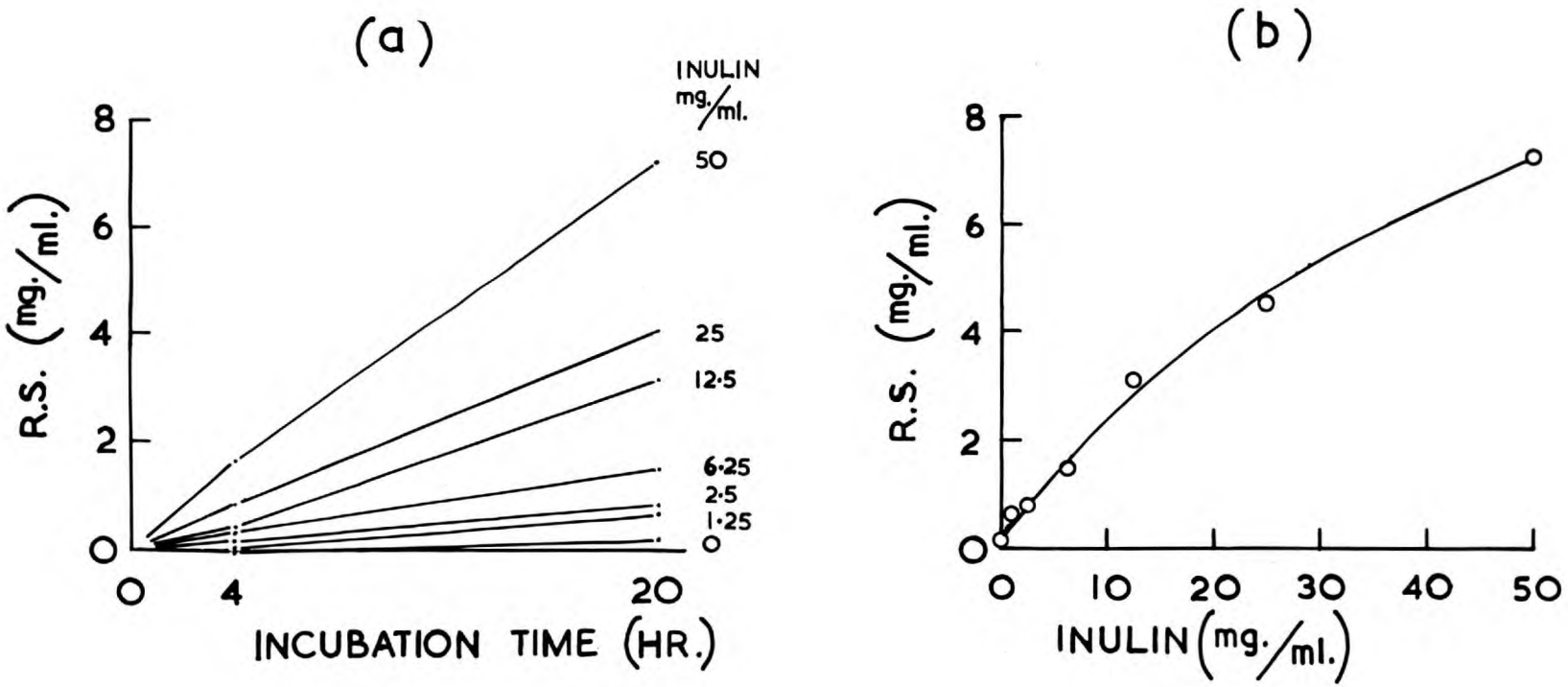


Figure 17. Effect of substrate concentration on the rate of hydrolysis of inulin.

(a) Time course of reaction in the digests.
(b) Extent of hydrolysis after 20 hr.

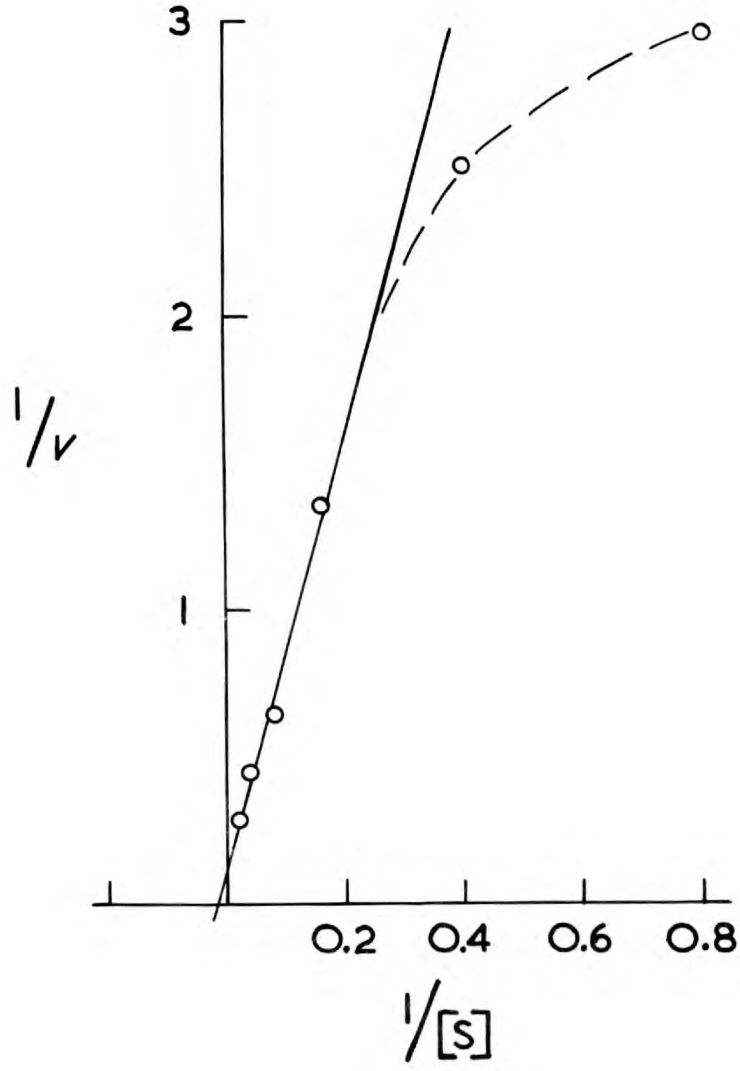


Figure 18. Lineweaver-Burk plot to determine the K_m of inulin hydrolysis. Values calculated from data in Fig. 17 (20 hr. values).

Table 20. Effect of enzyme concentration on the rate of hydrolysis of inulin.

The digests contained 0.01 M-inulin in 0.1M-acetate buffer pH 5; 28°C.

Concentration of acetone powder (mg./ml.)	Increase in reducing sugar, estimated as fructose (μM) 0–4 hr.	0–20 hr.	Rate of increase in reducing sugar μM/ml./hr./mg. powder 0–4 hr.		0–20 hr.	
24	9.40	44.3*	0.098		0.091	
16 (12)†	4.58	22.3	0.072	(0.095)†	0.069	(0.093)†
8	3.13	15.6	0.098		0.098	
4	1.33	6.67	0.083		0.083	
0	-0.17	0.06	-		-	

* this value represents hydrolysis of 16% of the inulin originally present.

† 0.3 ml. of enzyme solution (pipetted by error instead of 0.4 ml) would give these values.

the theoretical curves which may be expected for an enzyme acting on a single substrate. From the nature of the reaction, once an inulin molecule has been attacked a further substrate molecule of shorter chain length is released, without, however, changing the molar concentration of terminal fructose residues. The ease with which the terminal fructose of an oligosaccharide in hydrolysed by the enzyme is likely to depend on its DP; in fact, the form of the Lineweaver-Burk curve strongly suggests that the mixture of shorter chain compounds produced by extensive hydrolysis of inulin (viz. 40–50% at the lower concentrations) was more readily attacked by the enzyme. Moreover, if the reaction proceeded so far as to release sucrose from the chains, inhibition may be expected to have had a progressively increasing influence. The rates, therefore, at the lowest substrate concentrations in this experiment must

have been resultants of a complex interplay of factors. However, the rates at the four higher substrate concentrations lie on a reasonably straight line from which an estimate of Km can be made, viz. 66 mg/ml, which approximates to 1.3×10^{-2}M assuming the mol. wt. of inulin to be 5000. This value demonstrates low affinity of the enzyme for inulin, and makes it impossible to carry out experiments at saturating concentrations of this substrate.

The suggestion from this experiment that substrates of shorter chain length were hydrolysed more readily than inulin was investigated by comparing the rates of hydrolysis of the oligosaccharides of DP 3-6 with that of inulin. The oligosaccharides were separated from tuber extracts by paper chromatography; a sucrose sample was prepared in the same way. The inulin used was the commercial preparation from dandelion tap roots, in which the lowest detectable oligosaccharide is that of DP 15 (see p.76).

The results of the experiment are given in Table 21, and it is evident that the initial rates of hydrolysis of the oligosaccharides were considerably greater than that of inulin. There is also an indication that the rate rises with rising DP from DP 3 to 5 or 6. More precise analysis of the results is complicated by the following considerations:

1. The substrate concentrations were not the same (see column "a", Table 21) owing to arithmetical error.

2. The enzyme preparation contained substantial invertase activity (see sucrose data in Table 21) which not only released spurious reducing power from the sucrose appearing in the digests, but may have directly attacked the oligosaccharides: the invertase effect is clearly shown by

comparing columns "b" and "c" in Table 21.

Table 21. Rate of hydrolysis of oligosaccharides extracted from artichoke tubers.

The digests contained 24 mg./ml. of acetone powder in 0.1 M-acetate buffer pH 5; 30°C.

Substrate	Concentration	Increase in reducing sugar as fructose µM/ml				Concentration of extra-sucrosyl fructose µM/ml
		1 hr.	2 hr.	4 hr.	20 hr.	
	(a)				(b)	(c)
None	-	-	-	0.26	2.03	0
Sucrose*	4	0.72	1.02	1.72	5.83	0
DP 2 (sucrose)	4	0.67	0.58	1.82	5.48	0
DP 3	6	1.75	3.96	6.19	12.5	6
DP 4	4	1.71	3.14	5.25	12.5	8
DP 5	4	3.14	6.08	9.05	16.8	12
DP 6	4	2.96	5.88	10.1	20.2	16
DP 7	4.7	2.14	4.83	9.18	21.6	23.5
Inulin/	2.5	0.98	2.35	5.06	25.4	69.5

* Analar reagent
/ Biochemical reagent

The affinity of the enzyme for two of the oligosaccharides used in this experiment, viz. those of DP 3 and DP 6, was compared by setting up digests with a series of concentrations of substrate. The digests were incubated at 30°C, and sampled immediately and at times of 1, 2, and 4 hr. after the addition of the enzyme. The results are given in Fig. 19; in the case of the oligosaccharide of DP 6 the rate of hydrolysis increased over the range of concentrations used, whereas that of DP 3 was apparently constant. A Lineweaver-Burk plot of the data is shown in Fig. 20. Using the line of best fit calculated from the formula

$$y = \bar{y} + b\,(x - \bar{x}), \text{ where } b = \frac{xy - x.\bar{y}}{x^2 - x.\bar{x}}$$

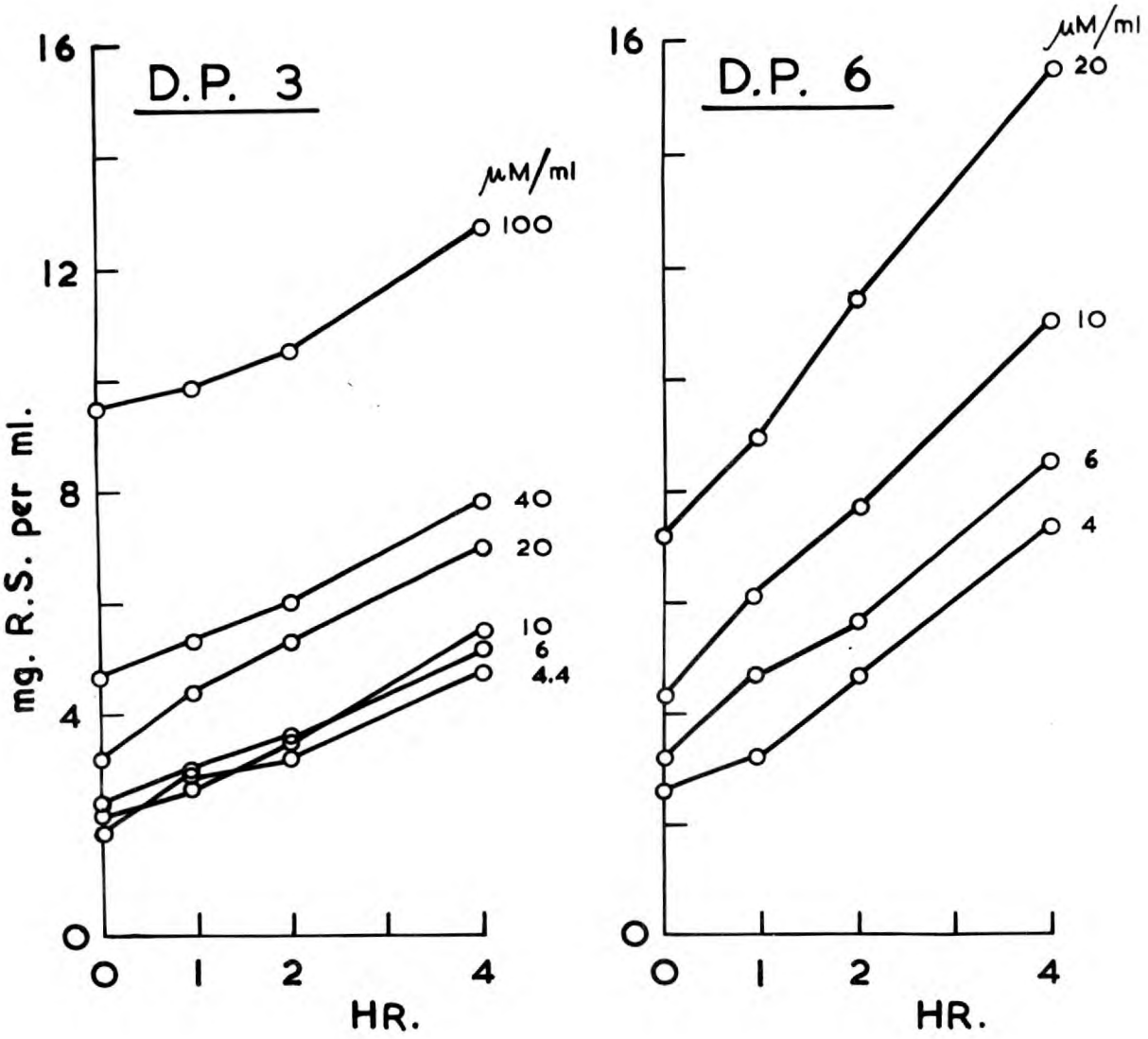


Figure 19. Effect of substrate concentration on the rate of hydrolysis of inulose-1 (DP 3) and inulose-4 (DP 6).

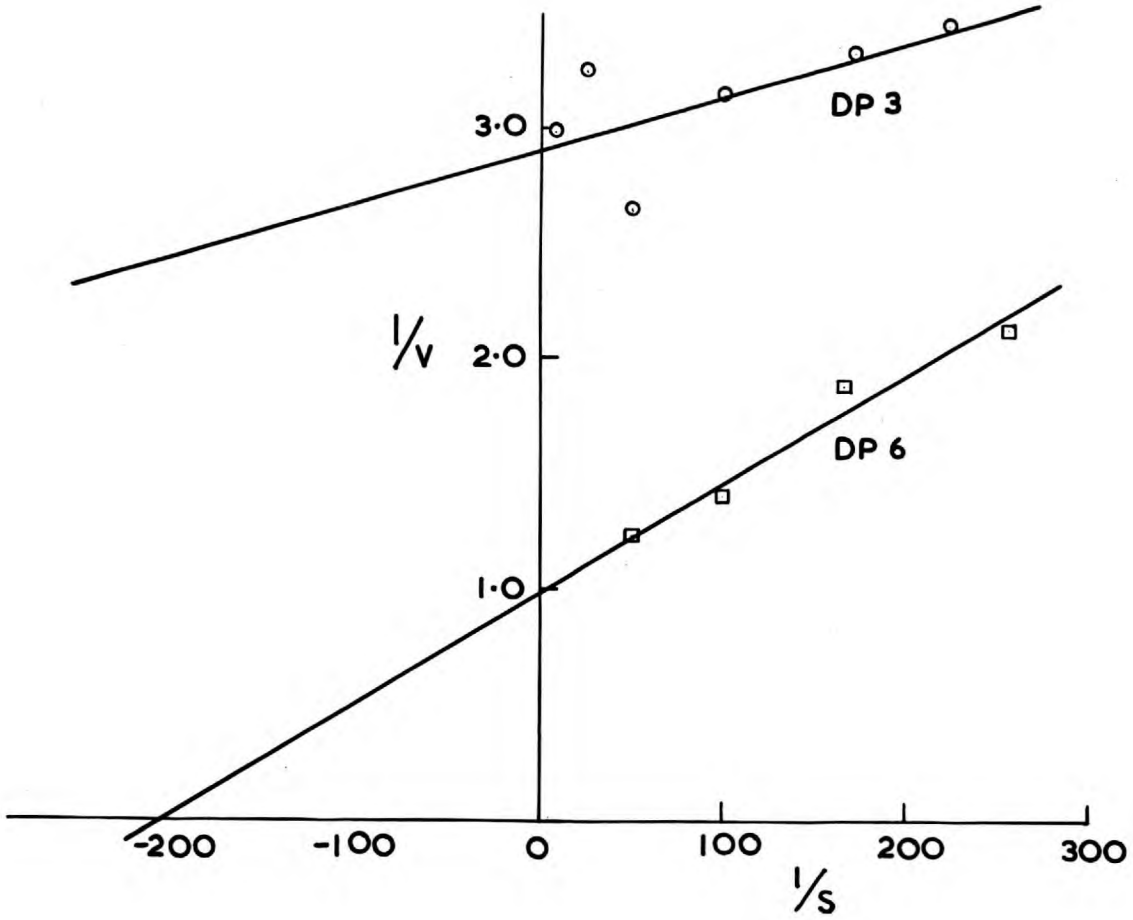


Figure 20. Lineweaver-Burk plots to determine the K_m of inulose-1 and inulose-4. Values calculated from data given in Fig. 19.

the K_m of the oligosaccharide of DP 6 is 4.7×10^{-3}. The values for the oligosaccharide of DP 3 do not appear to approximate to a straight line relationship, particularly at the higher concentrations; this may have been due to increasing inhibition with time owing to increase in the concentration of sucrose liberated as hydrolysis proceeded. No attempt has been made to take this effect into account in analysing the results owing to its complexity, but a simple line of best fit does pass through the points for highest and the three lower concentrations, and gives a value for K_m of 7.6×10^{-4}.

In view of the invertase and possibly other contaminating activities in the enzyme preparations used up to this stage, it was decided to attempt greater purification for further investigation into the properties of the hydrolase: to this end larger quantities of pure substrates were also necessary together with improved methods of analysis of the products of reaction.

The Preparation and Purification of Substrates

1. Individual members of the oligosaccharide series present in artichoke tubers were prepared by paper chromatography on Whatman 3MM or 1.6 MM sheets or on a L.K.B. "Chromax" column, developed with PEAW 7:1:2 or 6:1:3; the eluted sugars were rechromatographed before use. When the chromatographic mobilities of these sugars are plotted in the form of $\log_{10} \frac{R_f}{1-R_f}$ against the assumed D.P. up to 12, a straight line is obtained (Fig. 21); this is consistent with their being members of a homologeous series. In view of the remarks made on p.2 no further proof of their structure was sought.

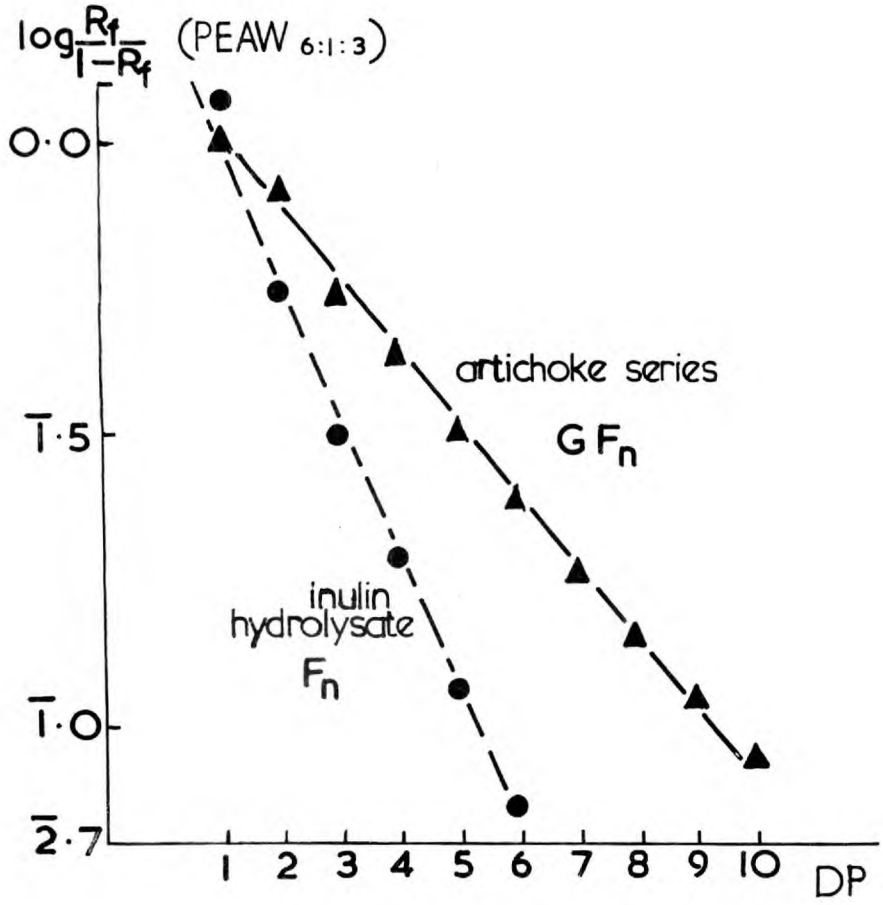


Figure 21. Chromatographic mobilities of oligosaccharide series. Data were taken by measuring the distance moved by the leading edge of each spot on chromatograms developed in PEAW 6:1:3.

A standard mixture of oligosaccharides for enzyme assay was prepared from artichoke tubers which had been stored in the cold room for about eight months. The tubers were washed, disbudded, and 217 g. disintegrated in a Waring blender with about 100 ml. 0.005 M-DIECA, which was added to suppress polyphenoloxidase activity. The homogenate was squeezed through muslin, boiled for 4 min. to denature enzymes, and the juice filtered while still hot through Whatman No. 1 filter paper. Chromatography of a sample of the extract showed that the oligosaccharides of lower DP predominated; there was some material of Rp-zero, and also free fructose. Fractional precipitation with ethanol was carried out to rid the extract of free fructose (in order to reduce the initial reducing sugar value), of sucrose (because of its inhibitory action), and of the sugars of DP greater than ca. 15, which were presumed to be attacked more slowly. A preliminary investigation indicated that a fraction precipitated between 75% (three volumes) and 89% (eight volumes) of ethanol was satisfactory. Accordingly, three volumes of ethanol were added to the 210 ml of extract; the mixture was left at 2°C. overnight and then the precipitate was separated by centrifugation at 9000 x g. for 10 min. The supernatant solution was still opalescent and was therefore filtered; a further five volumes of ethanol were added and the mixture was allowed to stand in the cold room overnight before separation of the precipitate by centrifugation at 9000 x g for 10 min. The precipitate was dissolved in distilled water and eight volumes of ethanol were added to the resulting solution; the material which was precipitated overnight in the cold room was separated as above, dissolved in water and made up to 50 ml: the solution contained

44.5 mg/ml of combined fructose. Chromatography of a sample of this solution showed that it was virtually free of fructose and sucrose, and that the bulk of the material was comprised in the oligosaccharides DP 5-12. This mixture of oligosaccharides will subsequently be referred to as "Substrate M".

The oligosaccharides of this series have not been given convenient trivial names with the exceptions of sucrose and the trisaccharide (1^Ffructosylsucrose). The successive members were called spot 1 (sucrose), spot 2 (trisaccharide), spot 3 etc. (Bacon & Edelman, 1951) but these names give no indication of structural relationships. It is intended here to refer to these sugars as the inulose series, with a suffix indicating the number of fructose residues in the chain attached to t the sucrose molecule; thus sucrose itself would be inulose-0, the trisaccharide, inulose-1, and the hexasaccharide inulose-4. Inulin obtained commercially will be referred to by this name which should be taken to describe a mixture of polymers with the general formula G F-F where the fructosyl residues are joined by 2:1' linkages. A sample of commercial inulin (Thomas Kerfoot & Co. Ltd., Vale of Bardsley, Lancs., batch no. ED 45348) was chromatographed to determine the lower limit of D.P. Successive developments in P.E.A.W. 6:1:3 followed by spraying with the urea-metaphosphoric acid reagent revealed no fructosan of D.P. $<$ 15. A sugar corresponding in Rp to that of D.P. = 15 in the artichoke extract was present but the amount was no more than 0.5% of the inulin applied as judged by the colour given with the spray. Sugars between this and the origin (D.P. $>$ ca. 20) were present in similar amounts and it may thus be assumed that at least 95% of the inulin was composed of polymers of

D.P.> 20.

2. Analogues of the inulose series of series of sugars differing by the absence of a terminal glucose residue were prepared by partial acid hydrolysis of inulin. In a typical preparation, 2.4 g. of inulin was incubated in 11 ml. of 0.1 N-oxalic acid for 30 min. at 65-70°C. The hydrolysate was cooled and put on to a column (3.3 x 14.5 cm.) composed of a mixture of 20 g. B.D.H. activated charcoal and 20 g. Celite 535, and the column washed with about 10 bed volumes of distilled water. Chromatography of a sample of the effluent showed that there was some disaccharide present with the glucose and fructose. This was recovered by paper chromatography. The sugars remaining on the C/Celite column were eluted with 25% v/v ethanol and the effluent was evaporated to dryness. The dried eluate (1.02 g.) was put on to the LKB Chromax paper column which was developed with PEAW 7:1:2; this column, like paper sheets, did not resolve sucrose and the reducing disaccharide (1-fructosyl fructose) satisfactorily; but it was found that this separation could be achieved by using a column of fuller's earth. The disaccharide fractions from the aqueous effluent of the C/Celite column and from the paper column were bulked and put on to a colume (2.8 x 12.5 cm.) of a mixture of 27 g. of fuller's earth and 7 g. of Celite; the column was washed with absolute ethanol, and the sugars applied in ca. 50% v/v ethanol. The column was eluted successively with absolute. 95% and 90% ethanol. A preliminary experiment had indicated that sucrose was eluted by 90% v/v ethanol, while 1-fructosylfructose remained absorbed on the fuller's earth; after all the sucrose had been eluted, the column was washed with

80% v/v EtOH; this did not elute the 1-fructosylfructose which was recovered from the column by eluting with distilled water.

The reducing disaccharide 1-fructosylfructose was named inulobiose by Pazur & Gordon (1953); this terminology will be used and extended to other members of this series, viz. inulotriose, inulotetraose etc.

The structure of the products of hydrolysis was deduced from that of inulin, and the following evidence:-

(1) A chromatogram of the inulin hydrolysate gave a series of spots each of which gave a yellow colour reaction with benzidine-TCA indicating the absence of glucose and a colour reaction with TSTZ spray reagent, which indicated that they were sugars with a reducing fructose group. The mobilities of the spots when plotted in the form of $\log_{10} \frac{R_f}{1-R_f}$ against the assumed DP gave a straight line, indicating that they were members of a homologous series (Fig. 21).

(2) Estimation of the quantity of various spots by the total reducing sugar and the total ketose methods supported the assumed structures (see Table 22).

3. Analogues of the inulose series of oligosaccharides in which the fructose chain was terminated by a melibiose group instead of a glucose group were prepared by transfer of fructose to raffinose, mediated by taka-diastase. Raffinose (4.53 g., final concentration 0.3 M) was incubated in 25 ml. 0.004 M-phosphate-citrate buffer pH 5.5 with 2% w/v of taka-diastase preparation (Parke, Davis & Co., Ltd., Hounslow, Nr. London: lot LT 274B) at 25°C for 21 hr. The digest was then heated in

Table 22. Reducing sugar and total fructose determinations on individual components separated by paper chromatography from the mixture of oligosaccharides obtained by partial acid hydrolysis of inulin.

