DISCUSSION

It was found that ATPase activity decreases after adrenalectomy similarly to alkaline phosphatase. Both enzymic activities can be restored by treatment of the animals with corticosteroids, within a short time. We were, however, unable to find a difference in hexokinase activity between normal and adrenalectomized animals.

Intestinal ATPase is obviously a different enzyme from the alkaline phosphatase. The two enzymes have a different pH optimum and behave differently towards fluoride. In spite of the decrease of ATPase activity caused by adrenalectomy, there was no change in the hexokinase activity, as might have been supposed on the basis of the observation that ATPase inhibition by fluoride increases the hexokinase reaction (Hele, 1950; Long, 1952) in the intestinal mucosa.

SUMMARY

1. The adenosinetriphosphatase (ATPase) activity of the intestinal mucosa of rats decreased considerably after adrenalectomy, roughly in proportion to the degree of insufficiency.

2. Injections of corticosteroids into adrenalectomized rats restored the ATPase activity. It was, however, impossible to restore the ATPase activity *in vitro* by the addition of deoxycorticosterone to the dispersion of the intestinal mucosa.

3. The hexokinase activity of the intestinal mucosa did not decrease after adrenalectomy.

4. Hexokinase activity in dispersions of intestinal mucosa was dependent on ATP concentration.

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The Oligosaccharides Produced by the Action of Yeast Invertase Preparations on Sucrose

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Yeast invertase, which was first prepared from the yeast cell by Berthelot (1860), has been investigated extensively since that time, both because of its use in industrial processes and because it provided a simple model for the investigation of enzyme kinetics. Its action was regarded as bringing about the hydrolysis of sucrose and other substances having an unsubstituted β-D-fructofuranose residue.

However, in 1950 it was discovered independently by Blanchard & Albon (1950) and Bacon & Edelman (1950) that carbohydrates other than sucrose, glucose and fructose could be detected while sucrose was being attacked by various preparations of the enzyme.

By the use of chromatography on cellulose powder and on charcoal, we have isolated one disaccharide and three trisaccharides from the reaction mixture in amounts sufficient to determine their general composition, and to measure their specific rotations. In addition, evidence has been obtained for the presence of two other disaccharides with similar properties and composition. These six substances are the major additional products of the action of yeast invertase on sucrose. The account of their properties given here, taken with the results of a number of other investigations (Fischer, Kohtès & Fellig, 1951; White & Secor, 1952; White, 1952; Albon, Bell, Blanchard, Gross & Rundell, 1953;

Whelan & Jones, 1953; Edelman, 1954; Gross, 1954; Gross, Blanchard & Bell, 1954) confirms and extends the original observations, and lends support to the hypothesis that yeast invertase is essentially a transfructosidase, its hydrolytic action being only one aspect of its transferring activity.

A preliminary account of part of this work was communicated to the Biochemical Society in London in October 1951 (Bacon, 1952).

EXPERIMENTAL

Materials

Yeast invertase. Two partially purified commercial preparations were used, viz. 'B.D.H. Invertase Concentrate' (British Drug Houses Ltd.) a colourless aqueous solution made from baker's yeast and, as The Distillers Co. Ltd. kindly informed us, preserved by the addition of about 50% (w/v) glycerol, and 'Wallerstein Blue Label Invertase Scales' (Wallerstein Laboratories, 180, Madison Avenue, New York) a solid preparation stated to be free from melibiase activity. In addition, a few preparations were made in the laboratory from pressed baker's yeast, and are described below.

Sugars. The absence of other carbohydrates from the sugar samples used was checked by paper chromatography.

Partly inverted sucrose solutions. For the isolation of the various products 20–50% (w/v) sucrose solutions were subjected to the action of an enzyme preparation at room temperature in the presence of sodium acetate:acetic acid buffer, pH 5·0, the course of the reaction being followed at frequent intervals on chromatograms pretreated with $HgCl_2$ solution (Bealing & Bacon, 1953). A time was selected at which the amount of sucrose remaining was comparable to the amounts of oligosaccharides formed; a reaction mixture containing 100–200 g. sucrose was then taken to this stage (usually 15–180 min. incubation) and the reaction stopped by raising the temperature to boiling point. Before fractionation of the oligosaccharides the monosaccharide was usually removed by the method of Whistler & Durso (1950) (see also below), samples of the partly inverted solution being put on a charcoal:Celite column, washed well with water, and the oligosaccharides then eluted with 25% (v/v) ethanol. In a typical experiment 10 ml. of 41% (w/v) sucrose after inversion yielded 1·1 g. of mixed oligosaccharides, including sucrose, i.e. about 75% of the sucrose had been converted into monosaccharide.

