Reproducibility of the substrate. This has been investigated by carrying out, in triplicate, estimations of the activity of a duodenal juice using four different substrate preparations. The results given in Table 2 show that there are only minor variations in the results obtained with the different substrates. Analysis shows that the variation between substrates is not significant ($P > 0.05$).

Accuracy of the estimation. From the experiment indicated in the last section, the twelve estimations on the given duodenal juice gave a result of 42.7 ± 0.4 (S.E.M.), expressing the result in terms of the colorimeter reading. This is accurate enough for the purposes for which the method was designed.

Correlation with Lagerlöf's method. The results of the estimations of tryptic activity of twenty-nine different duodenal juices by the present method and by that of Lagerlöf (1942) are shown in Fig. 3. The correlation coefficient for the two sets of results is 0·82, a figure which statistically is highly significant ($P < 0.001$). Fig. 3 enables the two sets of units to be interconverted; one Lagerlöf unit is equal to 70·5 units as defined in this paper.

RESULTS

The concentration of enzyme in juices obtained under different conditions is very variable. This is in part due to the variation in the output of juice per minute. The relationship between enzyme concentration, minute output and hormonal stimulation is to be reported in detail elsewhere, but the following are some typical values.

In seventeen determinations carried out on resting juice the range of activity was 1–75 units/ml., but twelve of these results lay in the range 8–38 units/ml. In the case of juice obtained after secretin administration the range for nineteen estimations was 19–93 units/ml. and fourteen of these lay in the range 25–75 units/ml. Juice obtained after pancreozymin stimulation showed a range of 18–90 units/ml. for sixteen juices, but eleven of the results lay in the range 50–80 units/ml.

SUMMARY

1. A method is described for the assay of tryptic activity in duodenal juices using a denatured preparation of dried human serum as the substrate and measuring the phenolic substances liberated by trypsin.

2. A unit of tryptic activity is defined, and a method for the calibration of any colorimeter is suggested.

3. The experimental basis of the method is presented and the overall accuracy is assessed.

4. The results from this method have been correlated with those from Lagerlöf's (1942) method.

5. The range of activity of varying types of juice is discussed briefly.

I wish to thank Prof. A. A. Harper and Dr H. T. Howat for their interest and for allowing me to use the duodenal juices obtained in their experiments. I am indebted to Dr S. Oleesky for the estimations by the Lagerlöf method and to Dr F. Stratton for the dried serum used in this work.

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The Action of Mould Enzymes on Sucrose

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The hydrolysis of sucrose by mould invertase preparations has been considered to result from α-glucosidase (glucosaccharase) activity (Kuhn, 1923), on the basis of observations that the reaction rate is decreased by the presence of free α-glucose, presumed to act as a competitive inhibitor. Modification of this view became necessary when mould extracts were found to hydrolyse part of the 'γ'-methyl fructoside mixture (Schlubach & Rauchalles, 1925; Purves & Hudson, 1934), and, in the absence of melibiase activity, to attack raffinose (Rohdewald, reported by Kuhn & Münch, 1927; Hestrin, 1940). These results indicated the presence of a β-fructosidase similar to that found in yeast invertase preparations; accordingly, it has been suggested that sucrose breakdown by moulds may

result from the combined action of an α-glucosidase and a β-fructosidase (Kuhn & Münch, 1927; Weidenhagen, 1932; Hestrin, 1940). However, the idea that a glucosaccharase is the typical constituent of mould invertase preparations has been restated in recent reviews (Neuberg & Mandl, 1950; Gottschalk, 1950).

The results presented here refer to the action of mould extracts on sucrose and lead to the conclusion that the enzyme concerned is a β-fructofuranosidase capable of transferring fructose residues to suitable acceptors. This theory affords an explanation for the appearance, first observed by Edelman (1950), of oligosaccharides other than sucrose as intermediates during the reaction.

Part of this work was communicated to the Biochemical Society at Sheffield on 21 July 1951 (Bealing & Bacon, 1951). Since this paper was submitted, Pazur (1952) has described the preparation from *Aspergillus oryzae* of a 'transfructosidase' which would appear to be the same enzyme as that described here.