A. 2.30 g. inulin in 11.0 ml 0.1 N-oxalic acid heated at 65-70°C for 30 min.

B. 2.45 g. inulin in 11.0 ml 0.1 N-oxalic acid heated at 65-70°C for 30 min.

Assumed sugar (a)	Preparation	Reducing sugar as free fructose (mg.) (b)	Total fructose as free fructose (mg.) (c)	Ratio of (c) to (b)	Theoretical ratio, from (a)
inulobiose	A	42.4	87.1	2.06	2
	A*	25.3	45.1	1.78*	
	B*	32.0	59.6	1.86*	
inulotriose	A	35.4	90.6	2.56	3
inulotetraose	A	22.5	88.6	3.94	4
	B	4.48	17.5	3.91	
inulopentaose	A	14.2	66.5	4.68	5
	B	4.79	23.0	4.81	

* after removal of sucrose by chromatography on fuller's earth.

a boiling water bath for 4 min., filtered while hot, and the volume of the filtrate reduced in a rotary evaporator to about 1 ml. of thick reddish-brown syrup. The syrup was put on to the LKB Chromax column and eluted with PEAW 6:1:3; chromatography of samples from the fractions on paper sheets showed that there was considerable overlapping of the sugars present. All the fractions containing sugars with $R_F >$ R_F raffinose were bulked, evaporated to dryness, and finally collected in a tared

beaker; the weight of sugar was 1.40 g. This material was re-run on the LKB column, using PEAW 7:1:2 for development: this solvent effected a satisfactory separation of raffinose from the presumed 1^F-fructosylraffinose, most of which was separated from a spot of lower R_F ($(1^F\text{fructosyl})_2$raffinose). These oligosaccharides were finally purified by chromatography on paper sheets.

4. Analogues of the inulose series which were terminated by a sugar alcohol instead of a glucose molecule were prepared from oligosaccharides with a terminal reducing fructose (see group 2). Solutions of the oligosaccharides from inulobiose to inulopentaose were treated with an excess of sodium borohydride at room temperature for about 30 hr., and the mixtures then poured through C/Celite columns. The columns were washed with ca 5 bed volumes of distilled water, and then with 25% v/v EtOH; material eluted by the latter was evaporated to dryness and then chromatographed on paper sheets, where the products of the reaction were located by spraying marker strips with urea-MPA. Time samples and qualitative chromatograms showed in each case the appearance of a single new spot of lower R_F than the substrate which gave a colour reaction with urea-MPA, but not with the TSTZ spray, indicating that the molecule did not possess a fructose reducing group.

When free fructose is reduced by sodium borohydride the product is a mixture of sorbitol and mannitol. No attempt was made to discover if both sugar alcohols were present in the substances made above.

5. Lactulose (4-β-D-galactosyl-D-fructose) was made from lactose

using dilute alkali to effect a Lobry de Bruyn-Alberda van Ekenstein transformation (Evans, Reynolds & Talley, 1951). Lactose (556 mg.) was incubated at room temperature in 4 ml. 0.035 N-NaOH (Sowden & Schaffer, 1952), and ketose content was estimated during the first 20 hr.; the results are given in Table 23. The mixture was left for a further 4 days, and then poured through columns of "Biodeminrolit". Chromatography of samples showed the appearance of a spot with $R_{lactose} = 1.34$. This was separated on the preparative scale by chromatography on a sheet of Whatman 1-6 MM paper: it was demonstrated to be a reducing compound containing fructose.

Table 23. Appearance of ketose on incubation of lactose in alkaline solution.

Lactose (556 mg.) was incubated in 4.0 ml. 0.035 N-NaOH at room temperature (about 20°C), and samples were removed at intervals.

	Time (hr.)	Ketose (fructose) µg./mg. lactose
Lactose		6.9
Lactose + NaOH	"0"	7.2
	1	9.9
	3.25	14.2
	20	37.3

6. β-methylfructofuranoside was prepared by the action of yeast invertase (0.625%) on sucrose (15% w/v) in 25% v/v methanol (Bacon, 1952). The yeast inverstase (B.D.H. invertase concentrate in glycerol) was dialysed overnight against distilled water, to reduce the concentration of glycerol which also acts as a fructose acceptor. The digest was

incubated at 20°C for 160 min. and then heated in a boiling water bath for 4 min. Chromatography of samples from the digest showed the formation of a non-reducing, substance containing ketose with $R_{fructose} = 1.52$ in PEAW 7:1:2: this compound reached a maximum after about 120 min. The mixture was chromatographed on the LKB Chromax column, and the β-methyl-fructofuranoside re-run on a sheet of Whatman 1.6 MM paper.

7. Irisin was extracted from a rhizome of Iris pseudacorus collected near Thornfalcon, Somerset. The rhizome was washed, peeled, and sliced, and the slices extracted with boiling 80% ethanol and then three changes of boiling distilled water. The extracts were bulked and a sample chromatographed; chromatograms sprayed with benzidine-TCA and with urea-MPA showed a large amount of fructose at the origin, and small quantities of sucrose and free fructose. The bulked extract was stood at 2°C overnight, after which only a small precipitate had formed; the addition of 4 volumes of ethanol produced a heavy white precipitate which was separated by centrifugation after standing overnight at 2°C. This was dissolved in hot water, and the solution centrifuged while hot; the small precipitate was discarded and the solution treated with 4 volumes of ethanol; the precipitated irisin was separated as before and dried in a vacuum desiccator to yield 2.73 g. of white powder, with an anhydro-fructose content of 88%.

8. Bacterial levan was separated from the medium in which a culture of Bacterium subtilis had been grown cf. Dedonder (1961). Nutrient broth (5 g. "Lab Lemco", 10 g. peptone, 5 g. NaCl per litre; pH 5) was mixed with half its volume of 30% w/v sucrose; the mixture was autoclaved,

inoculated with B.subtilis, and kept at 37°C for 24 hr. The bacteria were separated by centrifugation, and the medium then treated with 3 volumes of ethanol and left in the cold room for 2 days, after which the supernatant was decanted from precipitated material. Samples of the precipitate and of the mother liquor were chromatographed in PEAW 7:1:2 for 2 days; the precipitated material did not move from the origin, while the solution contained principally glucose and fructose, a small amount of sucrose, oligosaccharides with R_f's corresponding to those of β-O-D-fructofuranosyl (2→6)-D-glucose, inulose-1, and inulose-2, and some material at the origin. The precipitated levan was redissolved in hot distilled water to give a brown solution, which was again treated with 3 volumes of ethanol. The precipitate was separated by centrifugation as a brown gummy substance, which was dried by treating with several changes of absolute ethanol, and then with acetone. The final yield was 9.1 g. of pale brown powder; the fructose content of 69.3 mg. of the dried powder estimated by the total fructose method was 51.2 mg. of anhydrofructose (= 74% w/w). The levan was further purified by pouring a solution through a column of Zeokarb 225 (H^+ form); the dried levan obtained from the effluent was a white powder with an anhydrofructose content of 80%.

9. β-O-D-fructofuranosyl(2→6)-D-glucose was separated from the medium in which Bacterium subtilis had been grown (see under Bacterial levan). This sugar was present in the fraction soluble in 75% ethanol, and was recovered from this by chromatography on paper. On paper chromatograms this sugar reacted with the benzidine-TCA and urea-MPA sprays,

but not with the TSTZ spray. Estimation of a quantity of the sugar gave values of 367 μg with the total fructose method and 378 μg with the reducing sugar method. These results, together with the R_f values were taken as confirmation of its structure.

...

Assay for hydrolase activity

The estimation of hydrolase activity by determination of the increase in total reducing sugar in samples of the enzyme digests was unsatisfactory in many cases, especially where the initial values were high in comparison to the increases found. This situation would have been still more unfavourable if used for digests in which the substrates were themselves reducing sugars, as for example those obtained by the partial hydrolysis of inulin. Previous work e.g. Edelman & Bacon (1951) and the experiment described on p.61 indicated that the enzyme released single molecules of fructose. Free fructose could be effectively separated from oligo- and polysaccharides in the digests by paper chromatography but owing to the laborious nature of this method it was unsatisfactory for routine analysis. Experiments were therefore carried out to see if the fructose could be separated more simply by chromatography of the digests on small carbon-celite columns.

A mixture of equal weights of charcoal (Hopkin & Williams "M.F.C.") and Celite (Johns Manville Celite 535) was made, columns containing 1 g. quantities of the mixture were set up. The columns were washed with distilled water; the pH of the effluent was not under 5. Various known amounts of inulin and of fructose were put on the columns which were then eluted with distilled water. The amount of fructose,

free or combined, in the effluents was determined by the total fructose method. The results given in Table 24 show that the columns retained free fructose as well as inulin. The bed volume of the columns was about 2.5 ml., and thus 6 ml. of effluent represents 2-4 bed volumes.

Table 24. Behaviour of inulin and fructose on C/Celite columns.

Hopkins & Williams Charcoal M.F.C.

John's Manville Celite 535

Column	Load	1 g. 1:1 w/w per column µg F in effluent (consecutive 3 ml. fractions.)	
		1	2
1	none	5.1	-
2	10 mg inulin	6.5	2.0
3	40 mg inulin	9.6	0.9
4	2.31 mg fructose	6.2	3.8
5	23.1 µg fructose	5.1	1.7

A second attempt was made using columns of 0.5 g. of a 1:1 w/w mixture of B.D.H. Activated charcoal and Celite 535. The columns were washed with distilled water; the effluent from these columns was at first strongly acid, and washing with water was continued until the pH of the effluent was not under 5. The columns were charged with free fructose or inulin, and eluted with distilled water. The results given in Table 25 show that this type of charcoal yielded the free fructose almost quantitatively over the range 20-2000 µg. in the first 6 ml. of effluent; the effluent from the column charged with inulin also contained some fructose, but the amount was only 0.2% of the weight of inulin applied; no attempt was made to discover if this material was free or combined fructose.

Table 25. Behaviour of inulin and fructose on C/Celite columns.

B.D.H. Activated Charcoal M.F.C.

John's Manville celite 535

Column	Load	0.5 g. 1:1 w/w per column µg F in effluent (consecutive 3 ml. fractions)		
		1	2	3
1	none	0	0	0
2	10 mg inulin	13.6	8.2	-
3	2 mg fructose	1818	222	2.1
4	20 µg fructose	14.7	6.1	0

Although this system was entirely satisfactory in delivering free fructose and retaining inulin, a further experiment was made in an attempt to reduce the volume of effluent containing the free fructose to make its subsequent estimation more convenient. The bed volume of the columns was accordingly reduced, without changing the quantity of charcoal, by using 0.35 g. of a 3:1 w/w mixture of B.D.H. activated charcoal and celite 535. The columns were washed until the effluent was no longer acid. Inulin and fructose were put on to columns which were then eluted with distilled water. The amount of fructose in the effluents was determined by the total fructose method; the results (Table 26) show that the fructose was recovered quantitatively - the inulin again gave a value of about 0.2% of the weight applied. Although the fructose was not all eluted in the first 3 ml. of effluent, a comparison of the amounts of fructose in the first and second 3 ml. fractions in this and the previous experiment (see Table 27) shows that the reduction in the bed volume of the columns had resulted in an earlier emergence of the fructose.

Table 26. Behaviour of inulin and fructose on C/Celite columns.

B.D.H. Activated Charcoal

John's Manville Celite 535

0.35 g. of 3:1 w/w mixture per column.

Column	Load	µg fructose in effluent (consecutive 3 ml. fractions)	
		1	2
1	none	0	0.5
2	10 mg inulin	15.7	3.6
3	1.78 mg fructose	1680	87.0
4	17.8 µg fructose	14.5	3.4

Table 27. Elution of fructose from C/Celite columns.

Data from Tables 25 and 26.

C/Celite mixture	Wt. of fructose	Recovery of amount put on to column (% in consecutive 3 ml. fractions).		
		1	2	Total
1:1	2.0 mg	88.3	10.8	99.1
1:1	20 µg	70.8	29.3	100.1
3:1	1.78 mg	94.4	4.9	99.3
3:1	17.8 µg	81.6	19.1	100.7

These experiments showed that fructose could be eluted quantitatively from C/Celite columns under conditions in which oligosaccharides would be expected to be retained. It therefore became possible, in the absence of the substrates containing fructose, to measure free fructose released by enzyme action with the resorcinol-HCl method instead of the less specific reducing sugar method.

Mercuric chloride was used to stop enzyme action in digests before samples were applied to C/Celite columns; the suitability of this procedure was demonstrated by experiment. Three digests were made up

with hydrolase A, hydrolase B, and without enzyme respectively, using inulopentaose as substrate (0.0067 M in 0.1 M-acetate buffer pH 5). The digests were incubated at 25°C for 160 min. and then 0.01 M-mercuric chloride added to give a final concentration of 0.002 M. Samples were chromatographed immediately on C/Celite columns, and the digests were then incubated for a further 16 hr. at 25°C, after which samples were again chromatographed on similar columns. The amounts of fructose in the effluents (4.5 ml) are given in Table 28 which clearly shows that there was no significant reaction after the addition of mercuric chloride. As an added precaution, digests which had been treated with mercuric chloride were routinely stored in the deep freeze when it was not possible to perform the chromatography immediately.

Table 28. The effect of $HgCl_2$ on the activity of hydrolase A and and hydrolase B.

Substrate: 0.005 M-inulopentaose in 0.1 M-acetate buffer pH 5;

Enzymes: 0.1 ml. from DEAE-Cellulose column fractions (q.v.)

Digests (total vol. 0.4 ml.) incubated at 25°C.

Enzyme	Free fructose liberated µM/ml.	
	160 min.	160 min. plus 16 hr. in presence of 0.002 M-$HgCl_2$
none	0.14	0.10
hydrolase A	1.15	1.24
hydrolase B	2.70	2.79

Assay of hydrolytic activity by this method was compared with that made by estimating the increase in reducing sugar. Two sets of digests were made up, in each of which 0.01 M-inulin and 0.01 M-inulose-3 were incubated at 25°C with hydrolase A, hydrolase B, and without enzyme. In one set the total reducing sugar was estimated in samples taken from the digests at 1 and 160 min.; the other set was treated with mercuric chloride after 180 min. and the amount of free fructose in the digests estimated by the resorcinol-HCl method after samples had been chromatographed on C/Celite columns. The results are given in Table 29; it is evident that the more accurate method of fructose analysis is suitable for assaying the enzyme activity.

Table 29. Comparison of assay methods for estimation of hydrolase activity. Digests (0.4 ml.) contained 0.2 ml. of fractions from DEAE-cellulose columns, and were incubated in 0.1 M-acetate buffer pH 5 at 25°C. Two sets of digests were made up; one was assayed by estimation of the increase in total reducing sugar (I), and the other by estimation of total fructose content after chromatography on C/Celite columns, (II).

Substrate	Enzyme	I mU (mμM R.S.(as fructose)/ ml./min.)	II mU (mμM fructose/ml./min.)
inulose-3	none	0.22	0.83
	hydrolase A	14.6	14.2
	hydrolase B	37.0	31.8
inulin	none	1.89	1.67
	hydrolase A	6.45	7.95
	hydrolase B	19.2	21.5

Purification of Hydrolase by Chromatography on DEAE-cellulose.

Another batch of substrate-free acetone powder was prepared from tubers which had been stored in the cold room for six months. The tubers were disbudded, peeled, washed and dried. The weighed tuber material (590 g.) was macerated in small batches in a Waring blender in the cold room in a medium containing 0.1 M-cysteine, 0.05 M-DIECA, and 0.02 M-phosphate buffer, each reagent with pH adjusted to 7.2. The homogenate was filtered through four thicknesses of muslin and the filtrate added back together with a little cold distilled water and more tissue to the blender. The macerate was finally squeezed in the muslin, yielding 610 ml. of extract and leaving 95 g. of pulp. The extract was dialysed overnight against cold tap-water containing DIECA and phosphate buffer pH 7.2, and then centrifuged at 2800 x g for 10 min. at 0°C. Protein was precipitated from the supernatant by adding solid ammonium sulphate to the solution until it was 50% saturated with respect to the salt. While the salt was being added and dissolved, the solution was maintained at about pH 7.5 by the addition of N-KOH and using an external indicator. The precipitated protein was separated by centrifugation at 10,000 x g for 10 min. at 0°C after the solution had been stood for 20 min. The precipitate was washed with 50% saturated ammonium sulphate solution and then dissolved in 0.005 M-phosphate buffer pH 7.5. The solution was dialysed overnight against cold tap-water, centrifuged at 3000 x g for 10 min. at 0°C and then poured into 12.5 volumes of acetone at -15°C. A quantity of talc (2.13 g.) which had ben washed with distilled water was added to the suspension of precipitated protein in acetone to try to prevent the formation of hard lumps in the dried powder.

After standing for 20 min. most of the aqueous acetone was decanted and the mixture of precipitated protein and talc was separated from the remainder by centrifugation at 8000 x g for 10 min. at -10°C. The solid material was washed twice with dry acetone at -15°C, and dried under suction and then under vacuum to yield 2.4 g. of pale brown friable powder, containing 0.1% w/w of anhydrofructose and 35% of soluble protein estimated by the Neilsen method and calibrated against casein.

In the experiments described above routine estimation of hydrolase activity was carried out.using inulin as substrate; chromatography of the enzyme required assay of activity in numerous fractions and for this it was desirable to use a substrate which was attacked more quickly; inulin was also inconvenient since solutions containing enough of this substrate to permit a suitable rate of hydrolysis deposited inulin after a few hours when stored in the cold room. Oligosaccharides such as those of DP 3 to 6 were hydrolysed much faster than inulin (p.69) and a mixture of oligosaccharides (substrate M, see p.75) was used for subsequent enzyme assays; it is clear that a mixture of such oligosaccharides will serve to determine enzyme activity in relative units as long as the proportion of the sugars in the mixture is constant.

The presence of hydrolase activity was proved by incubation of a sample of the powder with substrate M (see p.75), and incubation with sucrose showed that invertase activity was also present in the powder. This material was applied to a DEAE-cellulose column. DEAE-cellulose (300 mg. Whatman's DE50, powder form) was made into a slurry in distilled water and poured into a glass tube plugged with glass wool. Distilled water was allowed to flow until the column of cellulose powder appeared

evenly packed. The column was washed with 1N-NaOH (about five bed volumes) and then with 0.005M phosphate buffer pH 7.5 until the pH of the effluent buffer was unchanged.

A protein solution for chromatography was prepared by suspending 109 mg. of the acetone powder in 2 ml. of 0.005 M-phosphate buffer pH 7.5; the suspension was centrifuged at 3000 x g for 1 min. at room temperature, and the precipitate of talc and undissolved material discarded. The solution was dialysed overnight against 0.005 M-phosphate buffer pH 7.5, and then transferred to a graduated centrifuge tube: from the 1.8 ml. recovered, 0.2 ml. was used for assay of the hydrolase activity. The remaining 1.4 ml. was put on the column at the rate of up to 40 mg. of protein per 100 mg. of cellulose; after elution with 0.005 M-phosphate buffer the column was eluted with solutions of successively greater ionic strength. The ionic strength was increased by using phosphate buffers uptto a concentration of 0.05 M, and then by using 0.05 M-phosphate buffer with increasing concentrations of sodium chloride. The buffers were made up to give a pH of 7.2 - 7.3, and then adjusted to pH 7.5 by the addition of 1N KOH.

Collection of the effluent was started at once; the solution was washed in with 0.005 M-phosphate buffer pH 7.5 and further volumes of this buffer were added until two 3.0 ml. fractions had been collected. The eluting buffer was then replaced by 0.02 M-phosphate buffer pH 7.5 and two further 3.0 ml. fractions were collected. The full elution procedure is shown in Table 30; the rate of flow of buffer through the column was 15-20 ml./hr. The fractions were assayed for hydrolase

activity by incubating samples of the fractions with substrate M, and for invertase activity by incubating with sucrose. The digests were incubated at 30°C, and sampled at 4 and at 24 hr. The results are given in the form of histograms in Fig. 22, and indicate that there was a separation of two peaks of hydrolase activity both separated from the peak of invertase activity.

Table 30. Elution procedure for DEAE-cellulose column.

Column (0.6 x 7.0 cm.) of 300 mg. DEAE-Cellulose as described in text. The phosphate buffers were made up with Na_2HPO_4 and KH_2PO_4.

Fraction number	Volume of fraction (ml.)	Eluting buffer	pH of buffer
1	3.0	0.005 M-phosphate	7.5
2	3.0		
3	3.0	0.02 M-phosphate	7.5
4	3.0		
5	3.0	0.05 M-phosphate	7.5
6	3.1		
7	3.1	0.05 M-phosphate + 0.10 M-NaCl	7.5
8	3.0		
9	3.0	0.05 M-phosphate + 0.20 M-NaCl	7.5
10	3.0		
11	3.0		
12	3.0	0.05 M-phosphate + 0.30 M-NaCl	8.0
13	3.0		

The residual volumes of the fractions were put in a deep freeze at -25°C. After two days in the deep freeze, certain fractions were removed, allowed to thaw, and bulked as follows; fractions 2 + 3 -

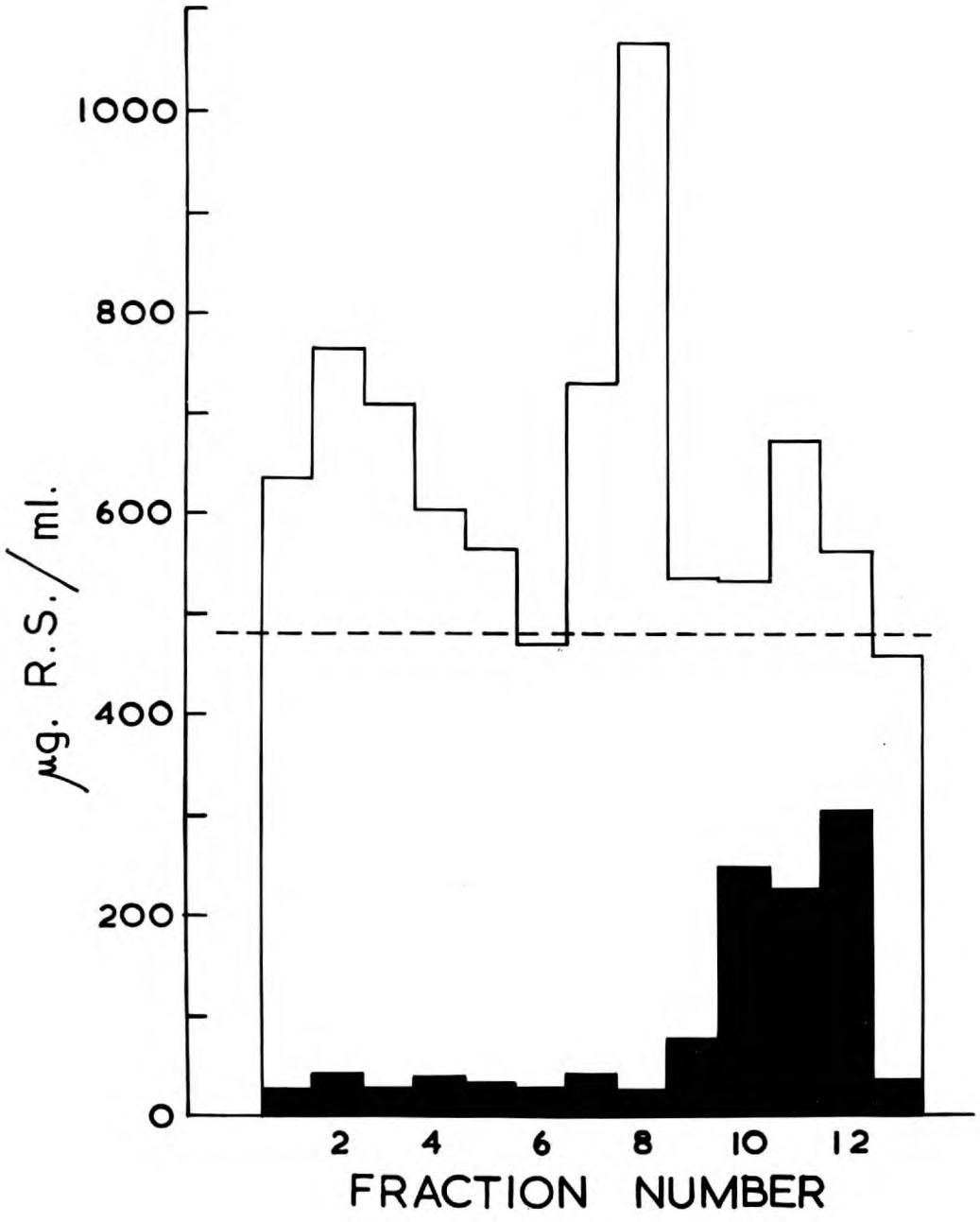


Figure 22. Chromatography of hydrolase preparation on DEAE-Cellulose; for details of the experiment see text.

Solid columns represent reducing sugar in digests with 0.1 M-sucrose (4 hr. at 25°C); open columns - reducing sugar in digests with substrate M (11.1 mg./ml.; 24 hr. at 25°C). The broken line represents the value obtained for substrate M incubated without enzyme.

(hydrolase A); fractions 7 + 8 - (hydrolase B); fractions 9 + 10 - (invertase peak). The activities of hydrolase A and hydrolase B against substrate M and of the invertase peak against sucrose were assayed as before; the results are shown in Table 31 as rate of increase in reducing power per hour, which are compared to the rates of the constituent fractions before storage in the deep freeze. This comparison shows that hydrolase A was largely unaffected but that the process of freezing and thawing caused the loss of about half the activity from the second peak (B) of the hydrolase.

Table 31. Effect of storage in the deep freeze on activity of various fractions from a DEAE-cellulose column.

Digests (0.8 ml.) contained 0.2 ml. of enzyme solution, and were incubated with substrate in 0.1 M-acetate buffer pH 5 at 30°C. Substrate-M was supplied at a concentration of 11.1 mg. combined fructose per ml., and sucrose at a concentration of 0.1 M. The activity of the fractions is expressed as reducing sugar liberated, estimated as fructose.

Substrate	Fraction number	Activity (μmoles/ml./hr.)		
		Before storage (a)	After storage (b)	(b) as percentage of (a)
Substrate-M	2	0.061		
	3	0.048		
	2 + 3 (A)	0.055*	0.057	104
Substrate-M	7	0.053		
	7	0.131		
	7 + 8 (B)	0.092*	0.047	51
Sucrose	9	0.042		
	10	0.380		
	9 + 10 (Invertase)	0.211*	0.181	86

* average of activity of constituent fractions

An attempt to concentrate the activities was made by reducing the volume of the fractions. The thawed solutions remaining from the previous experiment were first dialysed against 0.005 M-phosphate buffer pH 7.5 to remove the excess of phosphate and NaCl. The dialysed solutions were poured into tared 10 ml. beakers and their volumes estimated by weight. The solutions were reduced in bulk under a stream of air at room temperature for about three hours, and the concentration achieved estimated at the end of this period by re-weighing the beakers. The activity of the hydrolase was assayed against substrate M and also against inulin; the invertase fraction was assayed against sucrose and substrate M. The results, given in Table 32, show that the progressive loss of activity from hydrolase B noted after storage in the deep freeze continued. These results also indicate that the invertase does not hydrolyse the oligosaccharides present in the standard substrate mixture although there was some indication of such activity in the fractions from the column when sampled immediately. This indicates that such activity in the invertase fractions was due to the "tail" of hydrolase B which subsequently underwent denaturation.

The failure of the invertase to attack the standard substrate mixture at detectable rate could have been due to its not being a β-fructofuranosidase, but an α-glucosidase. The remaining part of the concentrated invertase peak was used to determine this: sucrose, raffinose or melezitose were supplied at a concentration of 0.1 M, and the pH of the digests was kept at 5 with 0.1 M-acetate buffer. The results are shown in Fig. 23; the hydrolysis of raffinose and the failure to hydrolyse

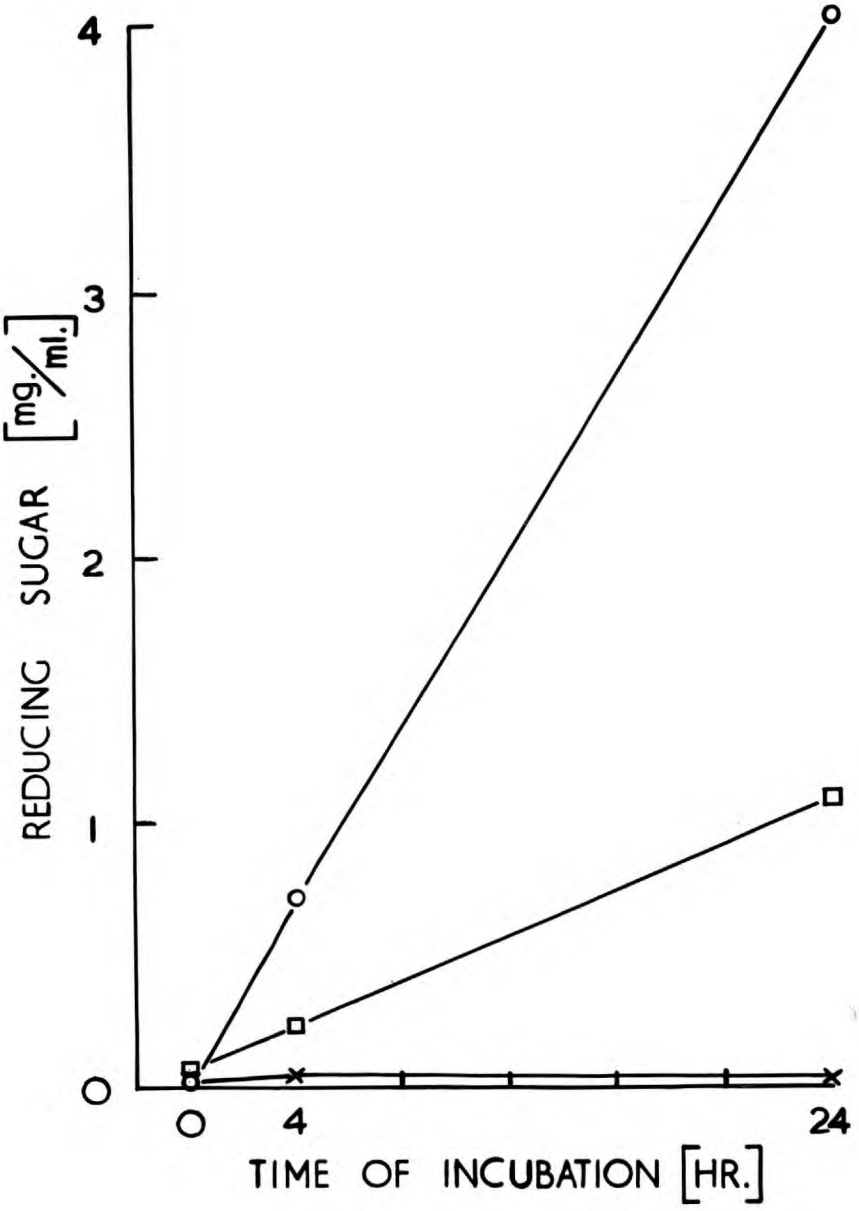


Figure 23. Activity of invertase fraction from DEAE-cellulose column against sucrose, raffinose and melezitose. Digests (0.4 ml.) contained 0.1 ml. of concentrated fraction (see text) with 0.1 M-substrates in 0.1 M-acetate buffer pH 5; incubated at 25°C. Activity was estimated by determining the reducing power in samples from the digests.