Methods of analysis

Most of the methods employed have been described in detail elsewhere (see Bealing & Bacon, 1953). We have reinvestigated the question of the relative reducing powers of glucose and fructose under the conditions of the method of Miller & Van Slyke (1936), and find the two sugars indistinguishable in this respect (cf. Bacon & Bell, 1948; Bacon & Edelman, 1951). Accordingly no corrections have been made when the reducing power of a mixture of glucose and fructose has been measured, as was the usual practice, against a fructose standard.

Chromatography on cellulose powder. The procedure was based on that of Hough, Jones & Wadman (1949). Whatman's Cellulose Powder (Ashless Powder for Chromatography (Standard Grade): W. R. Balston & Co. Ltd.) was packed dry into a glass tube of 29 mm. internal diameter, the lower end of the column being supported by a pad of absorbent cotton wool resting on a perforated stainless steel plate. Redistilled *n*-butanol was shaken vigorously with 0·25 vol. of water and allowed to stand until the butanol layer had cleared. Droplets of the aqueous phase still remaining (cf. Lester Smith, 1952) were removed by filtering through a large sintered glass filter (porosity 3), or alternatively 0·1 vol. of dry butanol was added to the butanol layer to render it homogeneous (R. Dedonder, personal communication). The solvent was run through the column, the first 0·5–1·0 l. of effluent being discarded. The mixture of sugars was applied to the top of the column as a weighed syrup and fractions of 2–25 ml. were collected, either by a drop-counting fraction collector (Technicon Chromatography Corporation, 215, East 149th Street, New York), or the same apparatus modified for collection at predetermined time intervals by the use of a short-interval process timer (Sangamo-Weston Ltd., Enfield, Middlesex). Selected fractions (2–20 ml.) were shaken with sufficient water to form an aqueous layer of 0·1–0·2 ml., which was then tested for carbohydrate by placing a drop on filter paper and spraying it with one of the reagents used for paper chromatograms of sugars. According to the intensity of colour produced, one or more 5 μl. spots of each fraction were applied to a paper chromatogram and run to determine the composition of the fraction. On the basis of the chromatographic evidence, groups of fractions were bulked and taken to dryness *in vacuo* with a bath temperature of not more than 50°, and in the presence of barium carbonate.

The effluents from these columns of 'Ashless Powder', as from columns of powder made from 'Ashless Tablets' (Hough *et al.* 1949), contained small amounts of degraded cellulose. This was removed by subjecting each homogeneous preparation from a cellulose column to chromatography on charcoal:Celite, as described below.

Chromatography on charcoal:Celite mixtures. The procedure of Whistler & Durso (1950) has been modified (as already described by Bacon & Bell, 1953) by the application of the 'gradient elution' technique (cf. Alm, Williams & Tiselius, 1952). The original procedure (of elution by stepwise increase in ethanol concentration) was employed in the early stages of the work described here, but the existence of some components of the oligosaccharide mixture, and the possibility of preparing others by this method, did not become apparent until a continuously increasing ethanol concentration was used to elute the oligosaccharides from the column. When samples of successive fractions obtained by this procedure are chromatographed side by side on large sheets of paper a comprehensive picture is obtained of the composition of the oligosaccharide mixture; such a chromatogram is shown diagrammatically in Fig. 1 (cf. Partridge, 1949).

Two preparations of activated charcoal were used with Celite no. 535 (Johns-Manville Co. Ltd., London, S.W. 1), B.D.H. Activated Charcoal (British Drug Houses Ltd.), and Active Carbon no. 130 (Sutcliffe, Speakman and Co. Ltd., Leigh, Lancs.). At first the latter appeared preferable because it gave a neutral effluent, while that from the B.D.H. charcoal was acid, but later experience showed that the B.D.H. product had a higher resolving power for the disaccharide mixtures encountered here. Fractions were therefore collected from B.D.H. charcoal on to solid barium carbonate and the homogeneous products of these fractionations subjected to gradient elution from Active Carbon

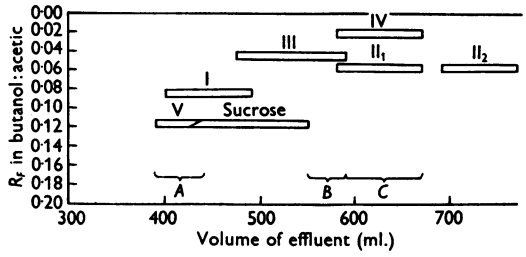

Fig. 1. Diagram showing emergence of oligosaccharides subjected to gradient elution on a mixture of B.D.H. Activated Charcoal and Celite no. 535. Details are given in the text. The size of the rectangles is not intended to indicate the amounts of the substances, only their distribution in the effluent.

no. 130 in order to free them from any soluble barium salts. Neither type of charcoal gave with Celite no. 535 an effluent altogether free from dissolved matter; this material was relatively insoluble after a fraction had been taken to dryness so that repeated evaporation and extraction served to remove at least some of this impurity. Further details of the procedure, as applied to the yeast-invertase oligosaccharides, are given below.