O—O sucrose, □—□ raffinose, X——X melezitose.

melezitose suggest that the enzyme is not an α-glucosidase. Chromatography of the raffinose digest showed the appearance of free fructose and melibiose, indicating that the enzyme is a β-fructofuranosidase.

Table 32. Concentration of activity by reduction in volume, of fractions from a DEAE-cellulose column.

Digests (0.8 ml.) contained 0.2 ml. of enzyme solution with substrates (inulin, 50 mg./ml.; substrate-M 11.1 mg./ml.; sucrose 0.1 M) in 0.1 M-acetate buffer pH 5, and were incubated at 30°C.

Fraction	Substrate	Activity as reducing sugar (fructose) liberated (μMoles/ml./hr.) (a)	Concentration factor by volume	Concentration factor by activity*
none	inulin	0	-	-
none	substrate-M	0	-	-
A	inulin	0.077		
A	substrate-M	0.160	3.5	2.8
B	inulin	(0.00)		
B	substrate-M	0.022	3.8	0.47
Invertase	substrate-M	(0.01)		
Invertase	sucrose	0.721	3.4	4.0

* calculated from (a) and Table 31 (column (b)).

The elution behaviour from DEAE-cellulose was re-examined on a larger scale using 900 mg. of cellulose and 1.0 g. of acetone powder for the preparation of the protein solution. The powder was suspended in 8.0 ml. of 0.005 M-phosphate buffer pH 7.5 and the talc and undissolved material centrifuged off at 3000 x g for one min. at room temperature; the precipitate was twice resuspended in buffer, and the supernatant and

washings bulked and dialysed overnight against 0.005 M-phosphate buffer pH 7.5 in the cold room. The dialysis sac was emptied into a measuring cylinder and 16.5 ml. of the total 17.5 ml. of dialysed solution applied to the top of the column. The elution procedure was the same as before except that four 5.0 ml. fractions were collected with each eluting buffer. Elution with 0.05 M-phosphate + 0.30 M-NaCl pH 8.0 was omitted as in the preliminary run (Fig. 22) all the available activity was eluted before this stage.

All of the fractions were assayed for their activity against substrate M; those eluted by 0.05 M-phosphate + 0.10 M-NaCl or 0.20 M NaCl were also assayed for their activity against 0.1 M-sucrose. The existence of two peaks of hydrolase activity suggested by the preliminary run was confirmed, as was the separation of the second peak from the peak of invertase activity. The relative activities found in the fractions are illustrated in Fig. 24.

Two fractions, one from each peak of hydrolase activity, were compared on the basis of their activities against substrate M inulin and sucrose. The result of the experiment (Table 33) showed that both enzymes attacked inulin and the mixture of oligosaccharides but had very little activity against sucrose; it also indicated that the hydrolase eluted in 0.05 M-phosphate + 0.10 M-NaCl had again lost considerable activity. It appeared however that the rate of hydrolysis of substrate M did not fall during the experimental period, suggesting that the change in pH from 7.5 to 5 may have been effective in stabilising the enzyme; it should be noted however, that the rate of hydrolysis of inulin did

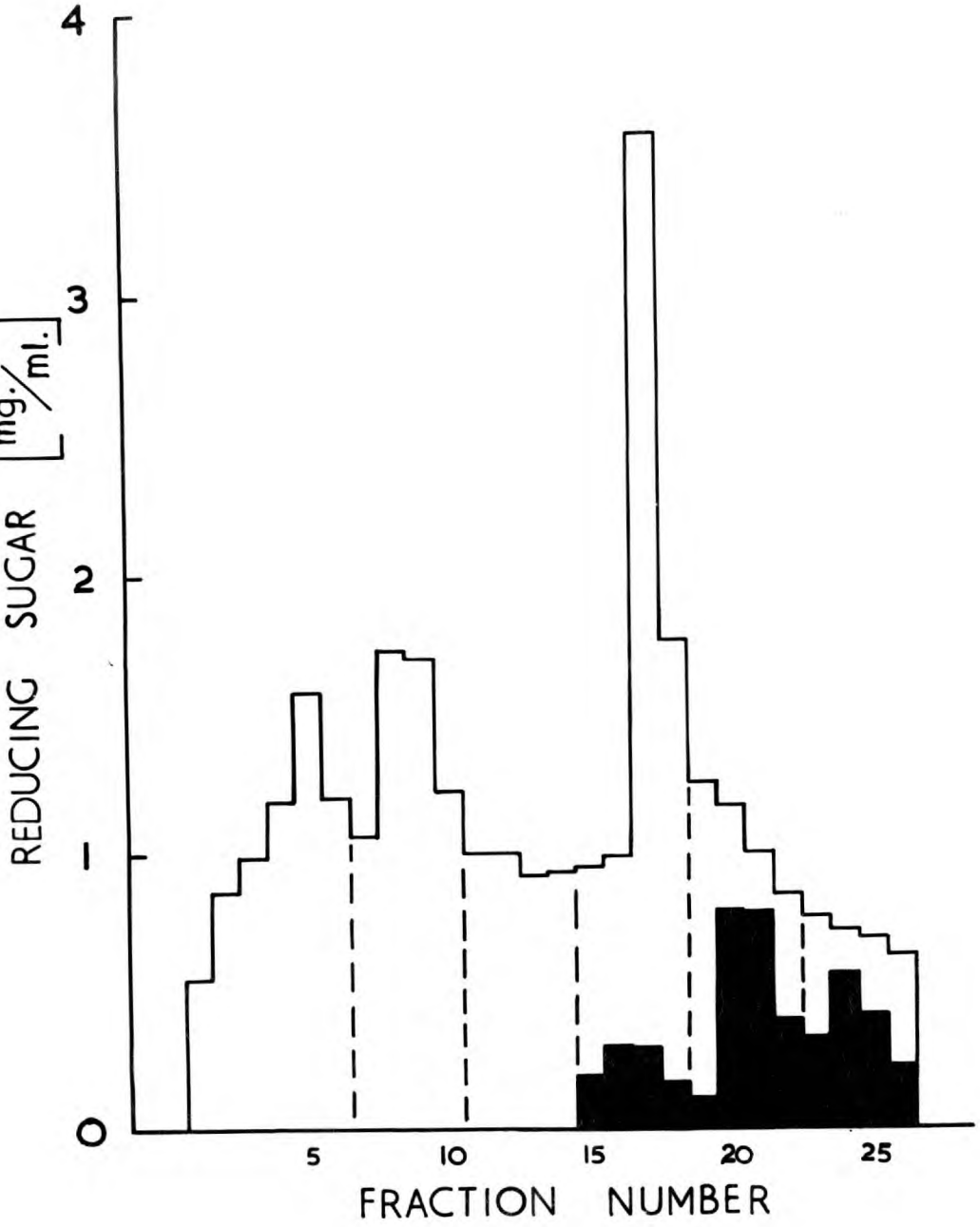


Figure 24. Chromatography of hydrolase preparation on DEAE-Cellulose. Open columns - hydrolase assay, closed columns - invertase assay. The broken lines indicate the points at which the eluting buffers were changed - for elution procedure see text. Digests for assays made up as described in Fig. 22, and incubated for 21 hr. at 25°C.

fall with time, although the amount of hydrolysis was not sufficient to make any significant difference to the substrate concentration.

Table 33. Comparison of activities of hydrolase A and hydrolase B against different substrates.

Digests (0.4 ml.) contained 0.2 ml. of enzyme solution with substrates (inulin 50 mg./ml.; substrate-M 11.1 mg./ml.; sucrose 0.1 M) in 0.1 M-acetate buffer pH 5, and were incubated at 30°C. Reducing sugar was estimated as fructose.

Fraction	Substrate	Reducing sugar liberated µMoles/ml.		
		1 hr.	4 hr.	22 hr.
9 (hydrolase A)	inulin	0.19	1.10	5.83
	substrate-M	0.51	2.12	12.6
	sucrose	-	-	0.02
17 (hydrolase B)	inulin	0.36	1.56	4.85
	substrate-M	0.56	2.80	13.8
	sucrose	-	-	0.63

The individuality of hydrolase A and hydrolase B.

The separation of a mixture of enzyme activities by chromatography on DEAE-cellulose depends on the physical properties of the proteins among which the character of the active site may or may not be relevant. Thus when two active fractions are produced by such a procedure it cannot immediately be assumed that two different enzymes are present: for instance a separation of equilibrating forms of the same enzyme may have been achieved. The presence of two peaks of hydrolase activity in the effluent showed that there were at least two protein species which could be differentiated by column chromatography; the possibility that these two species were an artefact of the experimental procedure was examined.

To this end another column was run, using 1 g. of cellulose powder and 950 mg. of acetone powder. The elution pattern was almost identical with previous runs when compared on the basis of the release of reducing power from substrate M as shown in Table 34.

Table 34. Distribution of hydrolase activity among fractions obtained by stepwise elution from DEAE-cellulose columns.

The activity eluted by each buffer is expressed as a percentage of the total activity eluted from the column.

Column I; data taken from Fig. 22

II; " " " " 23

III; " " " " 25

Eluting buffer (pH 7.5)	Column I	II	III
0.005 M-phosphate	21.3	20.4	17.1
0.02 M-phosphate	17.0	22.7	22.2
0.05 M-phosphate	3.7	11.3	7.8
0.05 M-phosphate + 0.10 M-NaCl	40.5	31.8	42.7
0.05 M-phosphate + 0.20 M-NaCl	14.5	13.8	10.1
0.05 M-phosphate + 0.30 M-NaCl (pH 8.0)	3.0	-	-
	100.0	100.0	99.9

The two most active fractions of hydrolase B, i.e. those eluted by 0.05 M-phosphate + 0.10 M-NaCl, were bulked, dialysed against distilled water for 2 hr. and then against 0.005 M-phosphate buffer pH 7.5 overnight. The solution was applied to a column of 300 mg. of cellulose powder, and

eluted with the same series of buffers as hitherto: the effluent was collected in 3.0 ml. fractions which were assayed for activity against substrate M.

The fractions forming peak A of the original separation, i.e. those eluted by 0.005 M- and by 0.02 M-phosphate, were bulked and dialysed in a similar manner. After dialysis, the solution occupied 12.2 ml. and an attempt was made to concentrate the activity by adding 1.15 g. "Sephadex G-50" coarse grade powder, stirring well, and then centrifuging at 6000 x g for 15 min. at 0°C. This treatment yielded a very small volume of supernatant solution; the Sephadex was transferred to a Buchner funnel and washed with 4 ml. of 0.005 M-phosphate buffer: filtration under suction yielded 5.8 ml. After removal of 0.1 ml. to determine if any hydrolase activity remained, the solution was chromatographed on a column of 300 mg. of DEAE-cellulose. The effluent was collected in a siphon which delivered fractions of 2.3 ml. The elution procedure was as above, and each fraction was assayed for its activity against substrate M.

The results of the first separation of hydrolase A and hydrolase B, and of their repurification are shown diagrammatically in Fig. 25. This shows that peak B (fractions 14 and 15 of the first separation), was again eluted as a homogeneous peak, and although this started to emerge from the column at an earlier stage than in the first separation, there was no separation of the activity to reproduce the original double peak elution pattern. The earlier emergence can probably be ascribed to the difference in the overall composition and amount of the protein mixture

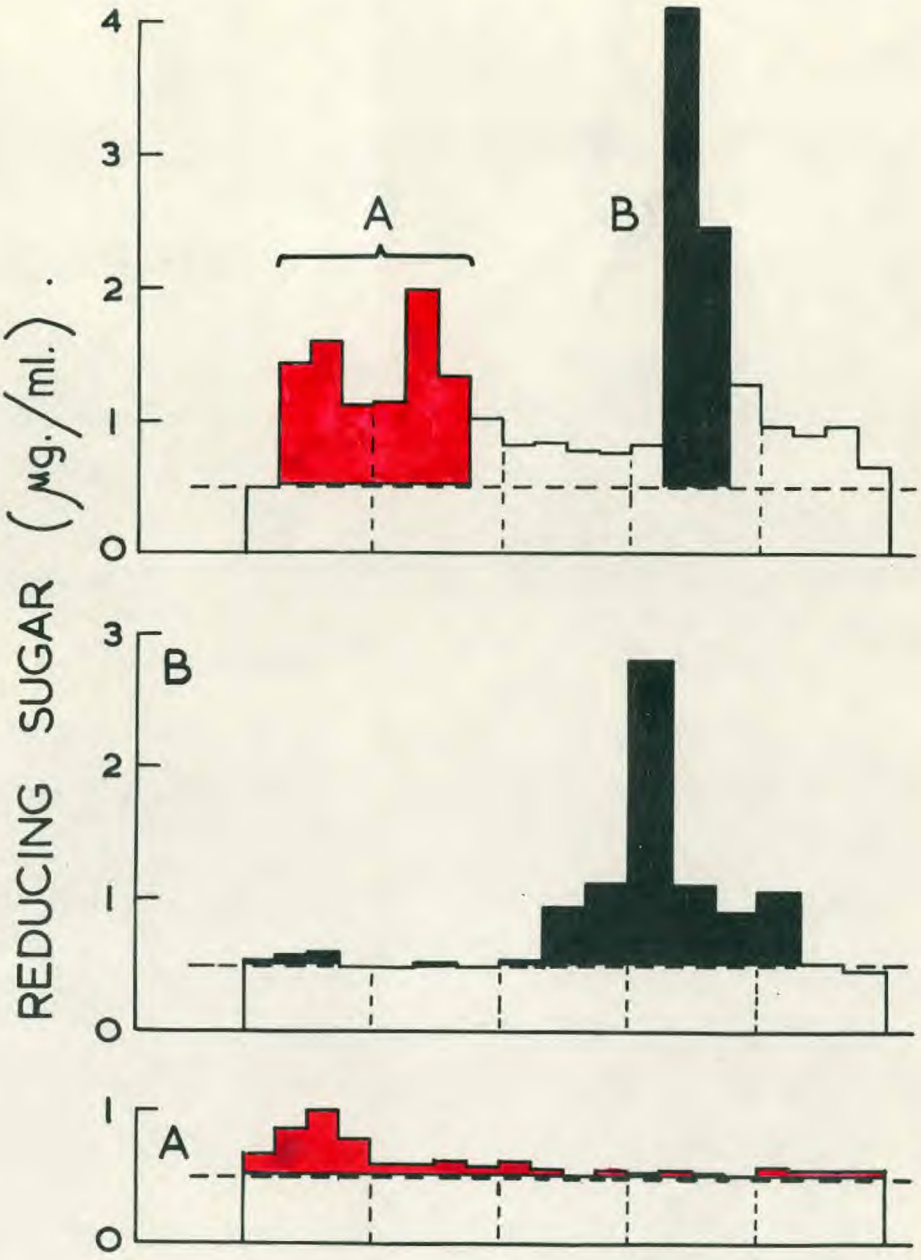


Figure 25. Re-chromatography of hydrolase A and hydrolase B on DEAE-cellulose.

put on the column. The results of the re-run of peak A are unsatisfactory because of the small amount of activity put on to the column (14.2 ml.), much of the original activity probably remaining in the bed-volume of the Sephadex, but although the increases in reducing power over that of the blank were small, the data demonstrate that hydrolase A, like hydrolase B, remains homogeneous on re-chromatography.

A further separation of the protein extract was made; a solution containing 548 mg. of acetone powder was put on to 1 g. of DEAE-cellulose, and the column eluted with the same series of buffers as before, four 5.0 ml. fractions being collected with each eluting buffer. As well as the usual assay for hydrolase activity, the protein content of each fraction was determined by the Neilsen method. The previous finding of two peaks of hydrolase activity recurred: the protein estimations permitted the calculation of the specific activity of the protein present in the various fractions. The results demonstrate that on this basis there are also two peaks as shown in Fig. 26.

...

Comparison of Hydrolase A with Hydrolase B

Although the two peaks have been shown to be different proteins, it remains possible that the two are identical in the properties of their active sites, and hence their enzyme activities, differing only in parts of the molecule which influence the elution behaviour from DEAE-cellulose, and the stability at pH 7.5.

Since the elution pattern from DEAE-cellulose was so reproducible, in the next separation the two peaks were collected in bulk. Approximately

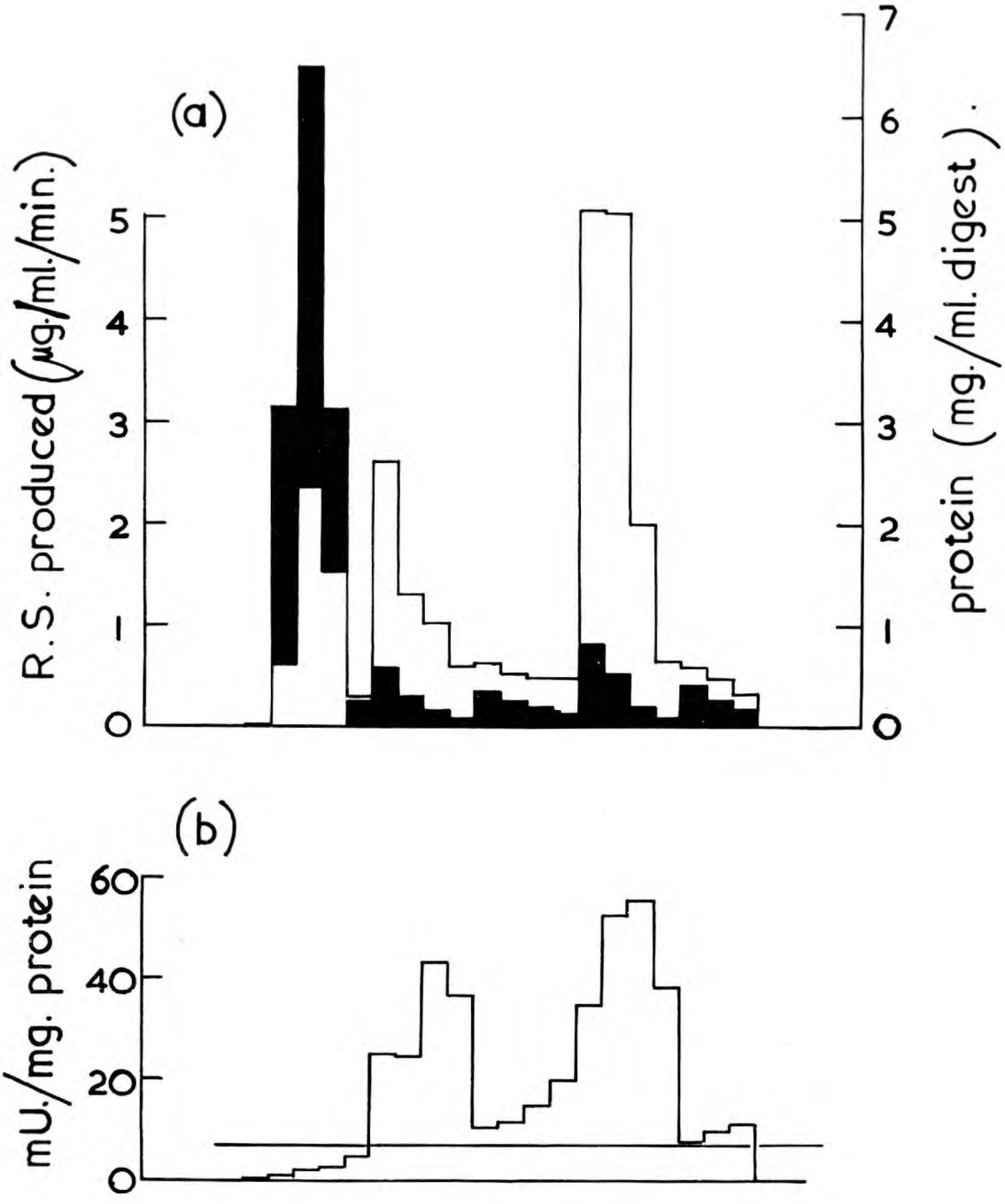


Figure 26. Chromatography of hydrolase preparation on DEAE-cellulose;

(a) elution patterns of total protein (solid columns) and of hydrolase activity (open columns)

(b) specific activity of hydrolase, calculated from (a).

the same quantities, namely 1 g. of DEAE-cellulose and 556 mg. of acetone powder, were used as in the last separation, and the same volumes of the eluting buffers were used. The fractions were collected as shown in Table 35. The peaks A and B were dialysed overnight against 0.005 M-acetate buffer pH 5, and then used to determine the influence of pH on their activity against substrate M. The pH of the digests was adjusted by using phosphate-citrate buffers. As acetate buffer was used in previous experiments, a digest made up in 0.1 M-acetate buffer pH 5.0 was included in each series. The results are given in Fig. 27; the activity in each digest is plotted as a percentage of the highest activity recorded: although the influence of pH on the activity of the two peaks differed, their optima were separated by only about 0.3 of a pH unit.

Table 35. Procedure for elution of hydrolase fractions in bulk from DEAE-Cellulose column (1.0 g. dry powder).

Eluting buffer (pH 7.5)	Volume of effluent collected (ml.)	
0.005 M-phosphate	20.0	
0.02 M-phosphate	5.0	
	15.0	
		hydrolase A
0.05 M-phosphate	5.8	
	14.2	
0.05 M-phosphate + 0.10 M-NaCl	5.0	
	15.0	
		hydrolase B
0.05 M-phosphate + 0.20 M-NaCl	5.0	

In an experiment described above, the activities of hydrolase A and B against inulin and substrate M were compared and the result indicated that the ratio of hydrolysis of inulin to that of substrate M

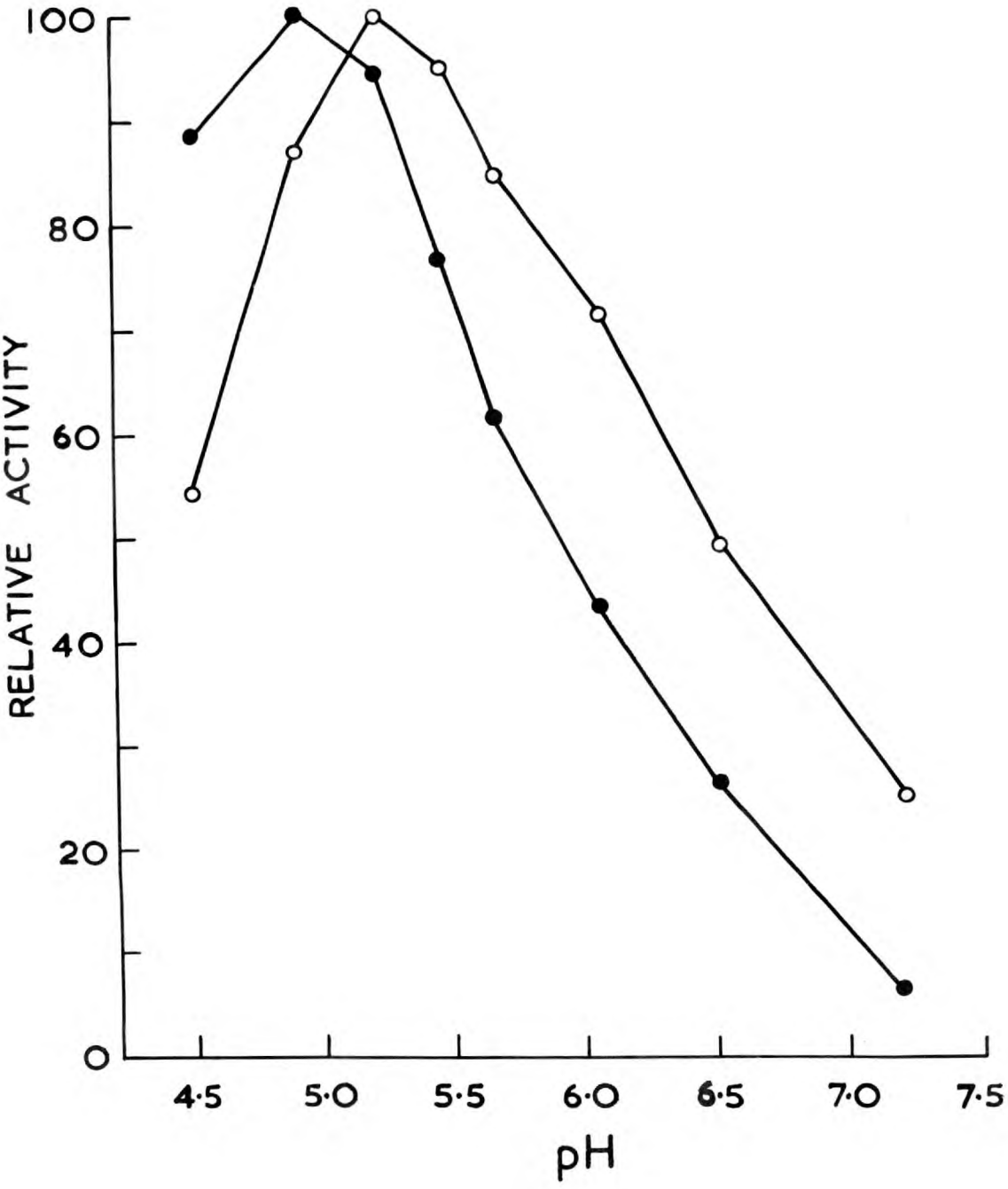


Figure 27. Influence of pH on activity of hydrolase A and hydrolase B against substrate M (11.1 mg./ml.). The activity is expressed as a percentage of the highest activity recorded for the respective fractions.

differed. The enzymic properties of the two peaks were now compared more extensively on the basis of their activity against a number of different substrates, which afforded a comparison of specificity and relative rate of hydrolysis. The substrates were inulin and substrate M containing β2→1 linked fructosyl residues, bacterial levan with β2→6 linked residues, irisin which has β2→1 and β2→6 linkages, and sucrose. In addition the inhibition of inulin hydrolysis by sucrose was examined. The activity of the enzymes was estimated by determining the reducing power in samples from the digests. Although the high blank value of many of the digests was undesirable (see Table 36), in most cases there is no difficulty in deciding whether a significant increase occurred. Thus, Fig. 28 shows that both enzymes were virtually inactive against sucrose or irisin, and showed little activity against bacterial levan. The inhibitory effect of sucrose is well marked with both enzymes. The digests containing inulin + sucrose were examined qualitatively by paper chromatography to see if there was any evidence of transfer of fructosyl residues from inulin to sucrose which could wholly or partly explain the sucrose inhibition. A photograph of the chromatogram is shown in Fig. 29; some transfer, with the probable synthesis of 1^F-fructosylsucrose could be detected in the digest with hydrolase B, but to a negligible extent in that with hydrolase A; this transfer could not account for the extensive inhibition by sucrose and since an enzyme carrying out such transfer, and distinct from the hydrolase, has been obtained from the artichoke tuber, it is likely that at least some of this activity can be ascribed to contaminating amounts of this enzyme rather than to the

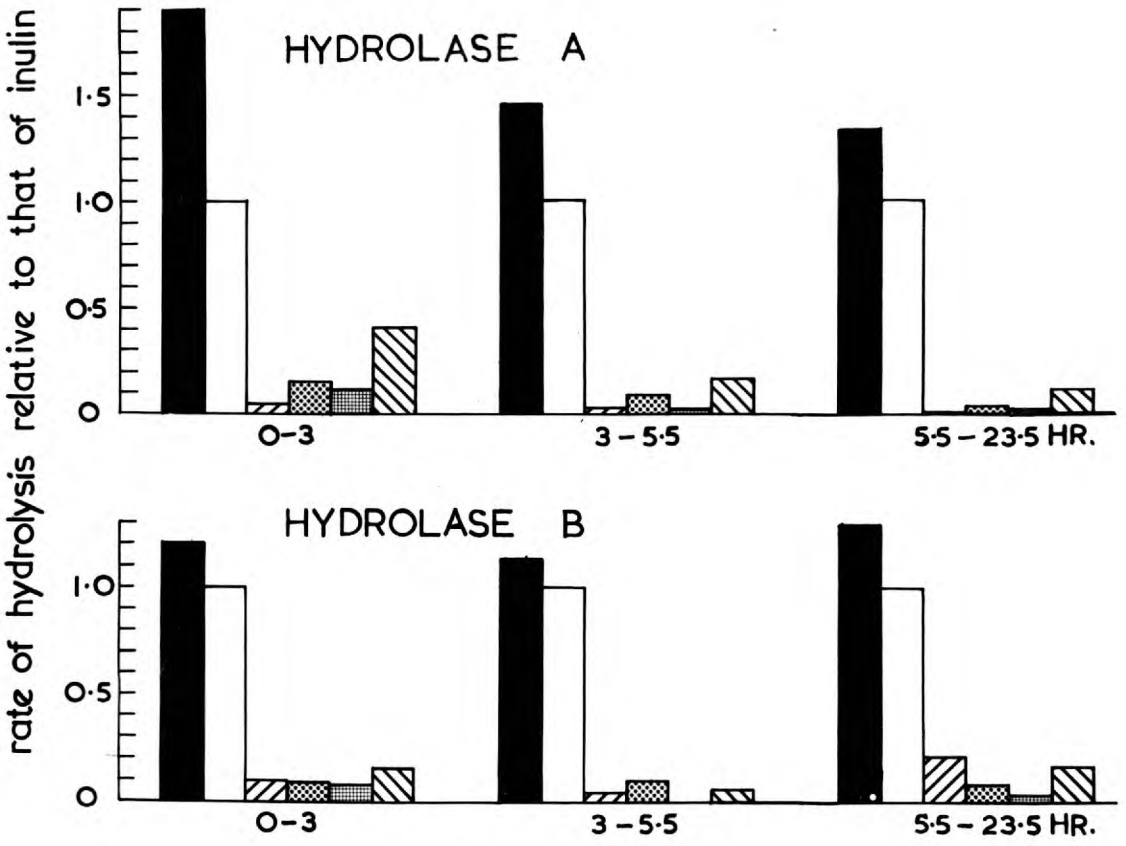


Figure 28. Action of hydrolase A and hydrolase B against various substrates. Details of the experiment are given in the text and in Table 36.

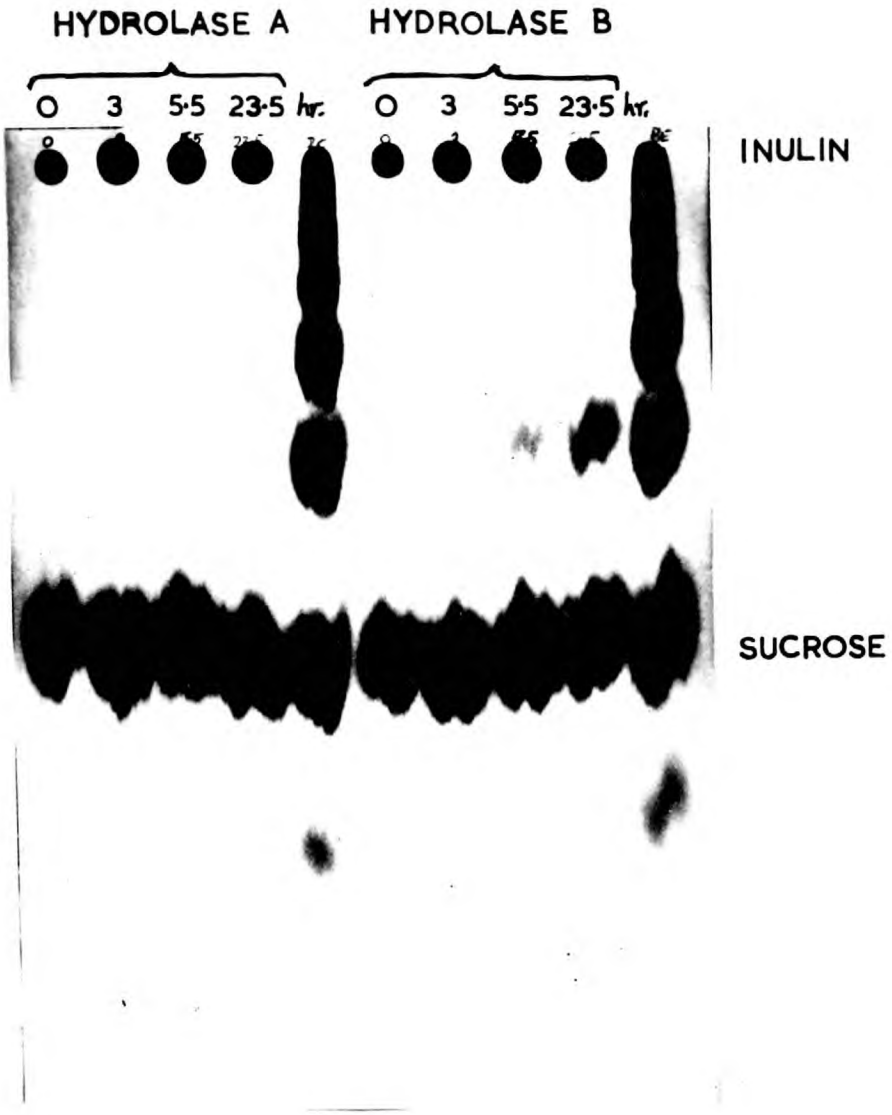


Figure 29. Incubation of hydrolase A and hydrolase B [redacted] with 0.005 M-inulin and 0.1 M-sucrose. For details of the experiment see Table 36. Initial time samples on chromatogram 5 µl, others 10 µl. Chromatogram developed for 2 days in PEAW 7:1:2 and sprayed with benzidine-TCA.

hydrolase: although the main peak of this transferase activity is eluted from a DEAE-cellulose column at pH 7.5 by 0.05 M-phosphate buffer some overlap with hydrolase B is to be expected (P.J. Roy; personal communication).

Table 36. Activity of hydrolase A and hydrolase B against various substrates.

Digests (0.4 ml.) contained 0.2 ml. of enzyme solution and were incubated with substrate in 0.1 M-acetate buffer pH 5 at 25°C. Activity was estimated by determining the increase in reducing sugar in samples from the digests.

Enzyme	Substrate	fructose content (µmoles per ml.)	Initial R.S. value µmoles/ml.	Increase in reducing sugar (as fructose) µmoles/ml.		
				3 hr.	5.5hr.	23.5hr.
hydrolase A	Inulin	278	3.60	0.93	1.82	7.83
	Substrate-M	61.7	2.33	1.78	3.07	11.1
	Sucrose	100	0.13	0.14	0.22	0.43
	Irisin	278	4.20	0.10	0.12	0.26
	Levan	278	2.71	0.38	0.53	1.25
	Inulin + sucrose	✓	1.68	0.04	0.08	0.16
hydrolase B	Inulin	278	3.55	3.77	6.71	16.7
	Substrate M	61.7	2.30	4.52	7.86	20.8
	Sucrose	100	0.16	0.36	0.62	1.51
	Irisin	278	4.30	0.30	0.30	0.40
	Levan	278	2.71	0.56	0.75	2.29
	Inulin + sucrose	✓	1.73	0.36	0.51	2.66

✓ inulin 139 µmoles of fructose/ml.
sucrose 100 µmoles of fructose/ml.

It is possible that the activities against inulin and against substrate M are those of two distinct enzymes in each of the two peaks.

An attempt was made to alter the ratio of these activities by partial denaturation: this was done by heating known volumes of samples

from each fraction in stoppered tubes in a water bath at 53.5°C for 10 min. The tubes were then removed and cooled. Digests of these fractions with the substrates were incubated at 25°C and samples removed for estimation of reducing sugar at times of 1 min., 4 hr. and 22 hr. after the addition of the enzyme. The results (Table 37) show that hydrolase B was more susceptible to denaturation than was hydrolase A. In the case of the latter, the activities against substrate M

Table 37. Effect of heat treatment on activity of hydrolase A and hydrolase B.

The activity of the enzymes against substrate M (11.1 mg./ml.) and inulin (50 mg./ml.) in 0.1 M-acetate buffer pH 5 at 25°C was estimated in digests (0.4 ml.) containing 0.2 ml. of enzyme which had been heated at 53.5°C for 10 min.; control digests with substrate and 0.2 ml. untreated enzyme were also set up. The activity was measured by estimating the increase in reducing sugar in the digests.

Substrate	hydrolase	Increase in reducing sugar (as fructose) (mμmoles/ml./min.)		Loss of activity (%)	
		4 hr.	22 hr.	4 hr.	22 hr.
Substrate M	A	8.44	6.39		
	A*	6.23	4.97	26.2	22.2
Inulin	A	3.48	3.61		
	A*	2.92	3.11	16.1	13.9
Substrate M	B	18.2	-		
	B*	4.19	2.91	77.2	84.0
Inulin	B	14.3	-		
	B*	2.27	1.80	84.2	87.3

* treated enzyme

and against inulin are denatured to about the same extent, suggesting that substrate M and inulin are hydrolysed by the same enzyme. In the case of hydrolase B both activities are reduced to a much greater degree; the denaturation of the activity against inulin appears to be greater than of that against substrate M, indicating that different enzymes are involved. Although there was an indication that activities A and B each consisted of a single enzyme, the amount of denaturation of A was unsatisfactory, and in the case of B, the amount of substrate M hydrolysed by the unheated enzyme was such that the rate determined was probably affected by the fall in substrate concentration and the rise in concentration of sucrose; the effect of this would be to lower the calculated percentage denaturation of substrate M hydrolysis.

The experiment was therefore repeated with a more specific substrate, namely inulopentaose being substituted for substrate-M. In the light of the results of the previous experiment, hydrolase A was heated for 15 min. and hydrolase B for 5 min. at 55°C, and in the case of the latter, the final concentration of unheated enzyme in the digest was half of that of the treated sample. The digests were assayed by the total fructose method after chromatography on C/Celite columns. The results (Table 38) again show that hydrolase B is more susceptible to thermal denaturation than hydrolase A, and that in each fraction, the hydrolysis of substrates of high and low D.P. are catalysed by one enzyme.

Because of the marked effect of temperature on depolymerization in dormant tubers, which resulted in the accumulation of oligosaccharides of DP 2-5 at low temperature (see p. 49) and because sucrose

Table 38. Effect of heat treatment on activity of hydrolase A and hydrolase B.

The activity of the enzymes against inulopentaose (0.01 M) and inulin (50 mg./ml. 0.01 M) in 0.1 M-acetate buffer pH 5 at 25°C was estimated in digests containing 0.2 ml. of enzyme which had been heat-treated (see text). Control digests with substrate and 0.2 ml. of untreated enzyme were also set up. The activity was measured by estimating the increase in free fructose.

Substrate	enzyme	Free fructose produced mμM/ml./min.	Loss of activity (%)
inulopentaose	A	15.7	
	A*	11.0	29.8
inulin	A	7.11	
	A*	4.88	31.4
inulopentaose	B	21.9	
	B*	2.68	87.6
inulin	B	16.5	
	B*	2.09	89.2

* heated enzyme

has been shown to be a potent inhibitor of the hydrolase at temperatures of about 20°C, an experiment was performed to examined the sucrose inhibition of hydrolase A and hydrolase B at lower temperatures. The low solubility of inulin in cold water made its use as substrate impractical, as even a saturated solution would be too dilute to give rates of hydrolysis which could be measured with accuracy: for this reason, inulotetraose was used. Series of digests were made up with 0.01 M-inulotetraose alone, 0.01 M-inulotetraose + 0.01 M-sucrose, and 0.01 M-sucrose alone;

these were incubated with hydrolase A and hydrolase B at temperatures of 3°, 16° and 25°C. The amount of free fructose was estimated after C/celite chromatography, and the results are given in Table 39; these show that the degree of sucrose inhibition is greater at the lower temperature; thus if inulotetraose is representative of the natural substrate the rapid depolymerization at low temperature cannot be due to a relaxation of sucrose inhibition of either hydrolase A or hydrolase B.

Table 39. Effect of the presence of sucrose on the rate of hydrolysis of inulotetraose by hydrolase A and by hydrolase B at different temperatures

Digests (0.4 ml.) contained 0.2 ml. of enzyme solution incubated with substrate in 0.1 M-acetate buffer pH 5 for 3 hr. at the temperatures shown below. The activity was measured by estimating the free fructose produced, and is expressed as μmoles/ml./min.

Enzyme	Temp. (°C)	Incubation Mixture			% age inhibition
		0.01 M-inulotetraose	0.01 M-inulotetraose + 0.01 M-sucrose	0.01 M-sucrose	
Hydrolase A	3	5.56	4.50	0.31	24.5
	16	11.3	9.62	0.28	17.4
	25	17.9	15.0	0.43	17.0
Hydrolase B	3	13.5	5.34	0.31	66.6
	16	26.8	11.7	0.31	59.3
	25	42.0	19.1	0.52	56.2
None	(25)	1.30	-	0.28	-

The results show that there is a marked difference in the effect of sucrose on the rates of hydrolysis by the two enzymes; this is

not due to the varying extents of hydrolysis and hence changes in concentration of the substrate reached in the different digests; this is shown in Fig. 30 where the percentage inhibition is plotted against the extent of hydrolysis of inulotetraose.

The results from the digests with inulotetraose alone also permit the calculation of the temperature coefficients of the two enzymes; these have been calculated as the average Q_{10} over the whole range from 3° to 25°C, from the relationship $\log_n Q_{10} = \frac{\mu}{2} \cdot \frac{10}{T_1 T_2}$, where the value $\frac{\mu}{2}$ is the slope of the graph of $\ln r_T$ plotted against 1/T (Fig. 31). The values obtained were $Q_{10} = 1.85$ for hydrolase A and $Q_{10} = 1.73$ for hydrolase B.

...

The characteristics of the two peaks were investigated more closely by examining the affinity of the enzymes for various sugars, as judged by the ease with which they were hydrolysed or by the extent to which they inhibited the hydrolysis of inulin. Sugars separated from artichoke tubers and from other sources were used. More emphasis was put on peak B than peak A; the amount of activity in this fraction was greater in the extracts used, and this preponderance was assumed to reflect the relative proportions, and hence physiological importance, of the two enzymes in the tuber.

Characteristics of hydrolase A.

The relative rates of hydrolysis of 0.01 M solutions of inulin, and members of the inulose and inulo-n-ose series of oligosaccharides were determined by incubating them with hydrolase A at pH 5 at 25°C.

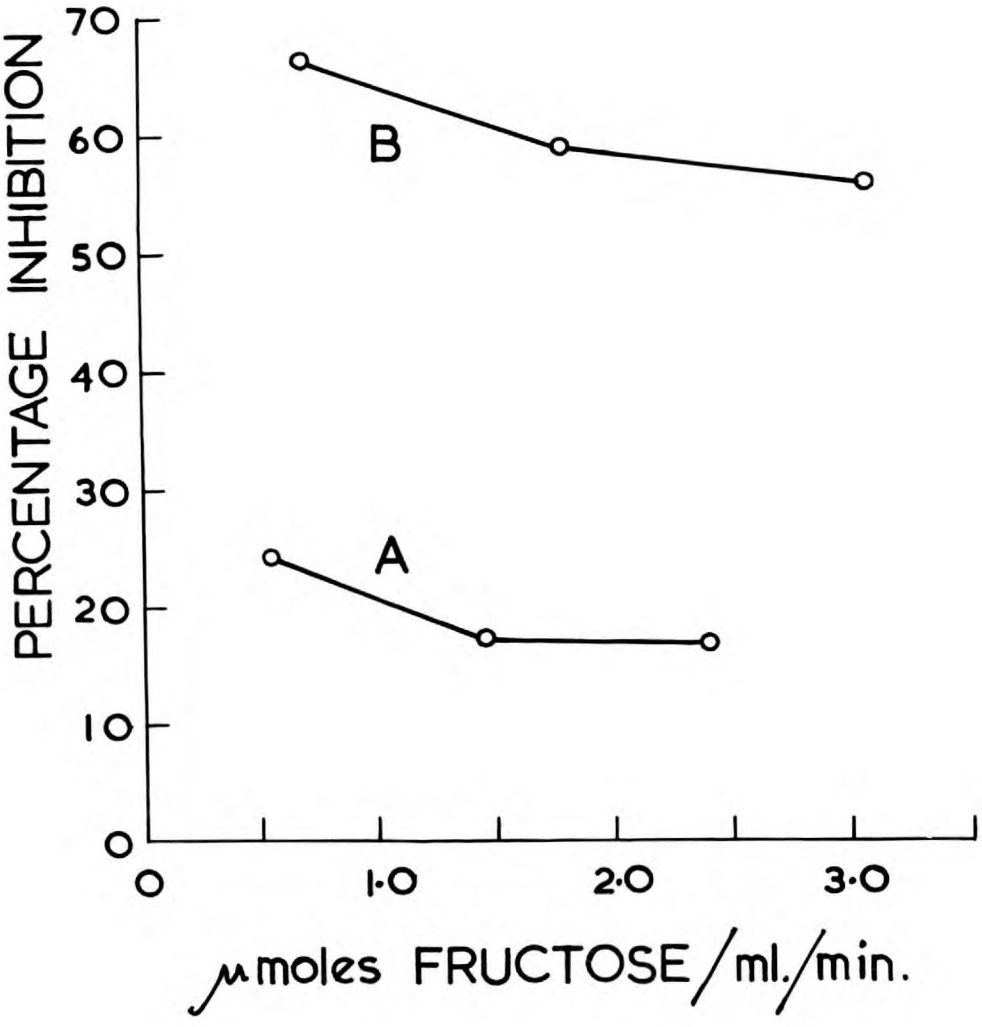


Figure 30. Sucrose inhibition of the hydrolysis of inulotetraose by hydrolase A and hydrolase B. The percentage inhibition is plotted against the extent of hydrolysis in the incubation mixtures; data from Table 39.

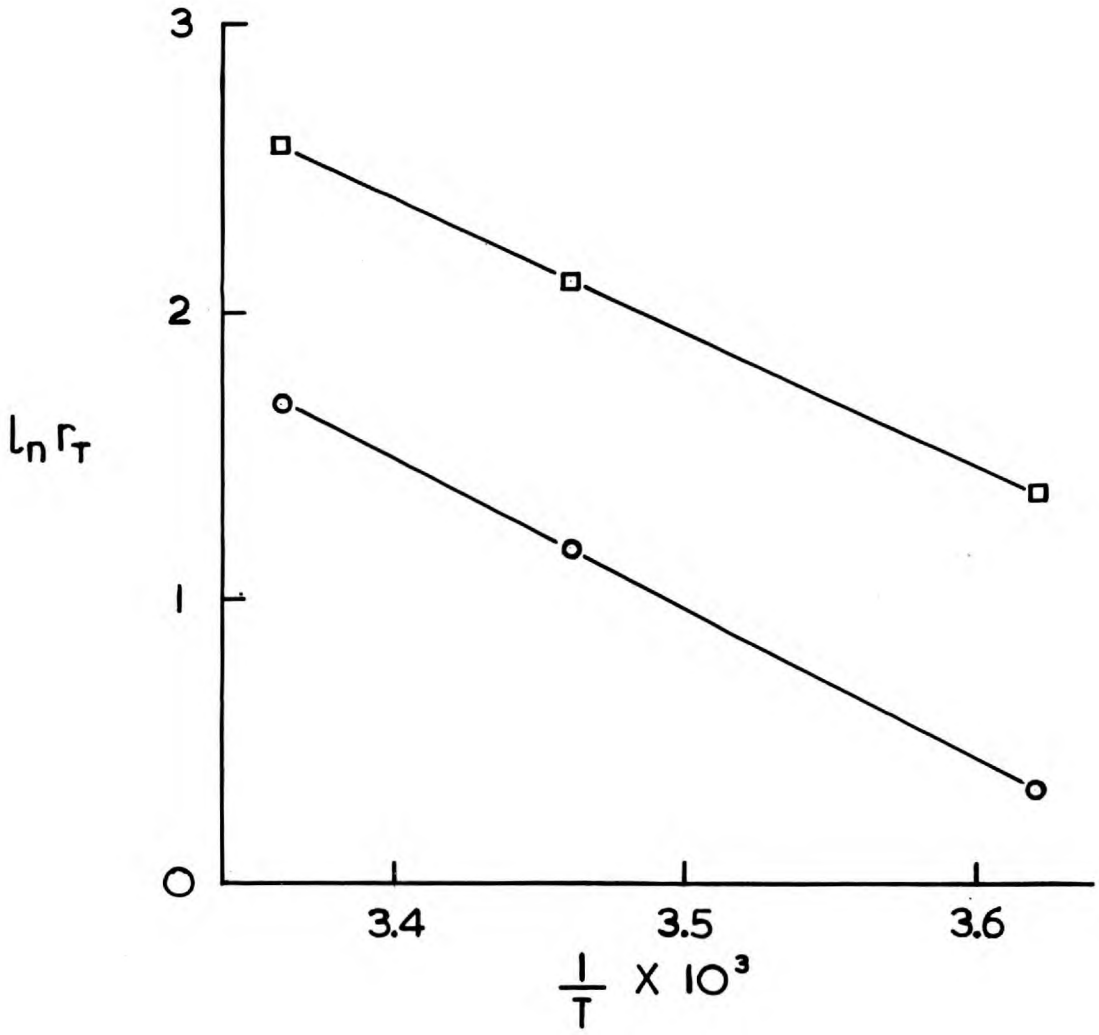


Figure 31. Graph drawn to calculate the average temperature coefficient of activity of hydrolase A and hydrolase B against inulotetraose, over the range 3-25 °C. Calculated from data in Table 39.

After 3.5 hr., the digests were treated with $HgCl_2$ and the fructose content of samples determined after chromatography on C/Celite columns. The results (Fig. 32) show that the oligosaccharides which are terminated by a reducing fructose group were hydrolysed at rates which increased rapidly with D.P. up to D.P. 5, and then fell again more slowly with further increase in D.P. The members of the inulose series were attacked at rates which increased with D.P. up to at least D.P. 6; this rate was comparable to the maximum rate obtained for hydrolysis of members of the fructose terminated sugars. Inulin was hydrolysed more slowly than this highest rate, the rate being close to that at which inulose-2 was attacked.

Characteristics of hydrolase B.

The inhibition by sucrose was examined using purified hydrolase B; the effect of raffinose was also investigated. Two sets of digests were made up, containing the individual sugars at a concentration of 0.01 M, together with a third control set; each set contained inulin at concentrations of 50, 25, 12.5 and 0 mg./ml. The digests were incubated at 25°C and samples taken at times of 1, 200, and 400 min. after the addition of the enzyme, for estimation of reducing sugar. The results are given in Table 40: in the case of sucrose it is clear that considerable inhibition occurs, and in the case of raffinose that there is no significant inhibition even though raffinose contains a sucrose residue. This is more clearly seen in the Lineweaver-Burk plot of the data (Fig. 33); it has been assumed in the calculation of these results that such hydrolysis of sucrose and raffinose as is indicated by the

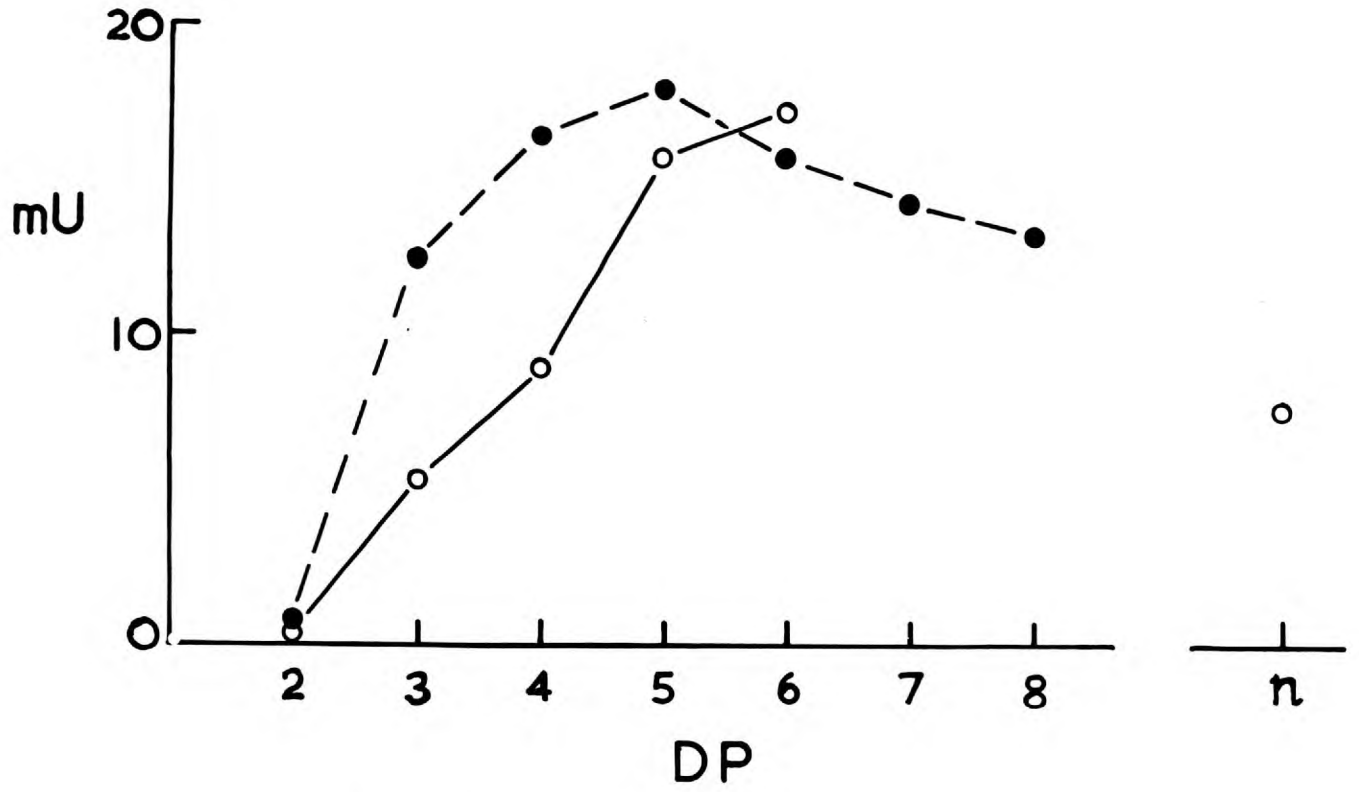


Figure 32. Relative activity of hydrolase A against inulin and members of the inulose (O) and inulo-n-ose (●) series of oligosaccharides.

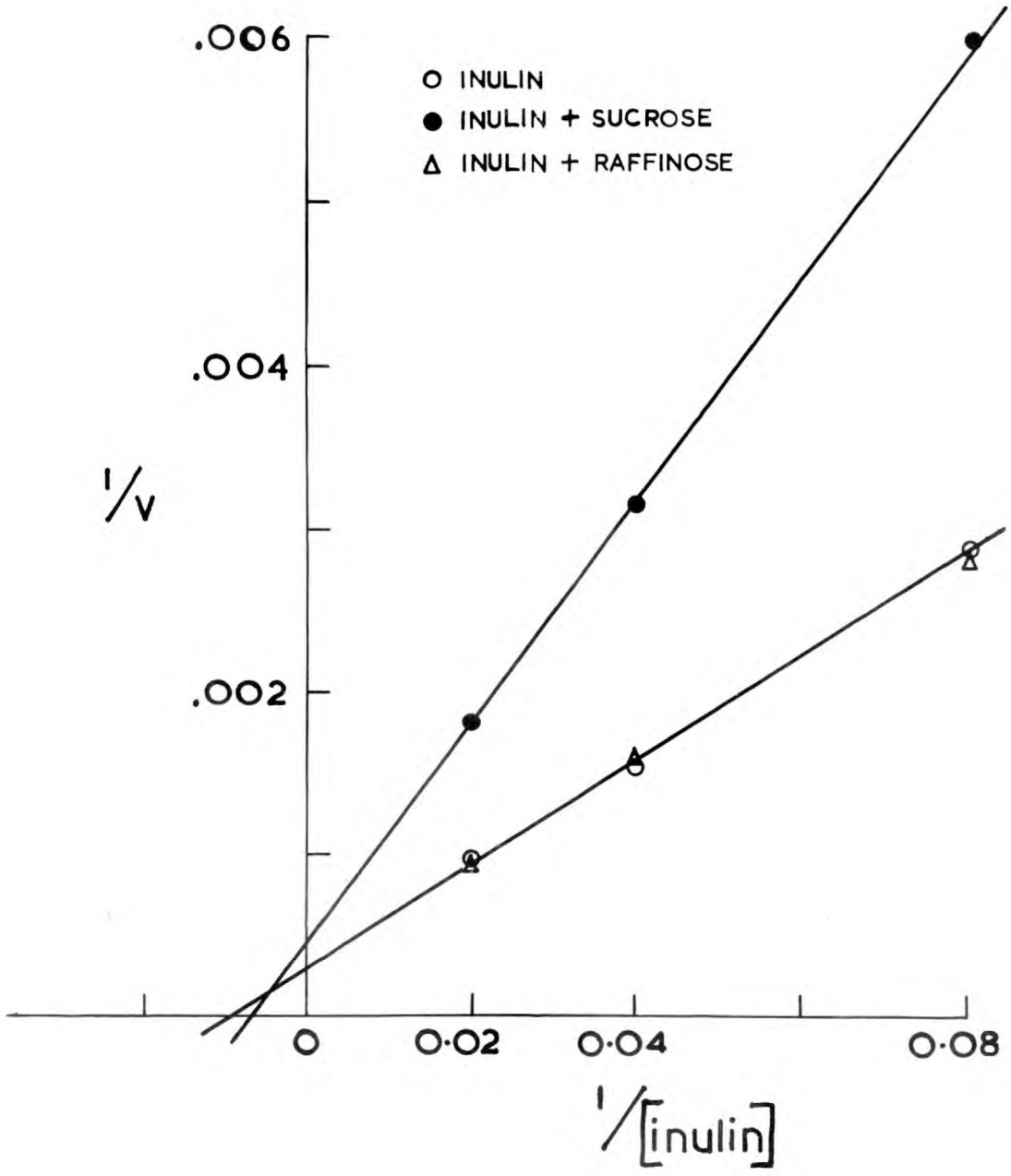


Figure 33. Lineweaver-Burk plots of activity of hydrolase B against inulin in the presence and absence of sucrose and of raffinose. Calculated from the data in Table 40.