RESULTS

The preliminary results on which this work is based have been published by Bacon & Edelman (1950). Some of their findings are repeated in the following account, which confirms all the statements then made, except that referring to raffinose (see below).

General characteristics of the action of yeast invertase preparations on sucrose

When a yeast-invertase preparation acts upon sucrose solution, and the course of the action is followed by paper chromatography, a number of carbohydrates other than sucrose, glucose and fructose are seen to be present, as long as some sucrose remains in the reaction mixture. The general term 'oligosaccharide' will be used to describe these additional substances. Details of experiments illustrating this phenomenon have been given by Blanchard & Albon (1950), Bacon & Edelman (1950), White & Secor (1952), and White (1952). The R_F values for the oligosaccharides with various solvent mixtures, obtained by these and other workers, have been tabulated by White (1952).

Enzyme preparations. In the present work, the oligosaccharides were formed by all the enzyme preparations examined, viz. five batches of B.D.H. 'Invertase Concentrate', one sample of Wallerstein 'Blue Label' Invertase Scales, autolysates of baker's yeast made with toluene, yeast cytolysed with ether (Neuberg & Lustig, 1942), and a sample of yeast crushed by the method of Hughes (1951), kindly provided by Dr P. M. Nossal. The B.D.H. preparation has been used for the experiments described below, unless otherwise stated. This preparation had no action on maltose, methyl α-glucoside, or αα′-trehalose.

Conditions influencing oligosaccharide formation. Oligosaccharide formation was detected under all conditions tested which permitted enzyme action on sucrose. The invertase preparation was tested on concentrations of sucrose ranging from 1 to 60% (w/v), and over the pH range 3–9, with citrate, acetate, phosphate, and veronal buffers. Oligosaccharide formation was seen whether the samples were inactivated by boiling, or by the use of $HgCl_2$.

Number of oligosaccharides. Bacon & Edelman (1950) noted the presence of three oligosaccharides, which they named I, II and III in order of decreasing R_F value (in butanol:acetic acid:water mixture (Partridge, 1948), hereafter referred to as butanol:acetic). This nomenclature has been accepted by White & Secor (1952) who described in addition IV, having an R_F less than that of III, and V, which was detected by the presence of reducing power in the leading edge of the sucrose spot. We have confirmed their observations, and in addition have found that II consists of at least two components (see below). Bacon & Edelman (1950) also described a further spot detectable on chromatograms run in phenol:water. Closer investigation has shown that this occurs only when the 'Invertase Concentrate' is used without dialysis, and probably depends upon the presence of glycerol for its formation (see below). Trace amounts of other carbohydrates have been seen when fractions from chromatographic procedures have been concentrated to small bulk (see components III and V, below).

Separation of the oligosaccharides. Partition chromatography on cellulose powder leads to the elution of oligosaccharides in the same order as their migration on paper, i.e. (sucrose + V), I, II, III, IV. It was found necessary to remove the monosaccharide from the reaction products before attempting separation on cellulose; even after this treatment only about 300 mg. of mixed oligosaccharides could be separated successfully on a column of 100 g. cellulose powder. Consequently, very small amounts of homogeneous fractions were obtained in primary separations by this method.

Gradient elution from charcoal:Celite produces a different order of emergence and enables V to be distinguished from sucrose, and II to be seen as consisting of two components (II_1 and II_2), the order being V, I, sucrose, III, (II_1 + IV), II_2. It also enables larger quantities of sugars to be separated, a column of 40 g. charcoal:40 g. Celite tolerating 2–3 g. of mixed oligosaccharide. This procedure proved capable of achieving separation of all but II_1, free from other components and in reasonable yield, the fractionation being carried out in the following way.

A sample of inverted sucrose solution freed from monosaccharide as described above ('Materials'), and containing 2–3 g. of mixed oligosaccharides was run on a column (29 × 400 mm.) containing a mixture of B.D.H. Activated Charcoal (50 g.) and Celite no. 535 (50 g.), with 1 l. water in the lower reservoir and 50% (v/v) ethanol in the upper (see Bacon & Bell, 1953). Fractions (about 4 ml. each) were collected at 5 min. intervals and examined by paper chromatography; Fig. 1 shows diagrammatically the results of such a separation. (When used under the same conditions Active Carbon no. 130 gave the same qualitative results, but the separation of the disaccharides V, I, and sucrose was not as satisfactory.)