Table 40. Effect of sucrose and raffinose on the hydrolysis of inulin by hydrolase B.

Digests (0.4 ml.) contained 0.2 ml. of enzyme solution and were incubated in 0.1 M-acetate buffer at 25°C. Activity was measured by estimating the increase in reducing sugar in samples from the digests after 200 and 400 min., and is expressed as mμmoles/ml./min.

Final concentration of inulin mg./ml.	added Sugar: none		0.01 M-sucrose		0.01 M-raffinose	
	200 min.	400 min.	200 min.	400 min.	200 min.	400 min.
50	15.3	14.2	8.45	9.03	22.3	16.2
25	11.2	9.22	3.55	4.39	10.8	10.2
12.5	4.39	4.00	2.44	2.81	6.88	6.37
0	0.12	0.31	0.33	0.56	1.28	2.14

reducing sugar values in the digests without inulin also occurs in the presence of inulin. From such a plot it is possible to deduce whether an inhibitor is competitive or non-competitive in action, according to whether the lines for substrate with, and without inhibitor intersect on the abscissa or on the ordinate. The number of points in this experiment was too small to calculate a statistically acceptable line, and moreover the lines of best fit of the inulin and inulin + sucrose data intersect neither on the ordinate nor on the abscissa which nevertheless suggests that sucrose inhibition is at least partly competitive: the statistical treatment however gives equal validity to all three points, whereas the accuracy of the measurement of the increase in reducing power would be expected to be greater in the digests with the greatest inulin concentration and the highest rate of hydrolysis. When these points

are given greater emphasis, which can be supported by the fact that the values for the inulin and inulin + raffinose series are coincident at the two higher concentrations, the indication is that sucrose is a competitive rather than non-competitive inhibitor; the Ki for sucrose can thus be calculated from the formula

$$Ki = \frac{i}{\frac{Kp}{Km} - 1}$$

giving a value of 7.3×10^{-3}. The Km of inulin is calculated to be 1.4×10^{-2}, assuming a M.Wt. of 5000; this value is very similar to that of 1.3×10^{-2} found previously for the unfractionated hydrolase (p.69). The values for the Ki of sucrose and Km of inulin indicate that the enzyme has a greater affinity for sucrose than for inulin.

As described on p.159 the amounts of the sugars of DP 3 - 7 found in tuber extracts bear such relationship to one another that the total fructose present in each is the same. The rate of hydrolysis of some of these oligosaccharides was investigated, to see if this enzyme is instrumental in such distribution of fructose residues. Oligosaccharides of DP 3, 4, 5 and 7 were thus supplied in such amounts that the concentration of total fructose in each digest was the same, namely 10 mg./ml. The digests were incubated at 25°C and sampled at 1 min., and 1, 4 and 6 hr. after the enzyme had been added. The results are given in Table 41 and show that the rates of hydrolysis in the digests differ extensively: it is thus unlikely that this enzyme plays any part maintaining the equilibrium position between these oligosaccharides in the tuber. It is apparent from the results that the effect of DP

Table 41. Action of hydrolase B on various oligosaccharides.

The digests (0.4 ml.) contained 0.2 ml. of enzyme solution with substrates at a concentration of 10 mg. of combined fructose per ml. in 0.1 M-acetate buffer pH 5; incubated at 25°C. Activity was determined by estimation of the increase in reducing sugar (as fructose) in the digests.

Substrate	Total fructose µmoles/ml.	µmoles of substrate per ml.	increase in R.S. µmoles/ml.		
			1 hr.	2 hr.	4 hr.
inulose-1	55.6	27.8	0.32	2.68	2.80
inulose-2	55.6	18.5	0.37	1.41	1.29
inulose-3	55.6	13.4	0.76	3.42	4.16
inulose-5	55.6	9.27	0.16	0.61	0.94

and concentration on the rate of hydrolysis of an oligosaccharide are both important at these levels; thus the rate of attack under the conditions used is DP 7 < DP 4 < DP 3 < DP 5.

Inhibition by sucrose was first interpreted as interference on the active site by a terminal fructose group linked in the "wrong" way, viz. to a glucose and not another fructose group. The fact, however, that raffinose did not inhibit inulin hydrolysis by peak B indicated that the active site of the enzyme could not accommodate a sucrose molecule with a substituent on its glucose moiety. The possibility that the glucose molecule is essential for the action of the enzyme was examined by determining the rate of hydrolysis of the oligosaccharides of DP 2-4 obtained in the hydrolysis of inulin, which are terminated by a reducing fructose moiety: the oligosaccharides (0.01 M) were compared with 3% inulin. The digests were incubated at 25°C for 4 hr. and then a

solution of $HgCl_2$ was added, to give a final concentration of 0.002 M which was assumed to stop further enzyme action. The amount of free fructose released during the incubation period was estimated after chromatography of samples from the treated digests on Charcoal/Celite columns, by the total fructose method. The results are given in Table 42, which shows that the oligosaccharide with DP 2 is scarcely attacked, but that those of DP 3 and 4 are hydrolysed by the enzyme, both at a faster rate than inulin. It is thus obvious that the terminal glucose residue of the artichoke oligosaccharides and of inulin is not essential for the activity of the enzyme. Moreover, the requirement is not merely for adjacent fructosyl residues joined by a 2:1' linkage as the difructose which contains such a linkage was not attacked by the enzyme (although it later became evident that the preparation of the reducing difructose contained a small amount of sucrose, this was totally inadequate to account for the failure of hydrolysis).

Table 42. Action of hydrolase B on members of the inulo-n-ose series.

Digests (0.4 ml.) contained 0.2 ml. of enzyme solution and substrates (0.01 M; inulin) 3% w/v, 0.06 M) in 0.1 M-acetate buffer pH 5, and were incubated at 25°C for 4 hr. Activity was estimated as increase in free fructose.

Substrate	Substrate concentration µmoles/ml.	µM fructose liberated	Percentage of terminal fructose hydrolysed
inulobiose	10	0.29	1.5*
inulotriose	10	1.59	15.9
inulotetraose	10	3.17	31.7
inulin	6	1.16	19.3

*calculated on the basis that the hydrolysis of one glycosidic bond yields two molecules of free fructose.

Various oligosaccharides of the artichoke series were incubated at a concentration of 0.01 M with peak B to determine the relative rates of hydrolysis; a digest with 3% w/v inulin was included. The digests were incubated at 25°C for 4 hr, and $HgCl_2$ solution was then added to the digest; samples from the treated digests were chromatographed on Charcoal/Celite columns and the total fructose in the effluents estimated. The results are given in Table 43, and confirm that sucrose was attacked very slowly by the enzyme. The rate of hydrolysis of the oligosaccharides increased with D.P. up to about D.P. 6 while the rate of hydrolysis of inulin was substantially lower than that of any of the oligosaccharides apart from sucrose. The hydrolysis of inulin which would be expected at a concentration of 0.01 M can be calculated from the Lineweaver-Burk plot in Fig. 33; this value (given in the table) is about the same as that for the oligosaccharide of D.P. 3, but the rate of hydrolysis of the latter may have been reduced by the increasing concentration of sucrose which is formed in the progress of the reaction. Samples from the treated digests were also chromatographed on paper; the sprayed chromatogram (Fig. 34) showed that in the digests of the compounds of D.P. 3 to 6, the oligosaccharide formed in largest amount by the hydrolysis of the one present at the start of the reaction was that of the next lower D.P., indicating that the enzyme does not degrade a substrate molecule completely to sucrose and fructose before attacking another (single chain attack), but that after the hydrolysis of a glycosidic linkage the association between the enzyme and substrate is broken (multi-chain attack).

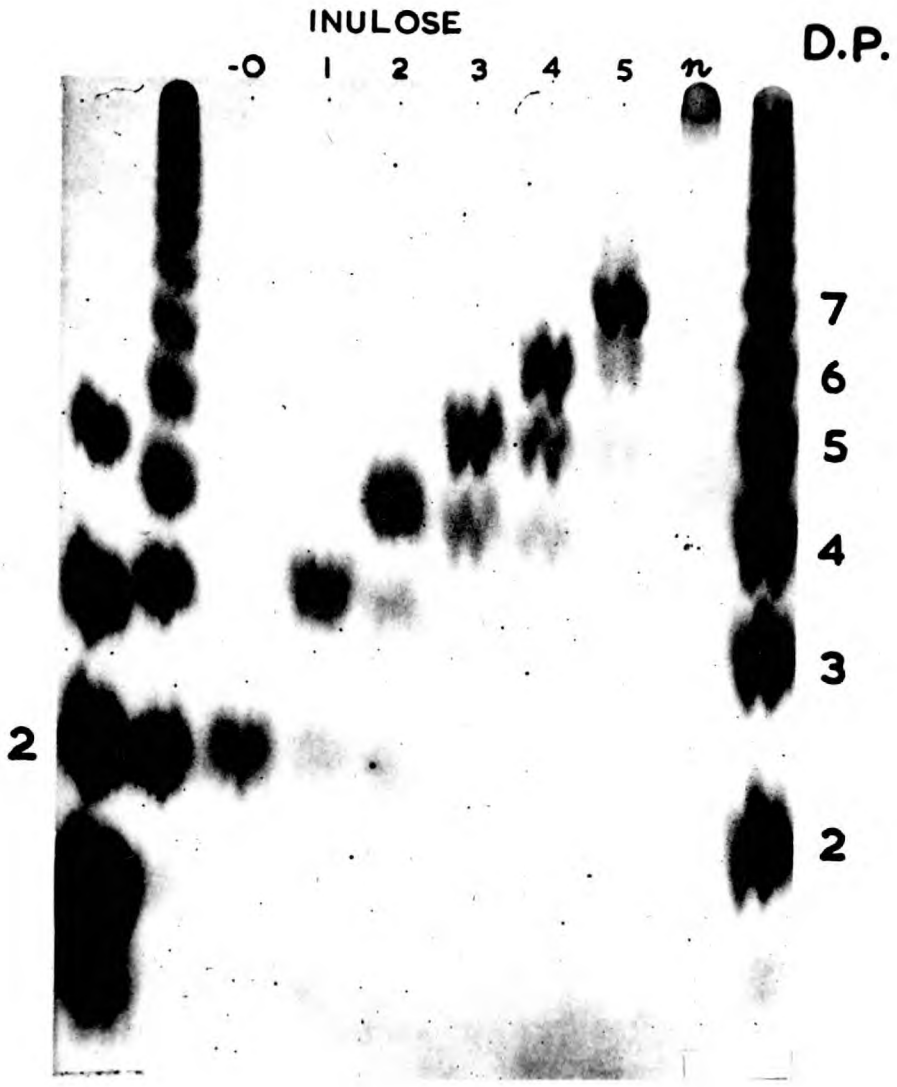


Figure 34. Action of hydrolase B on inulin and various members of the inulose series of oligosaccharides. For details of incubation procedure see text; chromatogram developed in PEAW 6:1:3 and sprayed with benzidine-TCA.

Table 43. Activity of hydrolase B against members of the inulose series and inulin.

Digests (0.4 ml.) contained 0.2 ml. of enzyme solution with substrates in 0.1 M-acetate buffer pH 5, and were incubated at 25°C for 4 hr. Activity was estimated by determining the increase in free fructose.

Substrate	Substrate concentration μmoles/ml.	μmoles fructose liberated	Percentage of terminal fructose hydrolysed
inulose-0	10	0.052	0.4
(sucrose)	10	0.11*	-
inulose-1	10	1.35	13.5
inulose-2	10	1.73	17.3
inulose-3	10	3.05	30.5
inulose-4	10	3.42	34.2
inulose-5	10	3.06	30.6
inulin	6	0.902	14.9
inulin	6	0.009*	-
	10/	1.24/	12.4/

* digests without enzyme
/ calculated value (see text)

The rates of hydrolysis by the enzyme of β2 : 1' linked chains of fructose units terminated by a glucosyl molecule can be compared with those terminated by a reducing fructose molecule using the results of the two previous experiments: in Fig. 35 the rates are plotted as a percentage of the rate of hydrolysis of 3% w/v inulin in the respective experiments. The comparison shows that the rates are related to the D.P. of the oligosaccharide as a whole rather than to that of the chain of fructosyl units.

The relative rates of hydrolysis of different oligosaccharides were examined again using a wider range of substrates; as well as the

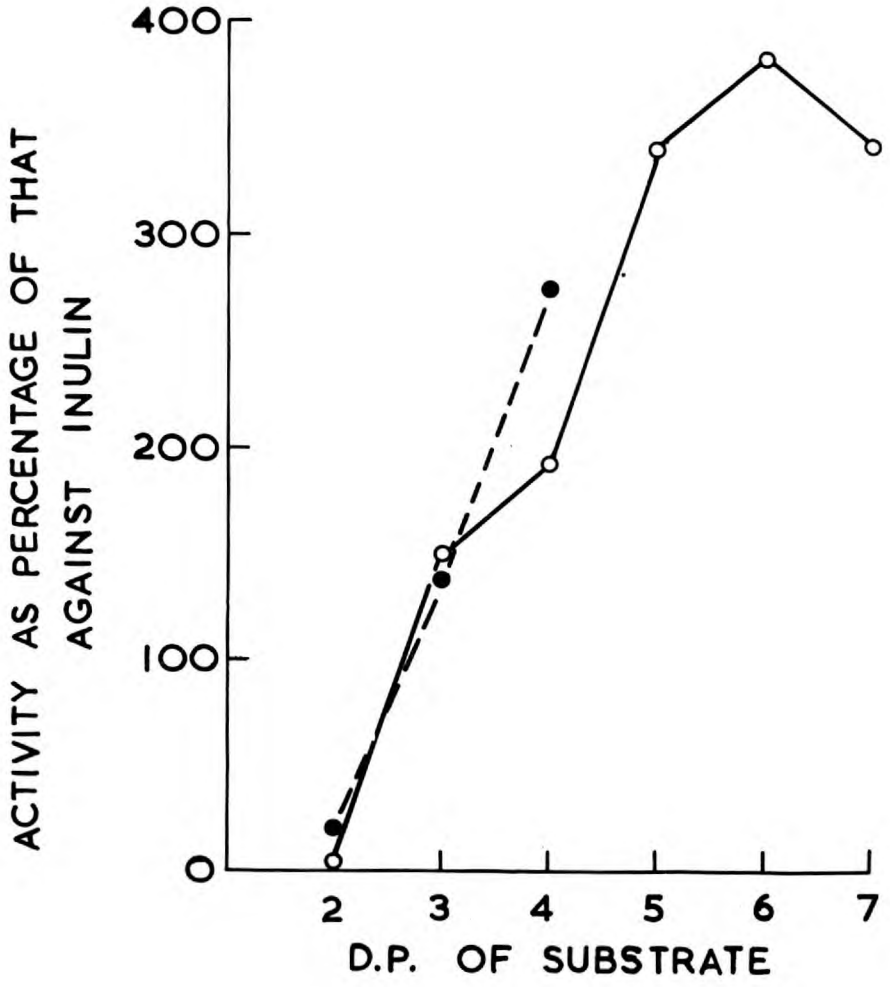


Figure 35. Comparison of rates of hydrolysis of members of the inulose (○) and inulo-n-ose (●) series of oligosaccharides by hydrolase B. Data from Tables 42 and 43.

sugars used above others extending these two series, and members of different series were examined. The substrates (0.01 M, inulin 5% w/v) were incubated with the enzyme in 0.1 M-acetate buffer pH 5 for 4 hr. at 25°C. The rates of hydrolysis estimated by the total fructose method after chromatography of samples from the digests on Charcoal/Celite columns are shown in Fig. 36. The results with those sugars used in the previous experiments confirm the differences found then; the results of hydrolysis of the series terminated by a raffinose group show that they behave as if the melibiose group were just a glucose residue.

The activity of the enzyme against β-methylfructofuranoside and against fructoside chains terminated by a sugar alcohol (see p.80) was determined; digests containing inulopentaose and inulin were included for comparison. All substrates were present at a concentration of 0.01 M, and the digests incubated at 25°C for about 3 hours. The results are given in Table 44, and show that the series of oligofructosides terminated by a sugar alchol are hydrolysed at a rate which increases with the length of the fructoside chain from 1-3 residues; that with one unit was practically inert, but the three-unit chain was hydrolysed at a rate comparable to that at which similar chains terminated by a glucose or a melibiose moiety are attacked.

The effect of the presence of various sugars on the rate of enzymatic hydrolysis of inulin was examined, together with the effect of sucrose on the hydrolysis of inulopentaose. The results are given in Table 45, where it is plainly shown that none of the other sugars used had an effect comparable to that of sucrose: the results indicate that the rate of hydrolysis of inulin was accelerated in the presence of

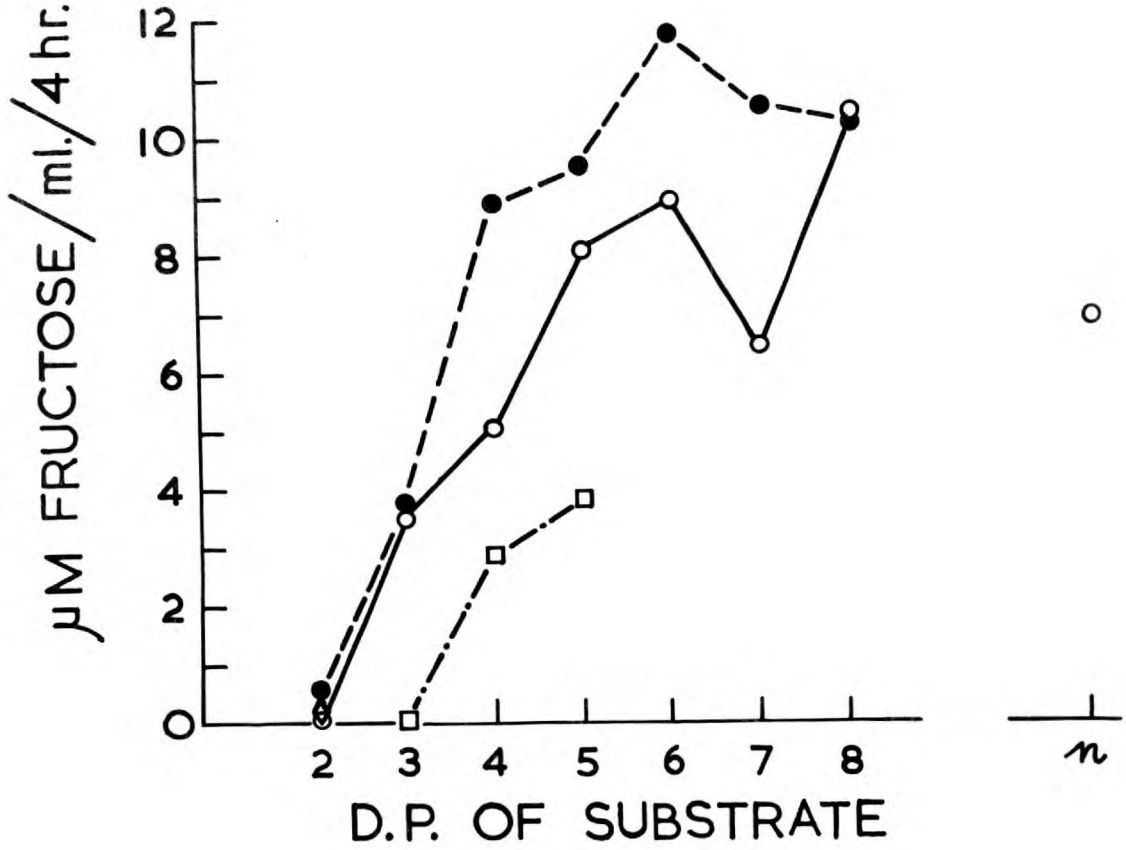


Figure 36. Action of hydrolase B on various oligosaccharides. For details of experiment see text.

○ inulose series ● inulo-n-ose series
□ raffinose (DP 3), 1^F-fructosylraffinose (DP 4), $(1^F\text{-fructosyl})_2$raffinose (DP 5). ◇ β-D-fructofuranosyl-6-D-glucose.

Table 44. Activity of hydrolase B against fructosyl chains terminated by a hexitol molecule, and against β-D-methylfructofuranoside.

Digests (0.4 ml.) contained 0.2 ml. enzyme solution with substrates (0.01 M) in 0.1 M-acetate buffer pH 5; digests containing inulopentaose and inulin were included in the experiment. Digests were incubated at 25°C for 3 hr.

Substrate	Fructose liberated (μmoles/ml./min.)
β-D-methylfructofuranoside	0.41
fructosylhexitol	0.18
$(fructosyl)_2$hexitol	2.26
$(fructosyl)_3$hexitol	6.51
inulopentaose	11.4
inulin	4.66

certain of the sugars. The hydrolysis of inulopentaose was inhibited by sucrose to about the same extent as the hydrolysis of inulin, the values being 43.8% and 46.6% respectively.

The effect of some other sugars was examined in a similar experiment; the effect of citric acid was also investigated as this compound was employed in the buffer mixtures used in the experiment in which the effect of pH on the activities of hydrolase A and hydrolase B was compared (p.107). The results are given in Table 46, and show that the rate of hydrolysis of inulin was unaffected by citric acid and by β-D-fructofuranosyl-6-D-glucose. Inulobiose inhibited the hydrolysis to about the same extent as did sucrose; it was also clear that the amount of free fructose released in the digest containing 1^F-fructosylsucrose and inulin was smaller than the sum of the amounts released

Table 45. Effect of various sugars on activity of hydrolase B against inulin, and of sucrose on activity of hydrolase B against inulopentaose.

Digests (0.4 ml.) contained 0.2 ml. of enzyme solution and substrate (0.005 M) in 0.1 M-acetate buffer pH 5; incubated at 25°C for 4 hr. Activity was estimated by determining the amount of free fructose in the digests.

Added sugar (0.01 M)	µmoles Fructose per ml.			Relative hydrolysis of inulin
	- Inulin	+ Inulin	Increase in fructose	
	(a)	(b)	(b) - (a)	
none	0.14	3.59	3.45	100
glucose	0.14	3.43	3.29	95.4
fructose	9.95	13.2	3.25	94.3
sucrose	0.18	1.92	1.74	50.4
melibiose	-	4.29	4.15	120.
cellobiose	-	3.53	3.39	98.2
maltose	-	3.91	3.77	110.
lactose	-	3.43	3.29	95.4
αα-trehalose	-	4.22	4.08	118.
raffinose	0.07	3.43	3.36	97.4
melezitose	0.03	4.23	4.09	119
	- inulopentaose	+ inulopentaose		
none	0.14	12.9	12.8	100.
sucrose	0.18	7.43	7.25	56.7

Table 46. Effect of various sugars and of citric acid on activity of hydrolase B against inulin.

Digests (0.4 ml.) contained 0.2 ml. of enzyme solution and 0.005 M-inulin in 0.1 M-acetate buffer pH 5; incubated at 25°C for 4 hr. Activity was estimated by determining the amount of free fructose in the digests.

Added substance	µmoles fructose per ml.			Relative hydrolysis of inulin*
	- inulin (a)	+ inulin (b)	increase in fructose (b) - (a)	
none	-	3.10	3.10	100
sucrose	0.16	1.58	1.42	45.8
inulobiose	1.18	2.92	1.74	56.2
inulose-1	3.52	4.03	0.51	16.5
β-D-fructosuranosyl (2 6)D-glucose	0.23	3.84	3.61	116.
citric acid	-	3.02	3.02	97.4

* calculated on the assumption that the rate of hydrolysis of the added sugar is identical in presence and absence of inulin.

from the two substrates incubated alone with the enzyme: this could have been caused by inhibition of inulin hydrolysis by 1^F-fructosylsucrose, inhibition of hydrolysis of the oligosaccharide by inulin, or both. These possibilities were examined by running chromatograms of samples from the digests with inulin alone, 1^F-fructosylsucrose alone, and a mixture of both. The positions corresponding to those of sucrose and fructose on marker strips were cut out for quantitative estimation of their fructose content. The results, given in Table 47, show that no sucrose was detectable in the digest with inulin alone; in that with 1^F-fructosyl-sucrose alone there was, as would be expected, a substantial amount of

Table 47. Inhibition of activity of hydrolase B against inulin by 1^F-fructosylsucrose (inulose-1)

Amounts of fructose and sucrose recovered from chromatograms of aliquots of digests (see text and Table 46). Results are expressed as μmoles per ml. of digest.

Inulin		Inulose-1		Inulin + Inulose-1	
fructose	sucrose	fructose	sucrose	fructose	sucrose
a	b	c	d	e	f
3.09	0	3.55	4.31	4.03	4.15

Derived data:

from c and d, excess of sucrose (present as contaminant in inulose-1 - 5% w/w) = 0.76 μmoles.

thus, from f, and assuming that no sucrose was liberated from inulin b, the amount of sucrose liberated by hydrolysis of inulose-1 in the presence of inulin = 3.39 μmoles, assuming equimolar enzymatic production of sucrose and fructose.

from e, amount of fructose produced by hydrolysis of inulin = 4.03 - 3.39 = 0.64 μmoles.

Therefore, inhibition of inulin hydrolysis by inulose-1, by comparison with a, = 79%.

sucrose. The quantity of sucrose in the digest with both inulin and 1^F-fructosylsucrose was comparable to that with 1^F-fructosylsucrose alone, indicating that the hydrolysis of this oligosaccharide was not affected by the presence of inulin; the calculations appended to Table 47 gives a value for the inhibition of inulin hydrolysis by 1^F-fructosylsucrose of 79%, which is very close to that obtained (Table 46) by assuming that the rate of hydrolysis of 1^F-fructosylsucrose was totally unaffected by inulin.

The effect of some of the sugars used above was re-examined, and the effect of β-methylfructofuranoside and β-fructosylhexitol were examined; inulin was incubated with these sugars in the presence of hydrolase B, and the amount of free fructose in chromatographed samples determined. The results (Table 48) indicate that inulobiose (sucrose-free, see p. 77) and β-fructosylhexitol inhibited the hydrolysis of inulin to about the same extent as did sucrose; melezitose and β-methylfructofuranoside appeared to have significant but smaller effects, while melibiose, maltose and αα trehalose did not inhibit the hydrolysis.

The inhibition of inulin hydrolysis by sucrose was re-examined to try to establish the nature of the inhibition; six concentrations of inulin were incubated with and without 0.01 M-sucrose at 25°C. The results of fructose estimations of samples chromatographed on Charcoal/Celite columns are given in Table 49; a Lineweaver-Burk plot of these values suggests that the inhibition by sucrose is non-competitive, as the two lines meet on the x axis. The experiment gives a slightly different value of Km for inulin, namely 3.6×10^{-2}, as compared with

Table 48. Effect of various sugars on the activity of hydrolase B against inulin.

Digests (0.4 ml.) contained 0.2 ml. of enzyme solution and 0.005 M-inulin in 0.1 M-acetate buffer pH 5; and were incubated at 25°C for various times (2.5 - 3 hr.). Activity was estimated by determining the amount of free fructose in the digests: values are corrected for fructose produced (if any) from the added sugar.

	Added sugar (0.01 M)	Rate of hydrolysis of inulin µmoles/ml./min.	Percentage inhibition
(a)	none	1.61	-
	sucrose	0.54	66.5
	inulbiose*	0.69	57.0
	melibiose	1.43	10.9
	maltose	1.44	10.3
	αα-trehalose	1.48	7.8
	melezitose	1.14	29.0
(b)	none	2.55	-
	sucrose	0.80	68.7
	β-D-methylfructofuranoside	2.08	18.4
	β-D-fructofuranosyl-hexitol/	1.42	44.3

* sucrose-free
/ 0.005 M.

the value of 1.4×10^{-2} (p.121); in the latter case however only three points were available to construct a line, and the estimation of activity was by the less precise reducing sugar method. The Ki for sucrose, assuming it to be a non-competitive inhibitor was 1.1×10^{-3}.

Table 49. Effect of sucrose on hydrolysis of inulin by hydrolase B.

Digests (0.4 ml.) containing 0.2 ml. of enzyme solution with a series of inulin concentrations in 0.1 M-acetate buffer pH 5, were incubated with and without 0.01 M-sucrose at 25°C. Activity was estimated by determining the free fructose in the digests.

Inulin concentration (μMoles/ml.)	Rate of fructose production μmoles/ml./min.		Percentage inhibition of inulin hydrolysis
	- sucrose	+ sucrose*	
10	2.84	1.38	51.4
5	1.61	0.82	49.0
3.3	0.91	0.51	43.9
2.5	0.78	0.37	52.7
2	0.68	0.33	51.6
1.7	0.52	0.22	57.7
0	0	(0.06)	-

* corrected for the value obtained for enzyme plus sucrose alone, given at bottom of column.

The Km for inulose-1, which is the member at the other end of the series attacked by the hydrolase was determined; as the hydrolysis results in the liberation of sucrose, the digests were made up to contain amounts of sucrose which were expected to swamp the effect of any sucrose liberated; thus a series of concentrations of 1^F-fructosylsucrose was incubated with the enzyme in the presence of 0.01 M-sucrose; the results are given in Table 50, and when a Lineweaver-Burk plot is made a straight

line is obtained. No digests were set up without sucrose; the Km was calculated on the assumption that the sucrose inhibition of hydrolysis of 1^F-fructosylsucrose was non-competitive, as in the case of inulin, and that such increase in sucrose as occurred by hydrolyis of 1^F-fructosylsucrose was insufficient to affect the extent of this inhibition significantly: on this basis, the Km was calculated to be 3.3×10^{-2}, that is, about the same as the Km for inulin (3.6×10^{-2}).

Table 50. Effect of substrate concentration on the rate of hydrolysis of inulose-1 (1^F-fructosylsucrose) by hydrolase B in the presence of sucrose.

Digests (0.4 ml.) contained 0.2 ml. of enzyme solution and a range of concentrations of inulose-1 in 0.01 M-sucrose and 0.1 M-acetate buffer pH 5, and were incubated at 25°C. Activity was estimated by determining the free fructose in the digests.

Concentration of inulose-1 (μmoles/ml.)	Rate of fructose production* μmoles/ml./min.
10	1.22
7.5	0.92
6	0.76
4	0.54
2.5	0.38
1	0.15
0	(0.06)

*corrected for the value obtained for enzyme plus sucrose alone, given at bottom of column.

The nature of the inhibition by sucrose was further investigated by incubating hydrolase B (0.2 ml. in 0.4 ml. digest) with 5% and 1% w/v inulin, with a series of sucrose concentrations in 0.1 M-acetate buffer pH 5, at 25°C; the results, obtained by the routine method, are given

in Table 51. In Fig. 37 the percentage inhibition of the hydrolysis of 5% and 1% inulin is plotted against sucrose concentration; the fact that the two curves are coincident shows that the extent of inhibition is determined by the absolute concentration of sucrose, rather than by the relative concentrations of inulin and sucrose; this situation suggests that the inhibition is non-competitive.

Table 51. Activity of hydrolase B against inulin in the presence of different concentrations of sucrose.

Three sets of digests were made up, with 0.01 M inulin (5% w/v), 0.002 M inulin (1% w/v) and without inulin respectively. Each digest (0.4 ml.) contained 0.2 ml. of enzyme solution with inulin and/or sucrose in 0.1 M-acetate buffer pH 5; and was incubated at 25°C for 4 hr. Activity was estimated by measuring the free fructose in the digests, and is expressed as mμmoles/ml./min.

Sucrose concentration (mM)	Inulin concentration mM		
	0	2	10
10	0.456	2.23	8.16
5	0.262	2.93	11.5
2	0.197	4.07	14.6
1	0.165	4.42	17.1
0.5	0.099	4.50	18.4
0.2	0.100	4.82	19.3
0.1	0.033	4.54	19.4
0	0.033	4.74	19.4

When each pair of results (i.e. 5% and 1% inulin) is expressed as a Lineweaver-Burk plot, non-competitive inhibition is again indicated, but it is possible that there is an element of competitive inhibition at the highest concentrations of sucrose. The plot of the two inulin digests

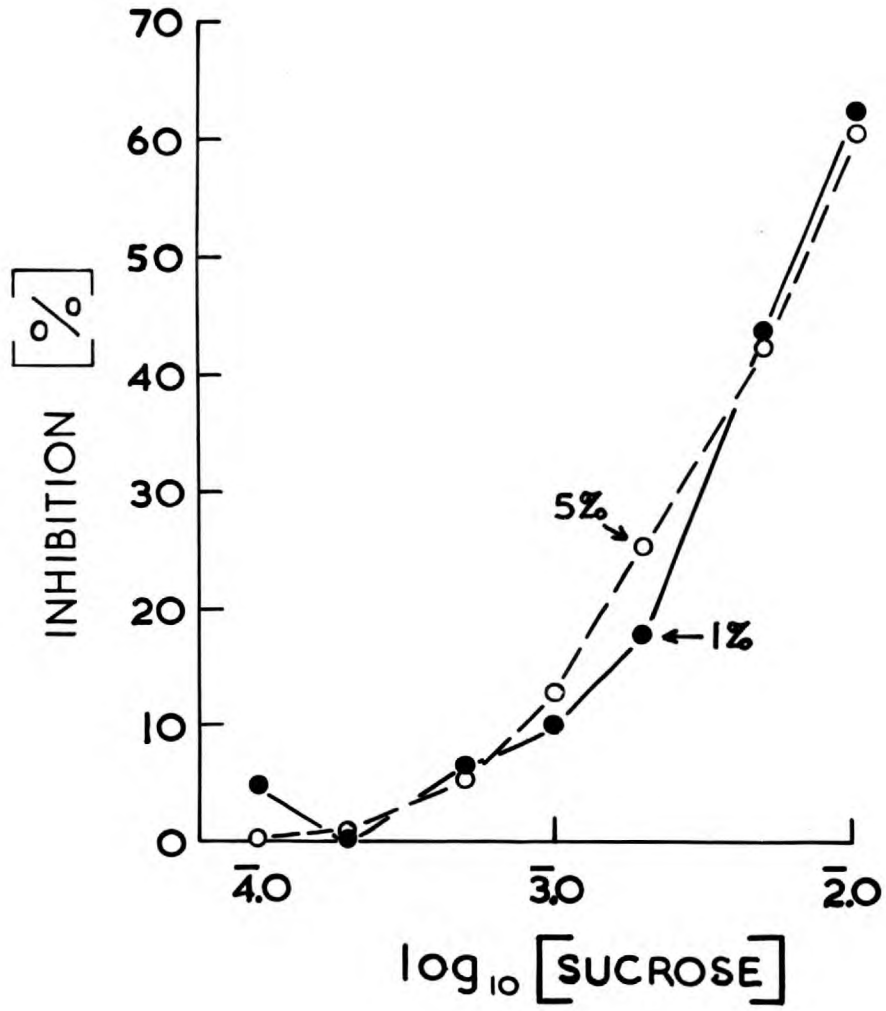


Figure 37. Sucrose inhibition of hydrolysis of 5% (0.01 M) and 1% (0.002 M) inulin by hydrolase B. Data taken from Table 51.

without sucrose gives Km = 3.6×10^{-2}. When the data are plotted as 1/v against i for the two concentrations of inulin (Dixon, cf. Dixon & Webb (1958) p. 21) the point of intersection which gives the value of Ki directly is on the x axis if the competition is non-competitive. It can be seen in Fig. 38 that this is not quite so, but the Km can be calculated from the fact that each line cuts the base at a value of $i = -Ki\left(\frac{s}{Km} + 1\right)$ in the case of competitive inhibition, gives values of 3.9×10^{-2} (5% series) and 1.9×10^{-1} (1% series): these values indicate that the assumption that the inhibition is competitive is invalid. Km cannot be calculated from this type of graph if the inhibition is non-competitive in nature.

An experiment was carried out to examine the effect of melezitose and raffinose; these sugars (0.01 M) were incubated with three concentrations of inulin in the presence of the enzyme. The results when expressed as Lineweaver-Burk plots gave values of 2.9×10^{-2} and 2.1×10^{-2} respectively when the Km of inulin was calculated from $-\frac{1}{Kp}$ by assuming that the inhibition by these sugars was non-competitive; the value for inulin alone (using six concentrations) was 4.6×10^{-2}. Owing to the variation in the value of Km obtained in different experiments, the difference between the values of Km in the presence of melezitose and raffinose, and that of inulin alone cannot be held to be significant. At the highest concentration of inulin (0.005 M) the effect of these sugars was compared with that of 0.01 M-sucrose; the results are given in Table 52. The inhibition by raffinose is at variance with an earlier result (see p.120).

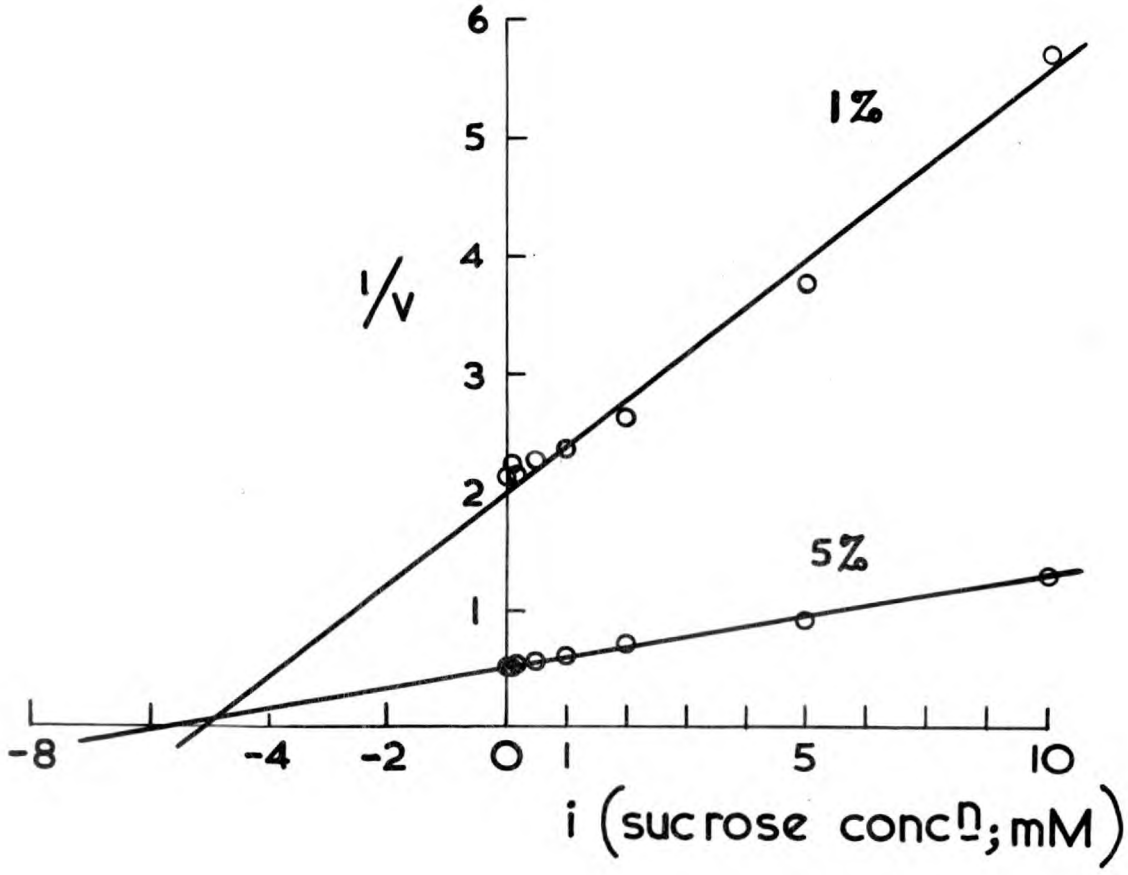


Figure 38. Nature of sucrose inhibition of inulin hydrolysis by hydrolase B. Values calculated from data in Table 51.

Table 52. Effect of sucrose, melezitose, and raffinose on the hydrolysis of inulin by hydrolase B.

Digests (0.4 ml.) contained 0.2 ml. of enzyme solution and 0.005 M-inulin in 0.1 M-acetate buffer pH 5, and were incubated at 25°C for 3 hr. Activity was estimated by determining the free fructose in the digests.

Added Sugar (0.01 M)	µmoles/ml./min.		Inhibition of inulin hydrolysis (%)
	- inulin	+ inulin	
none	-	2.55	-
sucrose	0.10	0.80	69
melezitose	0.03	1.87	27
raffinose	0.07	1.91	25

The Km of inulopentaose and of inulose-6 was determined in an experiment carried out at the same time as that described above (Table 53); the values, calculated from Lineweaver-Burk plots were 2.2×10^{-2} and 2.5×10^{-2} respectively.

Table 53. Effect of substrate concentration on rate of hydrolysis of inulose-5 and inulopentaose by hydrolase B.

Digests (0.4 ml.) containing 0.2 ml. of enzyme solution and substrates in 0.1 M-acetate buffer pH 5 were incubated at 25°C for 3 hr. Activity was estimated by determining the free fructose in the digests, and is expressed as m µmoles/ml./min.

Substrate concentration mM	Substrate	
	inulose-5	inulopentaose
10	5.47	63.3
7.5	4.49	47.8
6	2.77	39.1
4	2.57	27.2
2.5	3.35	18.6
1	1.76	8.12

DISCUSSION

The results in the experimental section show that during the growth of the daughter plant considerable changes in the carbohydrate content of the parent tuber take place. Differences in tuber size had no effect on the pattern of change; samples from the three series of tubers (on p. 18) can therefore be treated as replicates. Over the 7-week period from planting, some 80% of the dry weight disappeared from the tuber, and it was deduced from results of experiments performed in total darkness and from those experiments in which plants were removed from their parent tubers that most of this material was translocated to the daughter plant, some of it being later respired.

About 80% of the loss in dry weight was accounted for by a parallel disappearance of polymerized fructose; it is evident, therefore, that the fructosan of the tuber contributes markedly to the growth of the new plant. During this process the tubers changed very little in external appearance but when cut open gave every evidence of being "sucked out", the previously dense parenchymatous tissue being converted to loose stringy material containing many large gas spaces.

The dry weights of the daughter plants which were grown in the light were not measured, but a comparison can be made between the fresh weights of the shoots which grew in the light, and the fresh weight of shoot + root which grew in the dark. In Fig. 39 these values are plotted as a proportion of the dry weight lost from the tuber, and indicate that, at least during May and June, much of the energy and substance for the early growth of shoots in the light is supplied by

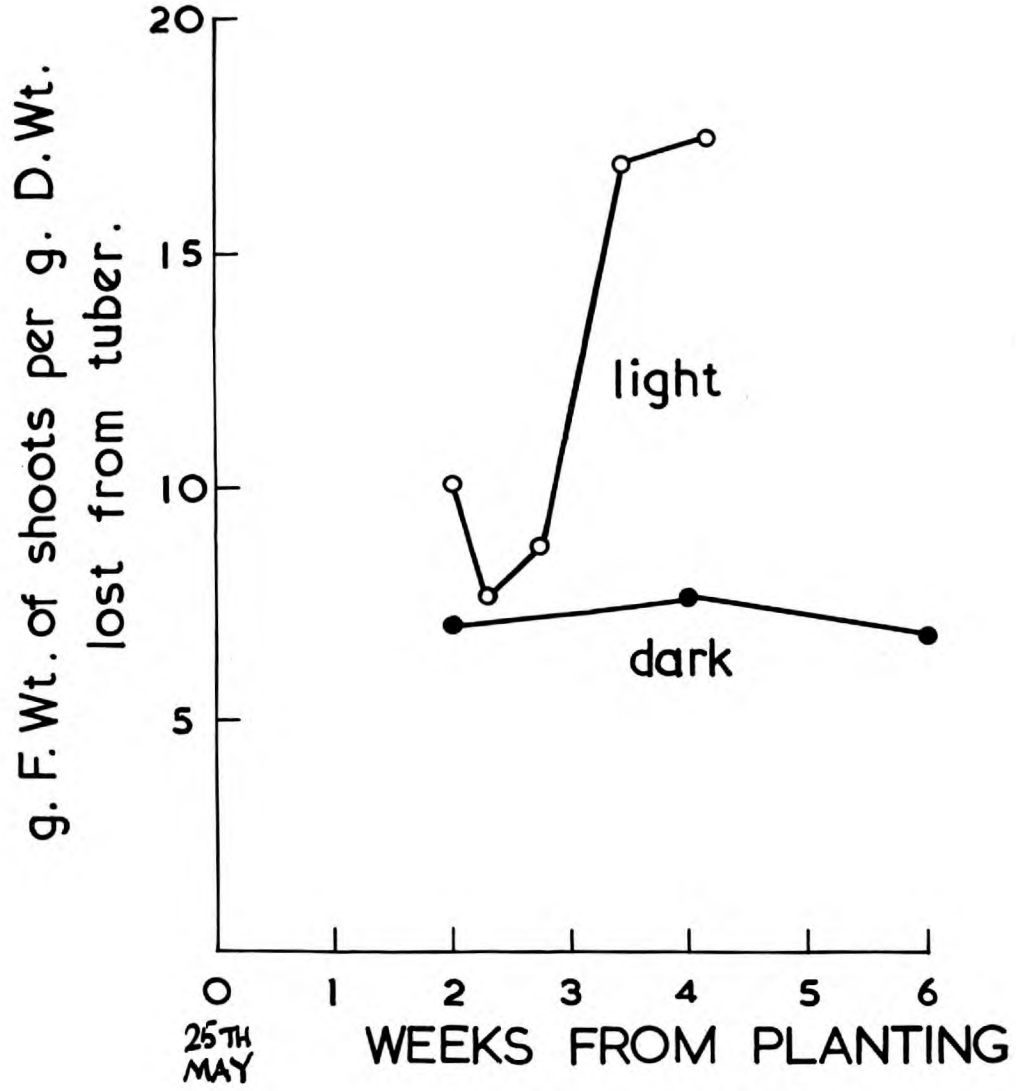


Figure 39. Fresh weight of shoots grown in the light, and of shoots plus roots grown in the dark, related to the dry weight lost from the tuber. Calculated from data given in Table 1 and Table 3.

material translocated from the tuber, rather than by photosynthesis.

The loss of material from the tubers is rapid: thus between two and four weeks after planting, the total fructose content fell from 73.2% to 32.3% of the original dry weight (average of three series); thus a tuber of 20 g. fresh weight would lose 1.73 g. of fructose over this period, or about 124 mg. a day.

Changes in the detectable monosaccharides and short chain fructosides, and also in the fructosan of D.P. $\geqslant$ 6, were followed individually. The following facts can be deduced from Table 53, where the sugar contents are given as averages for the three series.

1. All fractions with D.P. $\geqslant$ 2 decreased in amount with time, the material with D.P. $\geqslant$ 6 accounting for the bulk of the total weight lost as would be expected from the largest fraction. However, whereas the fructose content of the tetra-, penta- and polysaccharides fell at similar rates relative to their initial content, that of the trisaccharide fell more slowly, and that of sucrose more slowly still (see Fig. 40). The similarity of the curves for the tetra- and pentasaccharide and mixed polymer fractions indicates that all of the individual fructosides ranging from D.P. 4 to D.P. n were losing fructose at similar rates.

2. The amount of free fructose rose from practically none at the time of planting to a relatively high value at about the time of maximum breakdown of the polymerized material. This suggests direct hydrolysis of the combined fructose, although the amount of free fructose which accumulated at the highest level (110 mg./g. of the original dry weight) was very much less than the depolymerization which had occurred

Table 53a Changes in individual carbohydrates present in tubers during the growth of daughter plants.

Values are calculated as averages of three tubers, from data given in Tables 1 and 2 (series A, B and C).

Period of growth (days)	Glucose mg./g. of original dry weight	Fructose content : mg. free hexose per g. of original dry weight					
		DP 1 (fructose)	DP 2 (sucrose)	DP 3	DP 4	DP 5	DP ⩾ 6
1	-	4.2	41.7	34.1	47.5	48.5	618
14	3.7	10.0	32.2	26.0	39.7	42.3	598
28	2.1*	110	36.3	14.2	10.1	9.4	142
36	1.2	74.5	32.4	13.4	9.0	9.2	82.6
42	1.1	39.0	12.3	4.2	2.7	2.5	7.6
49	3.0	17.3	9.2	2.7	2.1	2.7	11.0

* average for two tubers

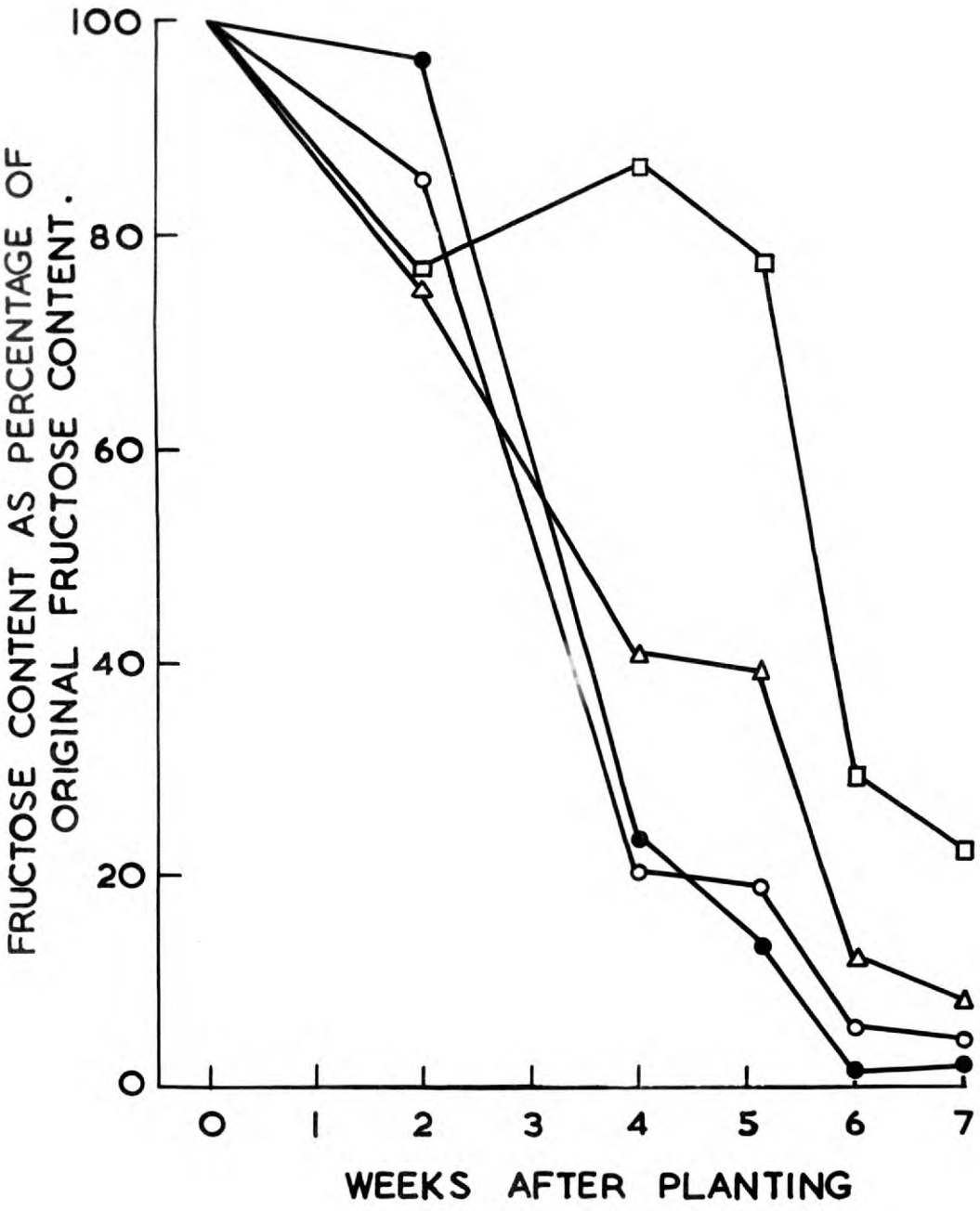


Figure 40. Relative changes in the fructose content of the fructosides of the tuber during sprouting at 20°C.

● DP≥6, ○ DP 5, ○ DP 4 (coincident with all values for DP 5), △ DP 3, □ DP 2 (sucrose). Calculated as averages of values for series A, B, and C in Table 2.

by that time (642 mg.).

3. The level of free glucose remained low throughout, being always less than 7 mg./g. of the original dry weight.

The quantities of monosaccharides are compared in Fig. 41; it is not likely that this situation is caused by the inversion of sucrose. The observations are consistent with the hypothesis that the process of carbohydrate breakdown is initiated by the hydrolase q.v.; it is assumed that sucrose is the major translocatory substance and although the possibility of direct translocation of polymerized material cannot be ruled out it has been shown that sucrose is the first detectable radioactive compound to appear in other tissues when leaves of the artichoke assimilate $^{14}CO_2$ (Edelman & Popov, 1962). Mayer & Porter (1960) have shown that in the case of rye leaves which had assimilated $^{14}CO_2$ into sucrose and fructosans, only radioactive sucrose was translocated to other parts of the plant while the fructosans remained in the leaf. If sucrose is the sole translocatory material, free fructose which is released from polymer must be converted to sucrose: it has been reported that radioactive fructose fed to tuber disks appears in the tissue in the form of sucrose, in which both the glucose and fructose moieties are radioactive (Recaldin, 1961). In the present work, an absolute increase in glucose content (combined in sucrose residues) is shown to occur when tubers are stored at 2°C, but although this appears to be derived from fructose, there is no direct evidence that free fructose is an intermediate in the process.

An attempt was made to see if the hydrolase is indeed involved

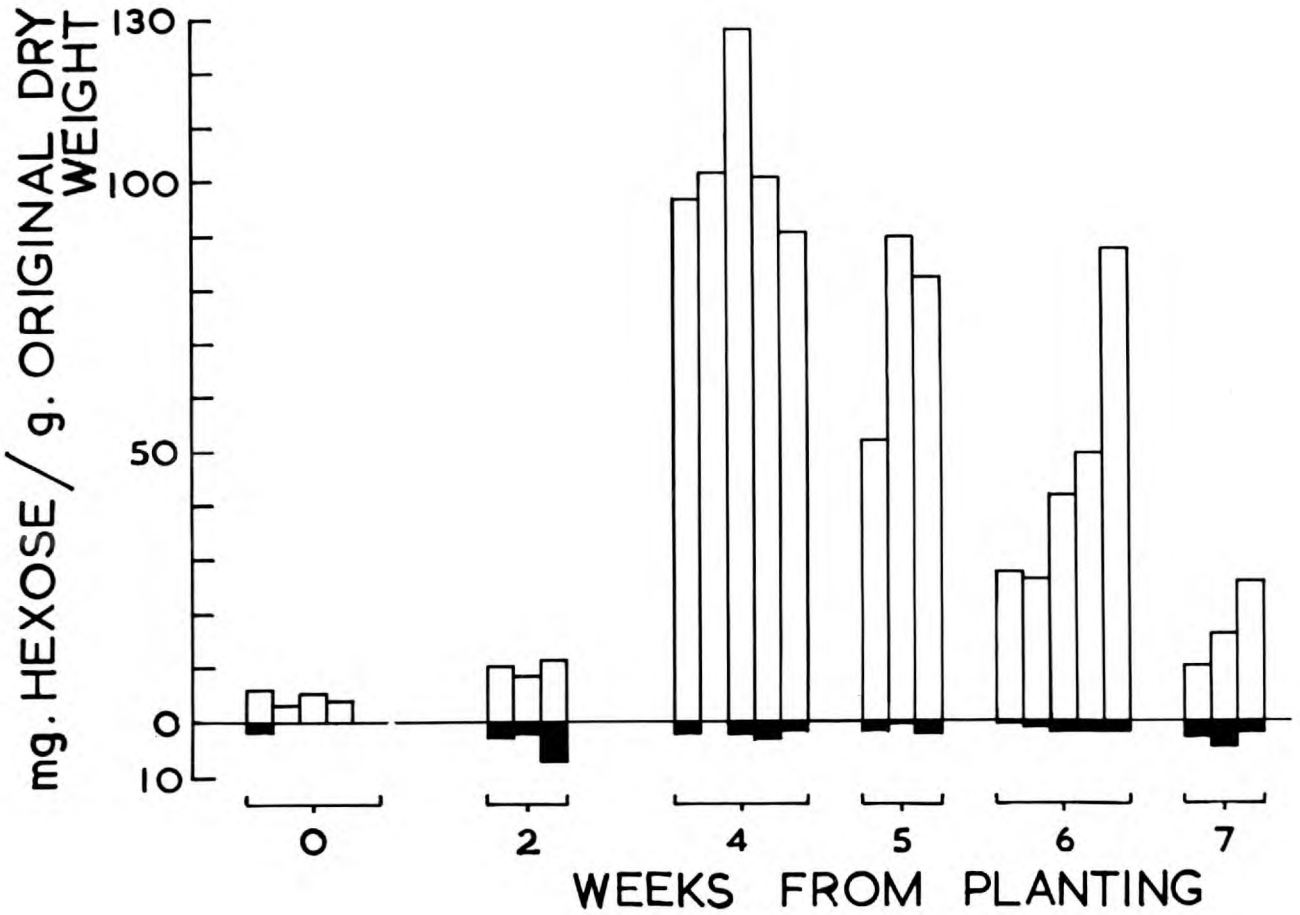


Figure 41. Monosaccharide content of tubers sprouting at 20°C. Solid columns - glucose, open columns - fructose. Calculated from values in Table 2; the letters indicate the series.

in the changes observed, by suddenly removing the carbohydrate sink, namely the daughter plant; continued enzyme activity would then result in increases in the lower members of the series and/or sucrose and free fructose. The results showed however that a controlling influence in the changes observed in the normally sprouting tuber is the daughter plant, as all detectable changes in the fructoside series stopped when material was prevented from leaving the tissue (Fig. 42).