The fraction II_2 was already homogeneous, but all other components were still contaminated to some extent. Fraction *A* (Fig. 1) when re-run under the same conditions yielded 25–30 ml. of effluent containing almost pure component V; the fraction of effluent immediately following this when re-run twice, each time rejecting the early (V-contaminated) and late (sucrose-contaminated) fractions, yielded pure component I. Fraction *B* (Fig. 1) re-run once yielded practically pure component III, and showed evidence of a minor component with R_F value close to component III but emerging later. Fraction *C* (Fig. 1), run on a cellulose column, yielded pure component II_1.

All these fractions were re-run on a 20 g.:20 g. column of Active Carbon no. 130 and Celite no. 535 before a final analysis.

Analysis of oligosaccharide fractions. By the application of these two methods small quantities of each oligosaccharide have been obtained and their properties and general composition determined (Table 1). The origin of all the fractions analysed (Table 1) is given briefly; all fractions from cellulose columns, without exception, were run on Active Carbon no. 130 before analysis.

Isolation and properties of oligosaccharides

Component I. As White & Secor (1952) found, contrary to the statement of Fischer *et al.* (1951), this is a reducing substance (Table 1). It was separated by chromatography both on cellulose powder and on charcoal and was homogeneous when examined by electrophoresis on filter paper in the presence of borate buffer (D. Gross, personal communication). Its specific rotation was so low (about +5°) that no accurate reading could be obtained with the quantities available. On hydrolysis it yielded equal amounts of fructose and glucose. Its reducing power by the ferricyanide: cerium method of Miller & Van Slyke (1936) was about 60% of the reducing power of the hydrolysis products (cf. White & Secor, 1952). The following method was used to show that this sugar is a fructosyl glucose (cf. Whelan & Jones, 1953).

A small sample (6·5 mg.) was oxidized with hypoiodite by the procedure of Moore & Link (1940): to the material dissolved in 1 drop of water and 0·5 ml. of methanol was added 0·7 ml. of 7·5% (w/v) I_2 in methanol, and then 0·65 ml. of 4% (w/v) KOH in methanol drop by drop over 15 min. at 40–45°. After standing 30 min. at 40° it was treated with a further 0·5 ml. of KOH solution over 15 min. Under these conditions 10 mg. of glucose or 20 mg. of maltose were found to be completely oxidized as judged by paper chromatography. After such treatment component I no longer gave a brown colour with the benzidine: trichloroacetic acid spray (Bacon & Edelman, 1951), but instead the yellow colour typical of fructose. On hydrolysis with dilute oxalic acid it now yielded no glucose; its ketose content was 95% of that before oxidation.

Component II_1. The samples of component 'II' obtained from chromatography on cellulose powder had to be fractionated on charcoal following the discovery that this component was not homogeneous. As expected, two components were revealed, the first to emerge being named II_1. Only very small quantities (approx. 10 mg.) of the substance obtained by this method were available for analysis, but a larger quantity was later isolated from fraction *C* (Fig. 1) by chromatography on cellulose powder. The latter was homogeneous under the conditions of electrophoresis in borate buffer (Gross, 1954).

Component II_1 is non-reducing, and the analyses suggest that it is a trisaccharide consisting of one glucose and two fructose residues (Table 1). Its $[\alpha]_D$ and R_F value are very close to those of 'α_1', the main trisaccharide produced from sucrose by mould invertase (Bacon & Bell, 1953). When run under identical conditions in the gradient elution procedure the two substances require the same volume of effluent for their elution. They were indistinguishable on electrophoresis (Gross, 1954).*

Component II_2. The analyses of this substance also suggest that it is a trisaccharide consisting of one glucose and two fructose residues (Table 1). It is non-reducing. It is distinguished from component III by its R_F value in butanol: acetic, and from component II_1 by its $[\alpha]_D$, which is +21–22°. It also differs from these two substances in giving a positive Raybin test. About 10 mg. dissolved in 1 ml. of 0·05 N-NaOH and shaken with 2 mg. of diazouracil at 8° (cf. Raybin, 1933) gave a deep yellow-green colour, and after addition of magnesium chloride a blue precipitate.