An attempt was then made to discover if depolymerization is affected by the presence of buds which are not growing, i.e. in tubers stored at low temperature (2°C). Depolymerization was followed by using the well-known phenomenon of the change in optical rotation which occurs when artichoke tubers are stored overwinter. Thus the optical rotation of the juice expressed from tubers of the artichoke early in the dormancy period (Autumn) is negative, but juice taken later in the period shows a more positive rotation, reaching its maximum positive value around February, the change from negative to positive usually occurring during December. The change is known to be accompanied by the disappearance of inulin from the tubers (Belval & Chollet, 1948), while Bacon & Loxley (1952) correlated the change with an increase in the proportion of oligosaccharides of D.P. 2–5 at the expense of more highly polymerized material. In previous work in which the optical rotation was studied the tubers all bore buds, but the present work shows that neither the rate nor the extent of the change which occurs when tubers are stored at 2°C is affected by the removal of buds (nor by wounding of the cortex performed as a control treatment); the buds made no

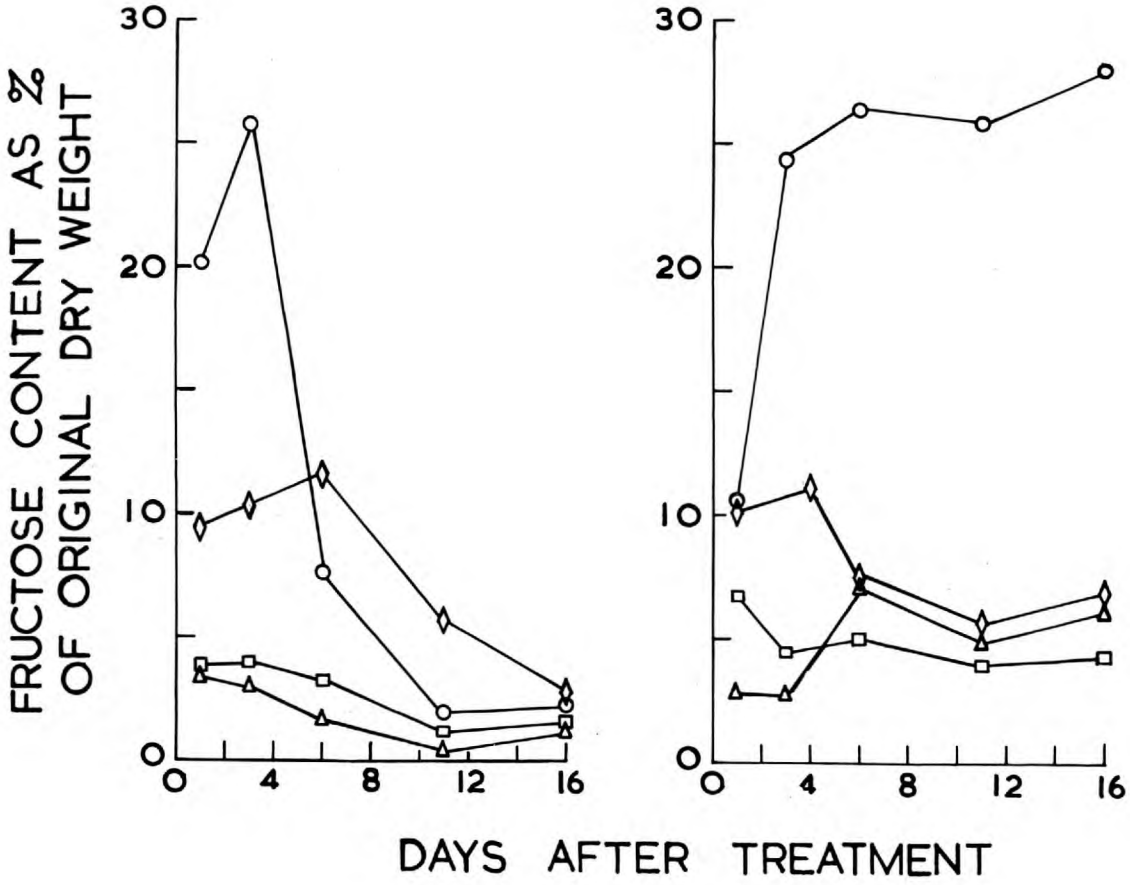


Figure 42. Changes in the fructosides after removal of growing tissue from the tuber. Controls on left, treated plants on right. ○ DP≥5, △ DP 3 plus DP 4, □ DP 2 (sucrose), ◇ free fructose. All values are in terms of fructose content calculated as free hexose, from Table 5.

perceptible growth over the experimental period and there was no significant difference in the amount of dry material lost by disbudded, wounded or untreated (control) tubers (see Fig. 6). Thus, if the depolymerizing process occurring during dormancy has the same enzymatic mechanism as that occurring in the parent tuber during the growth of daughter plants, it is obvious that the buds which give rise to the new plants do not initiate or control the process and, therefore, when the depolymerization in tubers sprouting at 20°C is arrested by cutting off the growing shoots, these can be considered to have been acting merely as sinks for the translocation of carbohydrate.

This experiment led to the discovery of a striking temperature effect viz. a considerable stimulation of depolymerization by low temperature alone. Thus the $[\alpha]_D^{20}$ of extracts of tubers stored at 2°C for seven weeks changed from -13.5° to +21.6°, whereas those at 20°C showed very little change, -13.5° to -11.4°.

The results from tubers with growing buds at 20°C indicated that depolymerization can occur at this temperature, as after 15 weeks such tubers lost 41.1% of their original dry weight; the specific optical rotation of the extracts from these tubers was however practically the same as from those tubers without buds which lost only 22.1% of their original dry weight after 14 weeks (although these tubers had been disbudded, a certain amount of new growth, in the form of a callus, did occur so that they cannot be regarded as fully dormant).

Direct measurements on the fructosan series present in the tubers stored at 2° and at 20°C showed that at the lower temperature the

fructosides of lower D.P. accumulated at the expense of the higher ones (cf. Bacon & Loxley); this is why the specific optical rotation of the soluble carbohydrate mixture changed in the positive direction, as the fructosides would appear to constitute a graded series with respect to optical rotation ranging from sucrose ($[\alpha]_D^{20}$ + 66.5°), through 1^F-fructosylsucrose (about + 30°, Bacon & Bell, 1953; Bacon, 1954).and higher members of undetermined but more negative specific rotation, to inulin (about -40°, McDonald, 1946). Little free glucose and fructose could be detected during such changes. At 20°C redistribution of fructose residues only occurred to a very limited extent, although depolymerization was inferred in those tubers with growing buds; here the depolymerization could be detected as a reduction of total sugar content, but it was not accompanied by the large and rapid change in specific optical rotation; this was not caused by extensive accumulation of free fructose, but because there was no accumulation of lower oligosaccharides.

The tubers stored at 8° behaved in a similar manner to those stored at 2°C; from the limited data available it is not possible to deduce whether the acceleration of depolymerization which occurs at 2° and 8°C is induced by storage below some critical temperature, or whether there is a gradual change over the range of temperature 2° to 20°C.

The change in rotation which occurs in the cold is apparently reversible to some extent, and although the optical rotation of tubers transferred from 2° to 20°C (after 7 weeks) never returned to the original negative value, it should be noted that tubers which were not exposed to

any cold treatment showed by this time a drift in optical rotation towards the positive; such reversal towards the negative could be the result of one or more of several processes - either a resynthesis of material of high D.P. from those of D.P. 2-5, or the release of free fructose, or the disappearance of oligosaccharides of D.P. 2 - 5. This question was not investigated further.

Previously reported ~~observations of~~ changes in optical rotation of tuber juice during the dormancy period must have been considerably influenced by the ambient temperature. The magnitude of the overall changes at 2°C found, namely $[\alpha]_D^{20}$ of total reducing sugar changing from about -20° to about +30°, are very much greater than those previously reported, for example, -2° to +8° (Bacon & Loxley) and -1.5° to +6° (Thaysen, Bakes & Green, 1929): this may have been due to varietal differences, but is more likely to have been a result of storage temperatures higher than 2°C.

Bacon & Loxley point out that as all the fructosides contain a glucose residue, the depolymerization implies a rising proportion of combined glucose to fructose, and they further conclude from indirect evidence that this occurs by conversion of fructose to glucose structures and not by preferential disappearance of fructose residues. This conclusion has been confirmed here by showing that there is an absolute increase in the combined glucose content of the soluble carbohydrate fraction during depolymerization.

When the fructose contents of the oligosaccharides in the dormant tuber were estimated it became apparent that although these

showed wide variation in absolute amount, they were always about unity relative to one another; calculations from results given in previous publications confirm this phenomenon (see Table 54). The similarity of these ratios implies an equilibration among the members during dormancy, and it appears likely that although sucrose and perhaps 1^F-fructosylsucrose do not conform during the sprouting of the tuber, this equilibration continues during the breakdown of oligosaccharides of D.P. $\geqslant 4$ (see Fig. 40). Such equilibration on the basis of fructose content by weight rather than on the basis of the molarity of the individual oligosaccharides has been reproduced in vitro by an enzyme extracted from artichoke tubers acting on 1^F-fructosylsucrose and higher oligosaccharides (Dickerson, personal communication). It is probable that this enzyme and not the hydrolase maintains this equilibrium in the tuber.

Table 54. Ratio of fructose content in oligosaccharides of dormant tubers.

Source	No. of estimates	Mean ratio of fructose content (by weight) to that of oligosaccharide of D.P. 5				
		D.P. 2 (sucrose)	D.P. 3	D.P. 4	D.P. 5	D.P. 6
Bacon & Edelman (1951)	10	1.16	0.90	1.11	(1.00)	1.06*
Bacon & Loxley (1952)	15	0.99	0.79	0.94	(1.00)	-
Present work	8	0.83	0.69	0.95	(1.00)	-
Total	33	1.05	0.80	0.99	(1.00)	1.06*

* Mean of 8 estimates

Among the oligosaccharides of D.P. 2-5 which were found to accumulate at low temperature, sucrose (D.P. = 2) is of particular interest as it is the form in which carbohydrate enters and probably leaves the tuber, and also because it is known to be a potent inhibitor of many of the enzymes involved in transformations of the fructosans. The data on p.50 where the sucrose content of tubers stored at 2° and 20°C for 7 weeks is given show that the concentration of sucrose was much greater at the lower temperature; the amounts of sucrose can be expressed on a number of bases, but with those used - e.g. per gm. original dry weight, per gm. current dry weight, per gm. current fresh weight, or per gm. of water present in the tuber - the ratios of the sucrose concentration at 2°C to that at 20°C are very similar, viz. 2.12, 1.89, 1.92 and 1.88 respectively.

...

If the method of depolymerization proposed above is correct, namely:-

$$G\sim F - Fn + nH_2O \longrightarrow G\sim F + nF$$

$$nF \longrightarrow \frac{n}{2}\, G\sim F,$$

then the overall formula is $G\sim F - F_n \longrightarrow G\sim F + \frac{n}{2}\, G\sim F$. This process is however subject to control which does not allow it to go to completion unless all the soluble carbohydrate is transported from the tuber to growing tissue; thus, one might expect a higher final concentration of sucrose if carbohydrate is prevented from leaving the tuber, but it was found from the experiment in which the growing daughter plants were removed from tubers that the process of depolymerization was halted;

this resulted in the concentration of sucrose at 20°C being approximately the same whether or not there was a net loss of carbohydrate from the tuber. The experiments where tubers were stored at 2°C show by the attainment of a more or less constant optical rotation after 7 weeks, that the process is again halted, although the "end-point" in this case gives a lower average D.P. The values obtained for the concentration of sucrose in the tissues in different experiments can be compared, as an estimate of its concentration can be made by calculating the weight of sucrose per gram of water that the tuber contained; this method cannot take account of differences in concentration in different parts of the cell or in different parts of the tuber, but permits comparison of tubers whose initial sucrose and total fructose contents were different on a dry weight basis. The values indicate that the sucrose concentration varies only between narrow limits as compared to that of the fructosan in tubers sprouting at 20°C, the greatest changes taking place when the tubers are depleted of carbohydrate (Table 55); thus, as stated above the amount of sucrose per original dry weight falls more slowly relative to its initial concentration than does that of the individual members of D.P. $3 \rightarrow n$. Comparison of tubers with and without growing shoots shows that apart from a possible increase one day after the shoots have been removed, the sucrose concentration in the tubers is maintained at a comparable level whether material is being translocated from the tuber or not. In this case there is an indication that in the tubers without shoots the concentration of free fructose remains high; this situation does not occur in tubers with

Table 55. Sucrose content of tubers.

The sucrose concentration is expressed as mg. of fructose per gm. of water.

(a) Tubers stored at 2° and 20°C.

Period of storage (weeks)	Storage temperature (°C)	Sucrose content
0	-	5.6 5.3
7	2	(17.0 (14.1
7	20	(6.7 (9.8

(b) Tubers sprouting at 20°C (data from Tables 4 & 5).

Days after planting	Temperature (°C)	Sucrose content	
0	(2)	(9.8 (12.3	
14	20	8.4	(15.9)*
16	20	9.8	(9.4)*
19	20	8.8	(10.3)*
24	20	4.7	(9.0)*
29	20	7.4	(9.6)*

* growing tissue removed after 13 days.

buds in which active depolymerization is not taking place and may be taken to demonstrate that this free fructose is not converted to sucrose under these conditions. From the loss of material in tubers with shoots it may be inferred that conversion of free fructose to sucrose is much more rapid, although the amount of fructose may exceed that found in the disbudded tubers.

It has already been pointed out that at least some of the other members of the oligosaccharide series are maintained at levels such that the amount of fructose present in each fraction tends to be the same (see p.158). In contrast to sucrose, however, the concentrations of the individual members fall steadily as fructose is lost from the tuber, i.e. the amounts are mainly determined by the total amount of fructosan present.

It should also be noted that substantial amounts of material must be lost from the sucrose fraction in dormant tubers; thus it is shown on p.50 that the fractions of D.P. 3-5 inclusive increase in tubers stored at 2°C for seven weeks: this change is of such a magnitude that the possibility of this increase being caused solely by hydrolytic degradation of oligosaccharides of higher D.P. can be excluded, and the process must thus involve transfer of fructose residues from material of high D.P. to sucrose. Furthermore, there is evidence for the synthesis of fructosans of high D.P. in the daughter tubers which developed from tubers stored at 20°C - this evidence is only valid as long as the earlier assumption that carbohydrate is translocated solely in the form of sucrose is accepted. No evidence is available to determine whether

this process can also occur in the parent as well as the daughter tubers; thus the fact that the latter are actively growing may be a significant difference in this respect. It is nevertheless evident that the composition of the fructosan mixture in the daughter tuber is typical of "dormant" tubers at the same temperature, as evidenced by its specific optical rotation and its appearance on paper chromatograms.

Thus it may be assumed that the distribution of fructose among the various fractions in the tuber is the result of a dynamic equilibrium which is affected by the ambient temperature and by the amount of carbohydrate present. A considerable number of enzymes are probably involved including those concerned with hydrolysis, transfer of fructose residues, synthesis of high from lower oligosaccharides, and whatever system is responsible for the interconversion of fructose and glucose residues. The concentration of sucrose, which appears to be constant at a given temperature but widely different at different temperatures may be the agency, through its known effect on certain enzymes, by which the process of establishing and maintaining the point of equilibrium is effected.

...

The depolymerizing effect of low temperature may be compared with the well-known sweetening of potato tubers which occurs at temperatures below 5°C; this is known to be due to production of sugar from starch, but the conversion mechanism is not properly understood (Arreguin-Lozano & Bonner, 1949; Porter & Rees, 1954). The extent of depoly-

merization in the potato is usually expressed in terms of the increase in sucrose and reducing sugar concentrations, so that comparison of this with breakdown of fructosan in artichoke tubers is difficult, although the extent of the latter seems to be much greater. The comparable conversion of starch reserves in trees into sugars and fats has been shown to be a reversible process controlled by temperature; thus, pieces of living wood and bark when transferred during winter to room temperature showed a definite formation of starch within two hours (Iraklinow, 1912 quoted by Bělehrádek, 1935). The depolymerization of starch in foliage leaves is, like the depolymerization of fructosan in artichoke tubers, affected by the concentration of the products of low D.P.; thus the overnight disappearance of starch is prevented when translocation of sugar away from the leaves is checked (cf. Curtis, 1935).

Denny, Thornton & Schroeder (1943-45) showed that depolymerization in artichokes takes place during low temperature storage (5°C) but these authors did not compare tubers stored at any other temperature. The method of analysis which they used makes it impossible to compare their results with those obtained here; duplicate samples of extracted juice were hydrolysed by invertase and by HCl for 24 hr., the values obtained in subsequent reducing sugar analyses being taken as sucrose and as sucrose plus inulin respectively. It is virtually certain that the invertase preparation used was able to hydrolyse some of the lower oligosaccharides which are now known to be present; thus the "sucrose" values which were obtained were 340-490 mg. R.S./g. D.Wt. compared with values of 46-141 mg. R.S./g. D.Wt. for sucrose separated by chromatography of

extracts of tubers stored at 2°C in the present work; the comparative values for sucrose and inulin in the present work; the comparative values for sucrose and inulin in the data of Denny et al. can probably only be taken as a rough indication of the relative amounts of oligosaccharides of low and high D.P. respectively. With this qualification, their data still show that this depolymerization was inhibited by storing tubers (at 5°C) in atmospheres with increasing concentrations of CO_2: oligosaccharide increased at the expense of starch or other polymerized material in potatoes, carrots, parsnips and lima beans in the cold.

...

Temperature also had a profound effect on the morphology of the newly growing tissue in sprouting tubers (Fig. 11). Tubers kept in damp sand in the dark at 20° after storage at 2°C for seven weeks, gave rise to sprouts which apart from etiolation, appeared normal. Buds of similar tubers kept at 20°C with no prior cold treatment developed into daughter tubers and never into etiolated shoots; other tubers which had not been subjected to cold treatment did not sprout into new plants even over a period of several months after they had been potted in soil.

The appearance of a red pigment around the vascular bundles on the cut surfaces of tubers was also a very noticeable effect of temperature, as it occurred at low but not at high storage temperatures (in darkness); red pigment did appear in the light in buds of tubers transferred from cold storage to 20°C. Apart from observation, no experiments were made to investigate this phenomenon: it is of interest, however, that Swanson et al (1956) have described the formation of a red

pigment on the surfaces of artichoke tuber slices in contact with filter paper impregnated with 2,4-D or with various other growth substances, and slices of stems of some of the plants produced pigment in the vascular tissue; the same pigment was produced by germinating lettuce seeds in the presence of 2,4-D, Cu^{++}, and coumarin, and by germinating seeds of Taraxacum officinale and Helianthus annuus in the presence of 2,4-D, but not by those of various other plants which were not members of the Compositae.

...

The occurrence in tubers of Jerusalem artichoke of an enzyme activity which liberates reducing sugar from inulin is fairly well documented, the earliest record being that of Green (1887). The action of such an enzyme from artichoke tubers on inulin and the fructosans of the tuber has been described by Edelman & Bacon (1951), who also give a summary of previous work on the enzyme. A standard mixture of fructosans from the tubers was attacked more rapidly than inulin, but sucrose was hydrolysed very slowly, so that the action of the enzyme may be expressed by the equation:-

$$G\sim F-(F)_n + nH_2O \longrightarrow G\sim F + nF$$

Preparations of the enzyme were found to have a little, and apparently limited, activity against grass levan and irisin; the activity was therefore characterized as "a fructofuranosidase with a moderate degree of specificity for such residues linked to position 1 of another fructose residue".

In storage organs such as those of potato, which contain starch,

free monosaccharide does not seem to be an intermediate in the process of utilization; thus although hydrolytic enzymes (e.g. α- and β-amylase, etc.) can be extracted from such tissues, they do not appear to be active in the living plant, where the utilization of starch is through glucose-1-phosphate, liberated by starch phosphorylase. Edelman and Bacon showed that the liberation of reducing sugar in aqueous extracts of artichoke tubers did not involve a phosphorolysis of fructosan and subsequent hydrolysis of sugar phosphate; as well as showing that the release of reducing sugar was due to a hydrolytic enzyme, their experiments gave no evidence of any phosphorolysis at all. The results of the physiological experiments, as described above, indicate that the fructosans of tubers are first hydrolysed to free fructose (and sucrose) in the process of their utilization in new growth; the enzyme can thus be assumed to be active in the living tissue, and its properties were therefore investigated further in the present work.

...

The initial experiments with unfractionated preparations of proteins in acetone-dried extracts from artichoke tubers showed that the enzyme described by Edelman & Bacon was present in the extracts, and that its activity was inhibited by sucrose as recorded by Recaldin (1961). These preliminary experiments will not be discussed further as interpretation of results is complicated by the presence of invertase activity and endogenous substrate in the acetone powders.

Acetone dried powders free of endogenous substrate were prepared by treating tuber extracts with ammonium sulphate; this salt

was added to give final saturation of 50-70% on different occasions; Recaldin showed that the major part (95%) of the hydrolase activity was precipitated from solutions 50% saturated with respect to ammonium sulphate. Precipitated protein was redissolved and, after dialysis, precipitated with acetone. Such powders contained less than 1% by weight of fructose, but still possessed invertase activity.

It was found that hydrolase could be separated from invertase activity by chromatography of the mixture on DEAE-Cellulose columns. The stepwise elution pattern which was adopted gave three discrete fractions which could be characterized as fructofuranosidases: of these, two corresponded in their properties with the hydrolase of Edelman & Bacon in their ability to hydrolyse chains of $\beta(2\rightarrow1)$ linked fructose residues and their inability to hydrolyse sucrose; these two fractions have been termed "hydrolase A" and "hydrolase B", the latter requiring buffer of higher ionic strength for elution from the DEAE-Cellulose column. The third fraction which was eluted by buffers of even higher ionic strength could be characterized as an invertase in so far that 0.1 M-sucrose was hydrolysed at a comparatively faster rate than the standard mixture of fructosides used to assay for hydrolase activity.

When the hydrolase fractions were re-chromatographed on DEAE-cellulose, both remained homogenous, showing that the segregation into two fractions was not an artefact of the method.

It is possible that hydrolase A and B are produced during the extraction procedure from some common precursor which is the form of enzyme present in the tuber. This possibility has not been investigated,

but it seems clear that the two fractions are not isomers, and that if one is produced by a modification of the other this process does not occur spontaneously during chromatography.

It is theoretically possible that in each fraction, every different oligosaccharide in the substrate mixture is degraded by a different enzyme; however, of all the sugars which were incubated with both fractions, those hydrolysed by one fraction were also hydrolysed by the other; thus in the situation proposed above, there would have to be paired specific enzymes, one in fraction A and the other in fraction B. In the experiments where the fractions A and B were partially denatured by heat, the loss of activity against substrate of high D.P. (inulin) was similar to the loss of activity against substrates of low D.P. (substrate M, or inulopentaose on the two occasions). Furthermore, there is an obvious similarity in the extent of the sucrose inhibition of hydrolysis of various substrates in the case of fraction B, namely inulotetraose 66.6%, inulopentaose 57.3%, inulin 50-65%.

This conformity in the properties of the activities of the fractions against various substrates indicates that the hydrolysis of substrates of both high and low D.P. is effected by a single enzyme in each fraction; in the subsequent discussion this will be assumed to be so.

Hydrolase A and hydrolase B were found to differ in other properties as well as in their chromatographic mobilities on DEAE-cellulose; thus hydrolase B was much more susceptible to denaturation during storage at pH 7.5, and by heat treatment at pH 5 than was hydrolase

A. The effect of pH on the activity of the two differed, although the pH at which optimum activity was found was about the same (hydrolase A, 4.9; hydrolase B, 5.2).

Since the advent of effective methods of protein fractionation the separation of one enzyme activity into several has become commonplace: such separations of different protein species are not artefacts of the experimental procedures followed by the various workers; thus, fractionation of crystalline ribonuclease has been carried out by a multitude of workers using several different methods, and at least six enzymically active components have been obtained; the main fraction, ribonuclease A, has however remained homogenous when material isolated by one procedure has been analyzed by another (Scheraga & Rupley, 1962). In some cases e.g. Lactic dehydrogenase, the active fractions have remained identical when compared by purely enzymatic criteria, cf. Markert and Møller) (1959), while in others fractions can also be differentiated by their enzymatic properties, as for example the series of β-glucuronidases extracted from ox-spleen by Mills et al (1953).

Markert and Møller (op. cit.) investigated the relative amounts of the various species (which they termed "isozymes") of lactic dehydrogenase in different tissues in embryonic and adult pigs, and found that these differed from tissue to tissue and in a single tissue at the different stages of development. No evidence regarding the distribution of the β-fructofuranosides in the artichoke is available except for Recaldin's (1961) statement that invertase is relatively greater in amount in the buds of tubers. It has also been stated that the outer parts of

the tuber contain more "inulase" than the inner (Susumu Murakami, 1954); this author also states that the activity of the enzyme is greater in sprouting than in dormant tubers, where the enzyme is present as a zymogen; thus it is claimed that its activity is increased by proteinases. The information on the contents of this paper has been culled from an abstract, and none of the points made have been examined in the present work. The tissue used for the preparation of acetone powders in the present work was composed principally of parenchyma with a little vascular tissue, from dormant tubers which had been disbudded and peeled. It should be noted that the extracts were made from a population of tubers, so that there is no direct evidence that hydrolase A and hydrolase B coexist in a single tuber, but the fact that different acetone powders gave an identical elution pattern makes it probable that this proportion of A to B reflects the situation in each individual tuber.

The invertase and the two hydrolases separated from the tubers obviously differ in enzymatic properties, but comparison of the two hydrolases shows that, although both are comprised in Edelman and Bacon's definition, they differ in details of their activity: thus, the ratio of hydrolysis of substrate-M and of inulin by the enzymes indicates that hydrolase A attacks the substrate of low average D.P. faster than hydrolase B, relative to the rates at which the two enzymes hydrolyse inulin. Neither fraction attacks irisin or bacterial levan to any significant extent, and transfer of fructosyl residues to sucrose by either enzyme was very limited, even if the same enzymes were responsible.

Comparison of the rates at which a number of purified oligosaccharides were hydrolysed relative to the rate of hydrolysis of inulin

has been made, and the values are given in Fig. 43. It is shown that hydrolase A attacks substrates of low D.P. (ca. 4 - 6) at a rate 2.5 times as fast as it does inulin; whereas in the case of hydrolase B, the maximum rate obtained is about 1.5 times as fast as on inulin. These results also indicate that the optimum D.P. differs, being about 5 for hydrolase A, and 8 for hydrolase B.

The experiment where the sucrose inhibition of hydrolysis of inulotetraose at different temperatures was examined (p.116) shows that hydrolase A is much less susceptible to sucrose inhibition than is hydrolase B. The temperature coefficients of the enzymes acting on 0.01 M-inulotetraose also appear to differ slightly (hydrolase A, $Q_{10} = 1.85$; hydrolase B, $Q_{10} = 1.73$) but this difference may not be significant.

These differences in enzymatic properties indicate that the two enzymes are not products of a common precursor.

...

Enzymes which hydrolyse polysaccharides can be classified as "endoenzymes" and "exoenzymes"; the former effect hydrolysis at points along a polysaccharide chain which are random within the limits of the specificity of the enzyme, e.g. α-amylase, whereas exoenzymes can only attack the ends of polysaccharide chains, e.g. β-amylase; exoenzymes need not necessarily release only monosaccharide residues from these ends.