Component III. Fractions consisting mainly of this component were obtained by both chromatographic procedures, but all the samples analysed contained a trace of disaccharide. This component has the same R_F as kestose (Albon *et al.* 1953), and the analyses of our fractions indicate the same

* Drs S. A. Barker, E. J. Bourne & T. R. Carrington (personal communication) have examined the infrared spectrum (720–980 cm.$^{-1}$) of component II_1, and found it to be identical (except for a small, and probably insignificant, shoulder at 872 cm.$^{-1}$) with that given by the trisaccharide 'α_1', *O*-α-D-glucopyranosyl-(1→2)-*O*-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside (cf. *J. chem. Soc.*, 1954, in the Press).

Table 1. *Analyses of oligosaccharide preparations*

In each case the sample was dissolved in 3·0 ml. of water and this solution was used for measurement of optical rotation (2 dm. tube), and for estimations of ketose and reducing substances (RS).

Component	Sample	Method of preparation	Dry wt. (mg.)	Optical rotation (°)	$[\alpha]_D$ calc. on dry wt. (°)	RS before hydrolysis* (mg./ml.) (i)	After hydrolysis with 0·5% (w/v) oxalic acid, 30 min. at 100°: Ketose (mg./ml.) (ii)	RS* (mg./ml.) (iii)	Ratio: (ii)/(iii)	Ratio: (i)/(iii)	$[\alpha]_D$ calc. from RS† (°)	$[\alpha]_D$ calc. from ketose† (°)
I	(*a*)	Cellulose	33·5	+0·06	+3	3·84	3·39	6·62	0·49	0·58	+5	+5
	(*b*)	Cellulose	18·3	+ve	—	2·89	2·30	4·68	0·49	0·62	—	—
	(*c*)	Charcoal	31·0	+0·02	—	6·47	5·87	10·5	0·56	0·62	—	—
II_1	(*a*)	Cellulose, then charcoal	10·5	+0·18	+26	—	2·50	3·60	0·69	—	+27	+26
	(*b*)	Charcoal, then cellulose	134·1	+2·45	+27	0	30·7	44·5	0·69	—	+29	+28
II_2	(*a*)	Charcoal	98·8	+1·16	+18	—	19·9	27·9	0·72	—	+22	+21
	(*b*)	Charcoal	66·5	+0·85	+19	0	14·7	20·8	0·71	—	+22	+21
III	(*a*)	Cellulose	27·2	+0·44	+24	—	4·50	6·58	0·68	—	+36	+35
	(*b*)	Charcoal	49·0	+0·89	+27	0	11·7	16·6	0·70	—	+29	+27
V	(*a*)	Charcoal	42·3	−1·15	−43	6·87	9·89	9·90	1·0	0·69	−61	−61
	(*b*)	Charcoal	19·6	−0·70	−54	—	5·95‡	—	—	—	—	−62
	(*c*)	Charcoal	19·4	−0·62	−48	—	5·42‡	—	—	—	—	−60
	(*d*)	Charcoal	23·8	−0·55	−35	—	4·79‡	—	—	—	—	−60
	(*e*)	Charcoal	26·6	−0·72	−41	—	6·63‡	—	—	—	—	−57

* Expressed in terms of the weight of glucose (or fructose) having the same reducing power. The figure 0 indicates that RS was not determined accurately, but did not exceed the error of the titration.

† On assumptions of structure stated in text.

‡ Measured on unhydrolysed solution.

hexose composition (2 fructose: 1 glucose) (Table 1). However, while the value of $[\alpha]_D + 28°$ found here for the sample from charcoal: Celite is in reasonable agreement with the value of $+27{\cdot}3°$ given by them, that for the fraction from cellulose is higher, recalling that given by Barker & Carrington (1953) for a similar, unidentified trisaccharide from the products of action of an extract of *Aspergillus niger* '152' on sucrose.

Component IV. No attempt was made to isolate or characterize this material; it appeared to be heterogeneous.

Component V. This fraction was isolated only by gradient elution from B.D.H. Activated Charcoal. It was readily distinguished from sucrose by its bright yellow colour with the benzidine: trichloroacetic acid spray; it could be recognized by this test when the products of a primary fractionation were examined. The purified fractions of component V showed some contamination with a substance of R_F (butanol: acetic) intermediate between those of sucrose and glucose. Electrophoresis on paper in borate buffer showed that three substances were present (Gross, 1954); one was a minor component, but the other two were present in proportions of about 1:3, judged visually. After hydrolysis with dilute oxalic acid, or with yeast invertase, the only detectable sugar was fructose. The reducing power was 0·7 times that of the hydrolysis products, but it seems unlikely that the major components can be other than disaccharides, judging from their behaviour on paper chromatograms, and on charcoal.

The effect of dialysis

Dialysis of the invertase concentrate in Visking synthetic cellulose casing (John Crampton and Co. Ltd., Wythenshawe, Manchester) in the presence of toluene against running tap water led to a variable loss of activity, which with some samples amounted to 90% or more. The dialysed solution lost activity further when stored in the refrigerator. For this reason in some experiments it was found more convenient to use the undialysed solution at a final dilution of 1:1000 or 1:2000, than to dialyse it.

When the dialysed solution was further dialysed in the refrigerator against distilled water for 24 hr., a preparation was obtained that no longer gave any colour with the phosphate reagents of Fiske & Subbarow (1925). As mentioned by Bacon & Edelman (1950), this preparation still formed oligosaccharides from sucrose. Inspection of chromatograms and preliminary quantitative studies indicated that the ratio of oligosaccharide formation to reducing sugar liberation was not changed by dialysis.

Quantitative aspects of the action on sucrose

The fact that two of the components (I and V) are disaccharides made it difficult to follow their formation by the application of quantitative paper chromatography to the reaction mixtures. However, a study of the course of the reaction showed that component I appeared later than components II and III, so that in the early stages of the reaction the sum of components II and III might be expected to give a fair estimate of oligosaccharide formation. (It is probable from its nature that component V also appears later in the reaction.) A preliminary investigation was therefore made of the formation of component II plus component III (measured as ketose) under various conditions. Those results which have a bearing on the mechanism of oligosaccharide formation are given briefly here:

Effect of substrate concentration. The undialysed invertase preparation, in a final dilution of 1:1000, was allowed to act on 2, 10, 30 and 60% (w/v) sucrose in 0·01 M sodium acetate buffer, pH 5·0, at 20° for 10 min. Samples were analysed as described by Bealing & Bacon (1953). The results of this (Table 2) and similar experiments show that while the rate of liberation of reducing sugar is at a maximum between 5 and 10% (w/v) sucrose (cf. Nelson & Schubert, 1928), the rate of formation of (II + III) continues to increase up to 30% (w/v) sucrose. At low sucrose concentrations the rate of formation of oligosaccharides is near the limits of detection of the method, but the formation of the same oligosaccharides at 1% (w/v) sucrose was shown by concentrating the reaction mixture ten times before chromatography.

Effect of overall rate of reaction. The same ratio of (fructose in II + III) : free fructose was found when ten parts of invertase were allowed to act on sucrose for 30 min. as when one part acted for 300 min., showing that the absolute rate of the reaction has no influence on the proportions of the products.

Effect of substances other than sucrose

Inorganic phosphate. The invertase concentrate (1:1250) was allowed to act on 12·5% (w/v) sucrose in 0·25 M phosphate buffer, pH's 6·8 and 7·2, for 45 min. at 30°. The reaction was stopped by raising the temperature to boiling point; samples of the two mixtures were chromatographed with *iso*-propanol: ammonia, and sprayed with the molybdate reagent described by Hanes & Isherwood (1949).

Table 2. *Oligosaccharide formation at various concentrations of sucrose*

For details see text. Reducing sugar and ketose are expressed in terms of fructose.

	Concentration of sucrose (%, w/v)			
	2	10	30	60
Reducing sugar (mg./ml.)	7·49	10·92	8·21	5·64
Ketose in components II and III (mg./ml.)	0·11	1·15	2·03	1·44

No spots other than those of inorganic phosphate could be seen.

Glucose. When glucose was added to the reaction mixture in quantities equivalent to those that would be produced by hydrolysing half the sucrose present component I appeared at the beginning of the reaction (cf. Edelman, 1954).

Fructose. The addition of fructose produced no qualitative effects detectable on paper chromatograms (but see Edelman, 1954).

Methyl α-glucoside. When methyl α-glucoside was added to the reaction mixture the phloroglucinol spray (Horrocks & Manning, 1949) showed that a ketose-containing substance was present in the glucose position of chromatograms run in butanol: acetic.

Glycerol. With butanol:acetic as solvent no additional spots could be seen when 20% (v/v) glycerol was added, but in phenol a fast-running spot (R_F about 0·65) was seen. When reaction mixtures made with undialysed invertase were analysed by gradient elution on charcoal a substance with the R_F of fructose in butanol:acetic emerged with component V. When run in phenol this mixture showed two spots with R_F values of about 0·4 and 0·5, corresponding to those seen with pure fractions of V, and a third spot with R_F 0·65; on hydrolysis the mixture yielded only fructose and a spot reacting with the silver nitrate spray of Trevelyan, Procter & Harrison (1950), and having the same R_F (butanol: acetic) as glycerol. It therefore seems probable that the 'fast-running spot in phenol' observed by Bacon & Edelman (1950) in incubations with undialysed invertase was a compound of fructose with glycerol.