Bacon & Edelman noted that during the earlier stages of inulin hydrolysis, a difructose appeared among the products but they attributed the formation of this compound to the activity of a transfructosidase (known to be present in their extracts) which transferred fructose residues

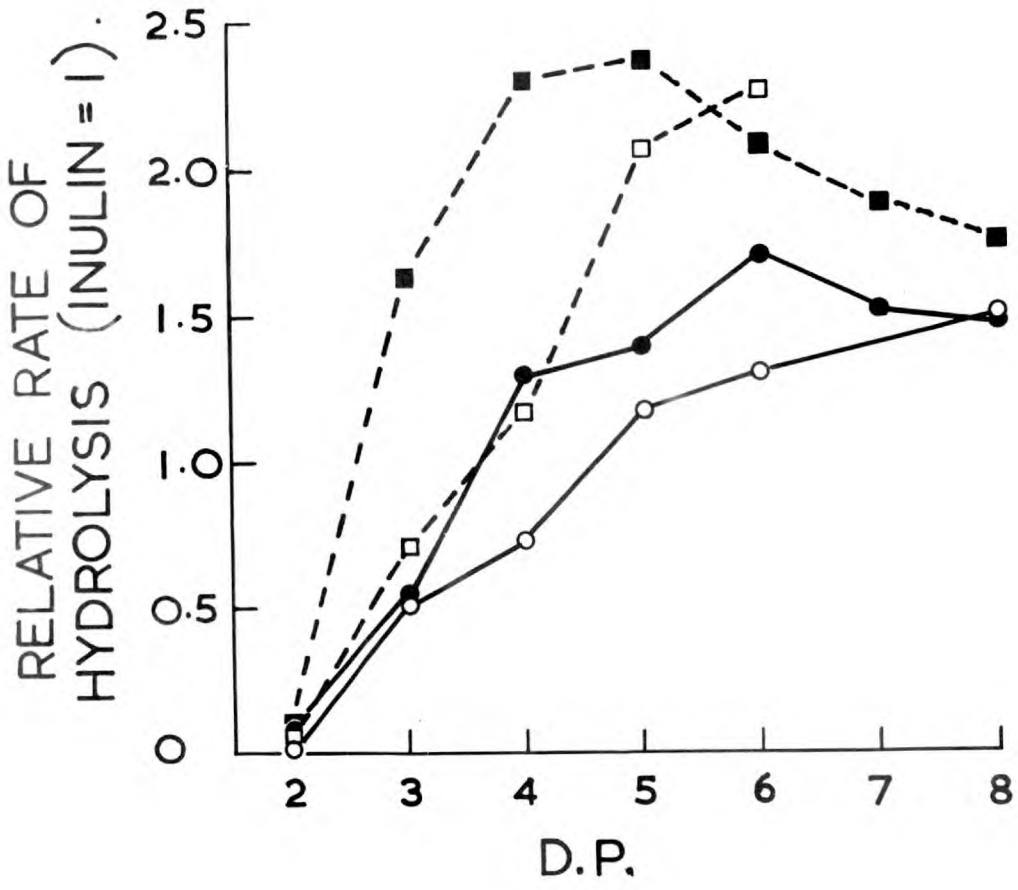
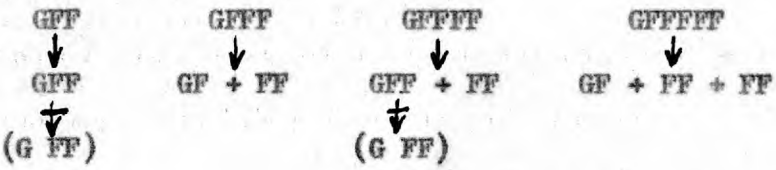


Figure 43. Comparison of activity of hydrolase A and hydrolase B against various substrates. All digests were incubated with 0.01 M-substrate in 0.1 M-acetate buffer pH 5 at 25°C.

□—□ inulose series, hydrolase A
■—■ inulo-n-ose series, hydrolase A
○—○ inulose series, hydrolase B
●—● inulo-n-ose series, hydrolase B.

from inulin to free fructose already produced by hydrolysis.

This conclusion is supported by results obtained in the present work, where a comparison of the two methods of assay for hydrolase activity was made. The Charcoal/Celite columns retain all sugars except monosaccharides; thus if either of the hydrolases released disaccharide units from the end of the chain, or attacked the chain at random points, the resulting reducing groups would be attached to chains of fructose units which would be retained by the column. Thus the value obtained for the increase in reducing sugar by direct estimation on samples from the digests would exceed that found for increase in free fructose after chromatography of samples on Charcoal/Celite columns; the results obtained however (p.89) show that these values are identical, and that the enzymes therefore act by releasing single fructose residues from the ends of the chains. This result would also be obtained if the enzyme liberated inulobiose molecules which were subsequently hydrolysed by the same or another enzyme in the fraction to free fructose, but this possibility is ruled out by the fact that inulobiose is hydrolysed more slowly than inulin when in equimolar concentration by both hydrolase A and B. Furthermore, oligosaccharides with odd or even numbers of fructose residues in the chain attached to sucrose are all degraded to sucrose <u>plus</u> free fructose, while if the enzyme released disaccharide or larger units from the chain substrates of different D.P. would be expected to yield different end products; thus if disaccharide units were released, the oligosaccharides of D.P. 3, 4, 5 and 6 would be expected to give final products as shown:-



It has also been noted (p.127) that the action of hydrolase B on some of the members of the inulose series yielded as the major products after a short incubation, free fructose and the oligosaccharide of next lower D.P. - this clearly implies that fructose residues are hydrolysed singly.

...

The Mode of Action of Hydrolase B

It has been shown above that the only bond broken by the enzyme at a significant rate is the linkage between a terminal fructosyl and its adjacent fructose residue providing it is of the β 2:1' configuration; thus the rate of hydrolysis of bacterial levan, which is a fructosan with β 2:6' linkages, was low compared to the rate at which inulin was attacked (see Fig. 28) and furthermore the initial rate of hydrolysis (about 15% of that of inulin) quickly fell to a much lower rate (about 6%), perhaps indicating the hydrolysis of a quantity of β 2:1' linkages which soon became exhausted.

This specificity for the type of linkage is accompanied by a specificity with respect to the size of substrate molecules; thus, results obtained when oligosaccharides of different D.P. were used showed that the rate of hydrolysis depended on the D.P. of the substrate, c.f. Fig. 26. It has not been shown whether the affinity of the enzyme for these substrates

differs, as Km values have been determined with only a few of them. The Km of inulin was estimated on a number of occasions but the values have been rather variable: the reason for this is probably twofold; in the first place, estimations of the activity of the enzyme against inulin can only be made using substrate concentrations very much smaller than that needed to saturate the enzyme because of the limited solubility of inulin at 25°C, and as a result, the intercept on the x axis when a Lineweaver-Burk plot is made must be plotted on a scale which brings it very near the y axis; in these circumstances a small shift in the slope of the line causes a relatively large change in the value obtained for Km. A second factor affecting the estimated value could be the tendency of inulin molecules to aggregate in solution (cf. Edelman & Bacon, 1951), the extent of which might have differed from one time to another. The Km values obtained with different substrates are given in Table 56. With the limited number of observations available, the differences are too small to show whether there is any change in Km with D.P. as has been shown for glucoamylase acting on the lower members of the maltose series of oligosaccharides (Hiromi Ono & Jimbo, 1961).

The reason for the change in rate of hydrolysis with D.P. is not known, but may indicate that the specificity of the enzyme extends further than the fructosyl residue which is hydrolysed. It is clear (from Fig. 36) that the series of oligosaccharides terminated by a fructose molecule are hydrolysed faster than those terminated by a glucose molecule; the difference however becomes less with increase in the chain length until at about D.P. 6 - 8 the rates are more or less equal; at this point,

Table 56. Km for hydrolysis of various substrates by Hydrolase B.

Inulopentaose	2.2×10^{-2} M
Inulose-1	3.3×10^{-2} M
Inulose-5	2.6×10^{-2} M
Inulin	3.3×10^{-2} M* ($\pm$ 0.3×10^{-2} M, S.E.)

* mean of 11 estimations, ranging from 1.9×10^{-2} to 4.8×10^{-2} M

presumably, the glucose molecule is too remote to affect the affinity of the active site for the substrate. There also seems to be an optimal chain length, as the enzyme hydrolyses substrates of D.P. 5 - 8 at a higher rate than it hydrolyses inulin (average D.P. about 30).

Of particular interest is the slow rate at which the enzyme hydrolyses inulobiose as this sugar can be regarded as representing the non-reducing end of more readily hydrolysed sugars since its structure is 1-O-β-D-fructofuranosyl-D-fructofuranose (Pazur & Gordon, 1953) and thus differs from such terminal residues only in its D.P. and in having a reducing group on the distal fructose molecule (it is possible that a proportion of these moieties also differ by adopting the pyranose configuration in solution). The activity of the enzyme against a number of compounds which may be regarded as inulobiose condensed with various residues, namely glucose, fructose, melibiose and hexitol, have been examined; the rates at which these substrates were hydrolysed can be compared in Table 57, where it is apparent that the addition of these residues has resulted in a considerable stimulation in rate compared to that at which inulobiose is hydrolysed. It is also clear that the increment is not the same for the different substituents. This result

Table 57. Comparative Rates of hydrolysis of various oligosaccharides by Hydrolase B.

Data from Fig. 36 and Table 44.

1^F-fructosylfructose	1.00
$(1^F\text{-fructosyl})_2$fructose	6.7
1^F-fructosylsucrose	6.3
$(1^F\text{-fructosyl})_2$hexitol	3.4
1^F-fructosylraffinose	5.2

indicates that it is not only D.P. (and the concomitant loss of the reducing group of inulobiose) which increases the affinity of the enzyme for these substrates. It is also clear that the rates of hydrolysis of inulose-1 and 1^F-fructosylraffinose (= 6^G-galactosylinulose-1) are similar, indicating that while the glucosyl residue increases the effective D.P. of the substrate, the further addition of the galactosyl residue has no influence on the rate of hydrolysis (except perhaps to reduce it a little). D.P. alone does not increase the affinity, therefore demonstrating that the enzyme shows specificity for the residues attached to the chain, but not necessarily susceptible to its attack.

The fact that inulobiose is not hydrolysed at a high rate makes it difficult to determine whether the hydrolysis of disaccharides in which fructose is condensed with other compounds in various ways is associated with a specificity of the enzyme for a Fβ2→1F linkage, or whether it is merely the D.P. which determines these very low rates. The rates of hydrolysis of sucrose, of O-β-D-fructofuranosyl(2→6)-D-glucose, of O-β-D-methylfructofuranoside and of O-β-D-fructofuranosylhexitol, have invariably been lower than that of inulobiose, but no method for assessing the relative effects of D.P. and of the linkage has been devised. The

fact that the fructosyl linkage in raffinose (D.P. 3) was hydrolysed more slowly than was that in sucrose holds little force in this respect since, as noted above, the 6^{G}-galactosyl residue of raffinose does not seem to increase the effective D.P. of an inulose chain, as 1^{F}-fructosyl-raffinose (D.P. 4) is hydrolysed at a rate similar to that of inulose-1 (D.P. 3) rather than to that of inulose-2 (D.P. 4). No model substrates in which the sucrose or other bonds can be presented in a configuration applicable to the enzyme of the type X-G~F where "X" effectively increases the chain length, have been found, and thus this question cannot be resolved at present.

Further investigation of the characteristics of the active site was made by examining the effect of various sugars (0.01 M) on the rate of hydrolysis of inulin: of these, the most notable were inulose-1, which is itself hydrolysed by the enzyme, (79% inhibition), sucrose (60%) and 1^{F}-fructosylfructose (57%); fructosylhexitol at a concentration of 0.005 M inhibited inulin hydrolysis by 44%, that is to the same extent as 0.005 M-sucrose. The inhibition by sucrose was examined in greatest detail because of its possible role as an inhibitor, and hence controlling factor, in the plant, and because of its position as the first member of the inulose series.

Sucrose inhibition was at first deduced to be competitive and this was interpreted as due to combination between the active site and the β-2-linked fructofuranosyl moiety of the sucrose. From kinetic and other data however, it later became clear that the inhibition by sucrose was principally non-competitive with a possible element of competition

at higher concentrations, and also that both raffinose and melezitose inhibited inulin hydrolysis, but to a lesser extent (25% and 27% respectively; 0.01 M). Inhibition by raffinose could be attributed to the unsubstituted fructosyl group which it contains, especially as melibiose was found not to inhibit; in the case of melezitose however this interpretation would require that the effect of this residue was not greatly affected by the substitution of a glucosyl residue on position 3. An alternative interpretation is that both monosaccharide moieties of sucrose can act as inhibitors (although the free monosaccharides do not) and that the masking of the glucose or fructose residues respectively in raffinose and melezitose still leaves the other with inhibitory powers. This interpretation might also explain to some extent the slower rates of hydrolysis of the inulose series of oligosaccharides (terminated by a glucose residue) compared with those of the inulo-n-ose series (terminated by a reducing ("free") fructose) but this possibility is vitiated by the lack of inhibitionary effects of maltose, cellobiose, and αα trehalose.

The enzyme does not appear to be inhibited by the other product of its action, namely free fructose; however, whereas the fructoside residue hydrolysed by the enzyme is in the furanose form, the liberated monosaccharide is mainly in the pyranose configuration. An attempt to determine the effect of D-fructofuranose was made by using 2-O-β-D-methylfructofuranoside; this compound inhibited the hydrolysis of inulin by about 19%, that is, to a lesser extent than melezitose or raffinose and to a much lesser extent than sucrose. However, it is a moot point

whether this compound is more comparable to free fructofuranose or to the fructofuranosyl group in sucrose.

The inhibition experiments which were performed with a variety of sugars yield little information on the nature of the active site because sucrose was shown to be a non-competitive inhibitor. This fact probably indicates that the effect of sucrose is at a site separate from, though able to influence, the active site for hydrolysis. Thus the comparison of the effect of sugars present at a single concentration (0.01 M) is not very meaningful if inhibition is detected, as there is no way of telling whether the sugar is affecting the active site or the "inhibition site" of the enzyme: moreover some sugars, e.g. inulobiose, inulose-1 and sucrose may affect both sites. Such a differentiation can only be made by experiments in which the nature of the inhibition can be deduced, and in view of the variation in the values obtained for the Km of inulin, it would be preferable to use a substrate of known D.P. Nevertheless, the data available provides a list of inert sugars which obviously do not form any combination with either site.

It is probable that hydrolase B degrades polymer by multi-chain attack. Bailey & French (1957) have claimed that β-amylase can hydrolyse starch by single-chain attack; here an enzyme-substrate complex is maintained while 1, 2, 3, 4 or even more maltose residues are successively liberated from the end of an amylose chain. The interpretation of the results is complicated by the fact that substrate molecules are not statistically comparable; thus in a digest with a large number of substrate and relatively few enzyme molecules it may be supposed that a

substrate molecule which has just dissociated from an enzyme-substrate complex is more likely to form another by virtue of its position; this situation is probably more and more applicable as the size of the substrate molecule increases and diffusion rates fall. In the present work it has been shown that the enzyme hydrolyses the oligosaccharides of D.P. 3-7 by multi-chain attack; thus chromatography of digests of the enzyme with these oligosaccharides showed that the major products were the oligosaccharides of next lower D.P., together with free fructose; hence the enzyme after hydrolysing the terminal fructose residue of a chain does not maintain linkage with a substrate molecule and degrade it further (this is impossible in the case of inulose-1, as the first product is sucrose). In the case of inulin, this observation could not be made as the chromatogram did not resolve sugars with D.P. greater than about 10. There was however an indication that oligosaccharides of D.P. about 10 were present in concentrations which although small, were probably greater than could be accounted for by a completely random hydrolysis of terminal fructose units; as suggested above, the effects of diffusion of enzyme and substrate molecules may account for this.

...

Classification of the hydrolases under the rules recommended by the Report of the Commission on Enzymes of the International Union of Biochemistry (1961) has been attempted; although they clearly fall into group 3.2.1 (hydrolytic enzymes acting on glycosyl bonds), within this group they would fit into either of two categories, numbered 3.2.1.7

and 3.2.1.26, which are described as inulin-1-fructanohydrolase (inulase, from yeast, hydrolysing β-1,2-fructan links in inulin) and β-D-fructofuranoside fructohydrolase (invertase, hydrolysing a β-D-fructofuranoside to an alcohol and D-fructose). No reference is given to the inulase in the Report, but the only entry under this name in the list of enzymes compiled by Dixon & Webb (1958) is an enzyme from yeast described by Adams et al (1943). This activity against inulin was very low compared to that against sucrose, but the authors believed that it was a separate enzyme because of different pH optima (inulin 3.2-4.0, sucrose 5.0-5.5) and because they found variations in the relative activities when enzymes were prepared from the same source by different methods; thus bakers yeast gave invertase: inulase ratios of 3250:1 and 2800:1, and brewers yeast 28300:1, 11800:1 and 16700:1. In view of the fact that the pH optima were very broad (e.g. at pH 5 the activity of inulase was 86% of the maximum, and invertase activity 88% of the maximum at pH 2.8), and that partial denaturation of enzyme activity by heat caused no marked change in the ratio of the two activities, the separate identity of the inulase may be doubled. In any event, in both brewers and bakers yeast, the inulase activity is insignificant compared to the invertase. The enzyme can be accommodated in 3.2.1.7, with a rider to the effect that only the linkage of the terminal fructose residues is broken. However, as stated above, because of the effect of D.P. on rates of hydrolysis, the possibility that the enzymes could hydrolyse the sucrose linkages in a substrate of higher D.P. cannot be excluded; if it were possible, the enzymes would be ineligible for this category.

Classification in the alternative group would indicate only the nature of the bond broken, without giving any further account of the specificity; the notes appended to 3.2.1.26 state that sucrose is among the substrates attacked by this enzyme; it would appear in fact that sucrose is the primary substrate as this group comprises a number of invertases (which differ widely in the relative extents of transfructosylation and hydrolysis which they catalyse). Furthermore, classification soley on the grounds of the nature of the bond broken can give a misleading impression of the specificity of the enzyme; thus Parrish & Perlin (1960) have described the case of a β-glucosidase which hydrolyses the β(1→3) linkages of laminarin but when acting on a wheat glucan with both (1→3) and (1→4) linkages, hydrolyses the (1→4) bonds, because its action is in fact to hydrolyse the bond of a β-D-glucosyl unit attached to adjacent residues through positions 1 and 3, rather than a (1→3) linkage as such. The same authors also describe a cellulase from Streptomyces sp. which acting on the same glucan, hydrolyses the glycosidic bond of residues which are identical to those in a cellulose chain; β(1→4) linkages from glucosyl units substituted at position 3 are not hydrolysed; that is, the →4 β-D-glucose relationship is as important as the β(1→4) linkage, and may explain why several known cellulases attack cellulose but not cellobiose.

At present the specificity of the hydrolases is not known with sufficient precision to make an unequivocal choice between the two possible categories, nor can the effect of the D.P. of the substrates be conveniently indicated.

...

The process of breakdown of polysaccharides in the artichoke

tuber seems to be quite different from the way in which many other polysaccharides are utilized, where phosphorylated intermediates are commonly produced; the process also differs from other instances of hydrolytic breakdown of polysaccharides where there are often two (or more) enzymes, one involved in breaking the polymer down to disaccharide or larger fragments (e.g. cellulase, α- and β-amylase), and others which hydrolyse oligosaccharides of low D.P. to monosaccharide, (e.g. cellobiase, maltase). Both the hydrolases distinguished in this work appear to be more comparable to the latter type of enzyme - it should however be borne in mind that the D.P. of inulin itself is small compared to most polysaccharides, and it would be of interest to know how fructosans of high D.P. (e.g. irisin, D.P. ca. 3000; Palmer, 1951) are enzymatically degraded.

Although the soluble fructosan must be principally located in the vacuole in view of its concentration in the tissue, (inulin crytallizes in the vacuole when sections of artichoke tuber are mounted in absolute ethanol) it is not known where the enzymes which act on these sugars are situated. The enzymatically-maintained equilibrium between various oligosaccharides (see p.159) may be taken to indicate that the transfructosylase responsible has access to sugars in the vacuole, either by being itself in solution in the vacuolar sap or perhaps by forming part of the tonoplast membrane. The same location may be proposed for the hydrolase on the grounds that its substrates are present, and presumably confined, in the vacuole; it has been noted above that, in the experiment where the growing shoots were removed from sprouting tubers, the tubers contained an amount of free fructose which was for reasons stated on p.161-2

presumed to represent an inert pool which could not be utilized under the prevailing conditions, rather than the constantly replenished pool postulated in the tubers which were supplying material to growing tissue; as free monosaccharides seem to be metabolised very rapidly when supplied to disks of artichoke tuber (Recaldin, 1961), this fructose may be confined in the vacuole: in this case, hydrolysis of fructosans would also be assumed to occur in the vacuole or on the surface of the tonoplast, transport from the vacuole possibly being necessary for the further metabolism of the fructose liberated.

Whatever the nature of inhibition by sucrose, it is quite possible that this sugar could affect the activity of both the hydrolases in the tissue; thus, although the relative concentrations of sucrose and substrates at the site of hydrolase activity is not known, the overall concentration of sucrose in the cells is high enough to inhibit both of the hydrolases in free solution. As noted on p.160 the amount of sucrose in tubers differs, particularly in accord with the ambient temperature; when the amounts are expressed as molarities they range from 2.9×10^{-2} to 9.4×10^{-2} M in tubers which still contain large amounts of fructosan. The high concentrations are found at 2°C, a temperature which induces extensive depolymerization, while tubers stored or sprouting at 20°C contain up to 5.0×10^{-2} M-sucrose. If sucrose exerts a simple controlling influence on depolymerization by its inhibition of hydrolase activity, these results are opposed to what might be predicted; these concentrations are all larger than those found to inhibit hydrolase B drastically (60%) and hydrolase A to a lesser extent (ca. 20%) <u>in vitro</u>.

The possibility of a decreased inhibitory effect of sucrose at low temperature must be excluded in view of the results given in Table 39, where it is shown that inhibition is in fact slightly greater at lower temperature. Thus it is clear that other factors must be involved in determining the extent of depolymerization of fructosan.

It is possible that free fructose is extracted from the vacuole into the cytoplasm, before being metabolised to sucrose; the conversion of fructose (and glucose) to sucrose by artichoke tuber disks has been demonstrated by Recaldin (1961); the autoradiographs which he shows are compatible with the formation of sucrose occurring by the series of reactions which terminate with the transfer of a glucosyl residue from UDPG to fructose or F6P. If this were so, the reactions involving phosphorylated sugars and sugar nucleotides would occur in the cytoplasm where ATP produced by mitochondria would be available. As noted above, the movement of free fructose into the cytoplasm might also be controlled by translocation of sugar out of the tuber and hence by sucrose concentration.

The enzymatic reactions visualised as occurring in the vacuole concern only transfer of fructosyl residues, either to water in the case of the hydrolases, or to the ends of fructose chains in the case of transfructosylases. The apparent importance of these enzymes, which do not use phosphorylated intermediates, in the carbohydrate metabolism of the artichoke may be a corollary of the fact that this form of storage carbohydrate is soluble and cannot be stored in large quantities in the cytoplasm in the manner in which starch can be synthesized and accumulated in plastids in potato tubers and similar organs.

Publications

Some of the results given in this thesis have been published, under the following titles.

"Changes in Content and Composition of the Fructose Polymers in Tubers of *Helianthus tuberosus* L. during Growth of Daughter Plants".
T.G. JEFFORD and J.EDELMAN (1960) J. Exp. Bot. 12, 177.

"Effect of Temperature on the Carbohydrate Changes and Morphology of stored Tubers of *Helianthus tuberosus* L."
T.G. JEFFORD and J. EDELMAN (1963) J. Exp. Bot. 14, 56.

"Characteristics of an Invertase-free β-Fructofuranosidase from *Helianthus tuberosus* L."
J. EDELMAN and T.G. JEFFORD (1963) Biochem. J. (in press).

Acknowledgements

I wish to record my gratitude to Dr. J. Edelman for his constant interest and encouragement.

I also wish to thank Professor H.K. Porter, Mr. V. Newbury, and other members of the Department of Plant Physiology for advice and assistance during the course of the work, Mrs. G. Adams for typing this thesis, and Mr. A. Horne who took the photographs.

LITERATURE CITED

Literature Cited

Adams, M., Richtmyer, N.K. & Hudson, C.S. (1943) J. Amer. Chem. Soc. 65, 1369.

Allen, P.J. & Bacon, J.S.D. (1956) Biochem. J. 63, 200.

anon. (1962) "Biologics from Calbiochem" No. 29. (Catalogue of California Corporation for Biochemical Research, 3625 Medford Street, Los Angeles 63, California).

Arreguin-Lozano, B. & Bonner, J. (1949) Plant Physiol. 24, 720.

Bacon, J.S.D. (1952) Biochem. J. 50, xviii.

Bacon, J.S.D. (1954) Biochem. J., 57, 320.

Bacon, J.S.D. (1959) Biochem. J. 73, 507.

Bacon, J.S.D. (1960) Bull. Soc. chim. biol. 42, 1441.

Bacon, J.S.D. & Bell, D.J. (1948) Biochem. J. 42, 397.

Bacon, J.S.D. & Bell, D.J. (1953) J. chem. Soc. p. 2528.

Bacon, J.S.D. & Edelman, J. (1951) Biochem. J. 48, 114.

Bacon, J.S.D. & Loxley, R. (1952) Biochem. J. 51, 208.

Bailey, R.W. & French, D. (1957) J. biol. Chem. 226, 1.

Bandurski, R.S. & Axelrod, B. (1951) J. biol. Chem., 193, 405.

Bělehrádek, J. (1935) "Temperature and Living Matter", Protoplasma-Monographien, volume 8, Borntraeger, Berlin.

Belval, H. & Chollet, M.M. (1948) Inds. Agr. et Aliment, (Paris) 65, 5.

Brooks, S.G., Evans, R.M., Green, G.F.H., Hunt, J.S., Long, A.G., Mooney, B., & Wyman, L.J. (1958) J. chem. Soc. p. 4614.

Burrows, S., Grylls, F.S.M. & Harrison, J.G. (1952) Nature, 170, 800.

Curtis, O.F. (1935) "The Translocation of Solutes in Plants". McGraw-Hill & Co., New York & London.

Dedonder, R. (1960) Bull. Soc. chim. biol. 42, 1745.

Denny, F.E., Thornton, N.C. & Schroeder, E.M. (1943-45) Contr. Boyce Thompson Inst. 13, 295.

Dixon, M. & Webb, E.C. (1958) "Enzymes", Longmans Green & Co., London.

Edelman, J. & Bacon, J.S.D. (1951) Biochem. J. 49, 446.

Edelman, J. & Bacon, J.S.D. (1951)a, Biochem. J. 49, 529.

Edelman, J. & Popov, K. (1962) C.R. Acad. bulgare Sci. 15, 627.

Evans, W.L., Reynolds, D.D. & Talley, E.A. (1951) Advanc. Carbohydrate Chem. 6, 27 (p. 54).

Folin, O. (1929) "Laboratory Manual of Biological Chemistry" 4th Ed., 99. Published by Appleton, (New York & London).

French, D. & Wild, G.M. (1953) J. Amer. chem. Soc. 75, 2612.

Green, J.R. (1887) Ann. Bot. Lond. 1, 223.

Hiromi Ono & Jimbo (1961) Koso Kagaku Shimpojiumu 14, 309.

Huggett, A. St.G. & Nixon, D.A. (1957) The Lancet p. 368.

Markert, C.L. & Møller, F. (1959) Proc. nat. Acad. Sci., U.S.A. 45, 753.

Mayer, A. & Porter, H.K. (1960) Nature, 188, 921.

McDonald, E.J. (1946) Advanc. Carbohydrate Chem., 2, 253.

Mills, G.T., Paul, J. & Smith, E.E.B. (1953) Biochem. J. 53, 232.

Nelson, N. (1944) J. biol. Chem., 153, 375.

Nielsen, H. (1958) Acta chem. scand. 12, 38.

Paladini, A.C. & Leloir, L.F. (1952) Biochem. J. 51, 426.

Palmer, A. (1951) Biochem. J. 48, 389.

Parrish, F.W. & Perlin, A.S. (1960) Nature 187, 1110.

Pazur, J.H. & Gordon, A.L. (1953) J. Amer. chem. Soc. 75, 3458.

Porter, H.K. & Rees, W.R. (1954) Plant Physiol. 29, 514.

Quillet, M. (1957) Compt. rend. 244, 2177.

Recaldin, D.A.C.L. (1961) "Studies on the Synthesis of Inulin by Tissues of Helianthus tuberosus L." Ph.D. Thesis, Univ. of London.

Rees, W.R. & Reynolds, T. (1958) Nature, 181, 767.

Report of the Commission on Enzymes of the International Union of Biochemistry 1961. (1961) Pergamon Press, Oxford.

Scheraga, H.A. & Rupley, J.A. (1962) Advanc. Enzymol. 24, 161.

Somogyi, M. (1952) J. biol. Chem., 195, 19.

Sowden, J.C. & Schaffer, R. (1952) J. Amer. chem. Soc. 74, 499.

Susumu Murakami (1954) - abstract in Chemical Abstracts (1954) 48, 12254.

Swanson, C.R., Hendricks, S.B., Toole, V.K. & Hagen, C.E. (1956) Plant Physiol. 31, 315-6.

Thaysen, A.C., Bakes, W.E. & Green, B.M. (1929) Biochem. J. 23, 444.

Wise, C.S., Dimler, R.J., Davis, H.A. & Rist, C.E. (1955) Analyt. Chem. 27, 33.