Alcohols. Bacon (1952) has described experiments in which various alcohols were added to the reaction mixture, and fructose-containing substances were detected. Spots additional to the oligosaccharides mentioned above were seen when 10, 20, or 30% (v/v) methanol or ethanol, 20% (v/v) *n*-propanol, 7·5% *n*-butanol, and saturated (< 5%, v/v) benzyl alcohol were added at pH 5; with *iso*propanol no additional spots were seen. Bealing (1953) has published a chromatogram showing some of these spots, which presumably correspond with the various alkyl β-fructofuranosides. Bacon (1952) has isolated the substance produced in the presence of methanol, and given evidence supporting the view that it is methyl β-fructofuranoside. Whelan & Jones (1953) have obtained confirmatory evidence for the action of a number of alcohols using this substance as the substrate for yeast invertase. Oparin & Bardinskaya (1953) have studied the conditions influencing the formation of the ethyl fructoside.

Dioxan. Addition of 25% (v/v) dioxan to the reaction mixture did not appear to retard the reaction, nor give rise to additional products.

Action of invertase on raffinose

The commercial preparations used were both devoid of melibiase activity, the products of their action on raffinose being chiefly melibiose and fructose. In preliminary experiments no other substances were observed but in later experiments using 15% (w/v) raffinose a ketose-containing spot with R_F less than that of raffinose was seen, and also a trace of a substance in the sucrose position of the chromatogram (cf. Pazur, 1952; Bealing, 1953). The phenomenon was not investigated further.

DISCUSSION

The significance of the occurrence of the oligosaccharides described above was first discussed by Fischer *et al.* (1951) who found that they were produced by a highly purified enzyme preparation. Blanchard & Albon (1950) had found the fructose: glucose ratio in one of the trisaccharides to be 2:1. Fischer *et al.* therefore suggested that yeast invertase (a β-fructofuranosidase) acted as a transglycosidase, proposing the following scheme:

sucrose + enzyme ⇌ fructose-enzyme + glucose,
fructose-enzyme + sucrose ⇌ trisaccharide + enzyme,
fructose-enzyme + water ⇌ fructose + enzyme.

Edelman & Bacon (1951) put forward a similar suggestion, viz. that a hydrolytic enzyme combines with its substrate, and that the enzyme-substrate complex may then react with water, or with some organic substance capable of acting as an acceptor for part of the substrate. The hypothesis of Fischer *et al.* would seem the more acceptable; each stage in the reaction requires only two reactants, and instead of the rather vague concept of an 'enzyme-substrate complex' it proposes an exchange reaction comparable with that demonstrated by Hassid & Doudoroff (1950) for sucrose phosphorylase. Morton (1953) has recently made similar suggestions in connexion with the transferring action of alkaline phosphatase.

The idea that transfructosidation is involved has been supported by the discovery that alkyl β-fructofuranosides are formed when primary alcohols are added to the reaction mixture (Bacon, 1952; Whelan & Jones, 1953. Whether the term 'transfructosylation' used by the latter authors is more correct than 'transfructosidation' cannot be decided with the evidence available; but see Koshland & Stein, 1953). More recently, Edelman (1954) has proved by the use of ^{14}C-labelled glucose and fructose that components I and V are formed, respectively, by transfer of fructose residues to these sugars.

The possibility that the formation of transfer products is not enzyme catalysed, but simply a

property of the 'nascent' glucose and fructose molecules liberated by hydrolysis is discounted by the following facts.

(1) The earlier studies of yeast invertase (cf. Neuberg & Mandl, 1950) have established it as specific for unsubstituted β-fructofuranosides and inactive towards α-glucosides other than sucrose and its derivatives.

(2) All the oligosaccharides observed, with the exception of component I, have higher ratios of fructose:glucose than the original substrate. There are good grounds for believing that component I is produced by fructose transfer to free glucose (Edelman, 1954).

(3) The oligosaccharide mixture produced by yeast invertase is qualitatively different from that produced by mould invertase (Bealing & Bacon, 1953) though both enzymes are fructosidases.

(4) The observations of White & Maher (1953*a*, *b*) show that there can also exist enzymes that produce oligosaccharides from sucrose by trans*gluco*sidation.

The first two observations suggest that both the hydrolytic and transferring activities of yeast invertase preparations are confined to the fructose residue of sucrose; (3) shows that a different fructosidase gives rise to a qualitatively different mixture of oligosaccharides from sucrose; and (4) shows that with other enzyme preparations the glucose residue of sucrose may be transferred instead of the fructose residue. It is conceivable that one, but only one, of the two transfructosidations mentioned could proceed independently of enzymic action; that this is not the case with yeast invertase is probably indicated by the fact that all the products are eventually hydrolysed by the enzyme, while only part of the products of chemical condensation, e.g. of treatment of fructose with methanolic hydrogen chloride, are hydrolysed (Schlubach & Rauchalles, 1925).

In view of the above considerations the simplest hypothesis covering the facts would seem to be that implied by Fischer *et al.* and tentatively accepted by most others investigating the phenomenon, viz. that a single enzyme is responsible for both hydrolytic and transferring activity. (The apparent contradiction of Aronoff's (1951) results has been resolved by Aronoff & Bacon, 1952.)

The testing of the specificity requirements of the transferring activity with respect to alcohols, free sugars, and sugar alcohols by Whelan & Jones (1953) has indicated that a primary alcoholic group is required. This rule evidently applies to the trisaccharides formed by fructose transfer to sucrose; three are possible, and all have been found in the present investigation. One, component III, is known to be formed by fructose transfer to position 6 of the fructose residue of sucrose. This is 'kestose' (Albon *et al.* 1953). Another, II_1, has properties similar to that of the mould trisaccharide 'α_1' (Bacon & Bell, 1953), which is formed by fructose transfer to position 1 of the fructose residue. Component II_2 has been shown by Gross, Blanchard & Bell (1954) to be the trisaccharide formed by fructose transfer to C-6 of the glucose residue; this explains why it gives a Raybin reaction (Raybin, 1933, 1937), while the other two, having a substituent in the fructose residue, do not (see also Levi & Purves, 1949).

The disaccharide I is presumably the same as that isolated by Whelan & Jones (1953) from the products of action of yeast invertase on a mixture of methyl β-fructofuranoside and glucose, and characterized by them as 6-*O*-β-D-fructofuranosyl-D-glucose. It would seem likely that the major constituents of component V are both fructosylfructoses, but the analytical data are not sufficient to confirm this. These could be the two disaccharides capable of being formed by transfer to a primary alcoholic group in fructose, viz. 1-*O*-β-D-fructofuranosyl-D-fructose, and 6-*O*-β-D-fructofuranosyl-D-fructofuranose. The fact that it may be formed by transfer to the more abundant pyranose form of free fructose might favour the formation of the former, but on the other hand yeast invertase forms more component III than component II_1 (Bacon & Edelman, 1950), i.e. more of the 6-substituted than 1-substituted fructosylsucrose. We have examined a single sample of a (presumed) fructosylfructose produced by mould invertase; this had a more negative specific rotation ($[\alpha]_D - 79°$) than component V, but showed the same three substances as component V on electrophoresis (Gross, 1954). However, only one was present in large amounts; if this were the 1-substituted fructosylfructose it would be consistent with the predominating transfer to C-1 by this enzyme, and also with the more negative optical rotation of fructopyranose as opposed to fructofuranose derivatives (cf. Bell, 1953). The specific rotation given by Pazur & Gordon (1953) for this substance seems to rest on unsatisfactory experimental evidence, and also seems unlikely on general grounds.

The discovery that mould invertase preparations have transfructosidase activity has led to a new evaluation of the significance of their inhibition by free sugars, especially by glucose. Bealing (1953) has concluded that this inhibition is due primarily to the glucose acting as an acceptor for fructose residues (cf. also Edelman & Bealing, 1953). Edelman (1954) has reported similar results with yeast invertase. The same considerations must evidently be applied to the reported inhibition of yeast invertase by ethanol (O'Sullivan & Tompson, 1890), glycerol (Bourquelot, 1917), and methyl α-glucoside (cf. Nelson & Freeman, 1925).

The question of the classification of the various invertases must also be examined from a new point of view but this will have to await a more precise characterization of the products of their action on sucrose. Of particular interest in this connexion is the difference shown by Edelman (1954) between the products of fructose transfer from sucrose to free glucose; yeast invertase forms the reducing disaccharide I, while mould invertase forms sucrose.

SUMMARY

1. Partition chromatography on cellulose powder and gradient elution from columns of charcoal–Celite mixture have been applied to the products of action of yeast invertase on sucrose.

2. Two disaccharide fractions (other than sucrose) and three trisaccharides have been isolated and their specific rotations and contents of glucose and fructose determined.

3. The nature of these substances is considered in relation to those products of the action of yeast and mould invertases that have so far been characterized.

4. Some quantitative aspects of the formation of the trisaccharides, and the qualitative effects of adding to the reaction mixture various substances of an alcoholic nature, are described.

5. These findings are discussed in relation to the hypothesis that yeast invertase acts by transferring the fructose residue from sucrose either to water, or to substances possessing a primary alcoholic group.

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