MATERIALS AND METHODS

Organisms and media. Enzyme preparations were obtained from *Aspergillus oryzae* (Commonwealth Mycological Institute 17299), *A. niger* (National Collection of Type Cultures 594), and *Penicillium spinulosum*. The strain of *Aspergillus niger* used had been subcultured on malt agar in this laboratory for 4 years: the *Penicillium* appeared as a contaminant of one of these cultures. Other species used were *P. chrysogenum* (strain Q-176), subcultured on malt agar for 2 years; and *Aspergillus flavus*, *Eremascus fertilis* and *E. albus*, which were kindly supplied by Mr J. Webster of the Botany Department of this University.

Roux bottles containing 70–100 ml. of Czapek-Dox medium (5% sucrose; 0·2% $NaNO_3$; 0·1% K_2HPO_4; 0·05% $MgSO_4.7H_2O$; 0·05% KCl; 0·001% $FeSO_4.7H_2O$) were inoculated with spores and incubated at room temperature (*Penicillium*), or 30° (*Aspergilli*). Mycelial felts were usually harvested within 8 days of inoculation, before the beginning of active sporulation.

When spores were required the mould was grown for 1–3 weeks on a solid medium (as above, with the addition of 2% agar), and the spores washed off with water. The same solid medium was used for the slopes on which the fungi were subcultured during the course of the present work (from October 1950).

Preparation of mould extracts. The term 'mould extract' will be used to describe any extract of mycelium or spores derived from the fungi used, irrespective of species or treatment.

Frozen material was crushed in a press (Hughes, 1951) cooled by contact with solid CO_2, the product being a mobile fluid (20°) which still contained a few viable cells, as indicated by the fact that a little growth occurred if the material was stored for 2–3 days without $CHCl_3$ or toluene. The suspension was centrifuged and the supernatant liquid dialysed for 2 days in the presence of toluene against running tap water, the dialysis being carried out in Visking synthetic cellulose casing (John Crampton and Co. Ltd., Wythenshawe, Manchester). A flocculent precipitate usually developed during dialysis: this was removed by filtration to give a clear, slightly coloured extract.

Extracts were also prepared by the autolysis of mycelium under toluene for 6–7 days at room temperature, sometimes after a short preliminary treatment of the material in a Waring Blendor. The extract was finally filtered under suction, dialysed as above, and filtered a second time.

The dry matter of extracts prepared by these methods was variable, in most cases lying between 2 and 4 mg./ml. The commercial preparations used (Parke, Davis and Co. 'Takadiastase') contained large amounts of lactose, so that after dialysis the dry matter fell to concentrations, for 5–10% (w/v) solutions, comparable with those of the extracts prepared in this laboratory.

Sugars. The sugars were chromatographically pure commercial products, except for turanose which was acquired through the courtesy of Dr C. S. Hudson.

Paper partition chromatography. The apparatus and methods employed were those of Bacon & Edelman (1951) and Bacon & Loxley (1952), with the following modifications.

Quantitative chromatograms were usually run four together in the same tank, the two sheets on each side of the trough being separated by glass or stainless-steel rods (Lindan & Work, 1951). Development of the outer chromatograms was retarded by this procedure, but adequate separation of the spots on all chromatograms was obtained after 2·5 days.

Sugars separated on these chromatograms were extracted by heating appropriate areas of the paper with a known volume of water in glass-stoppered tubes at 70–80° for 30 min. Each tube was shaken to incorporate condensation water in the extract, the paper compressed with a glass rod, and samples for estimation taken from the supernatant liquid. Extracts of regions of each chromatogram which contained no detectable sugar were used as controls in the estimation.

The recovery of fructose was 95–103% of that expected from the concentration of sucrose employed.

Treatment of two-dimensional chromatograms with yeast invertase solutions was used to elucidate the monosaccharide composition of oligosaccharides (Bacon & Edelman, 1951). Spraying with an aqueous enzyme solution was avoided because it permitted diffusion of the spots: instead, a filter-paper pad soaked in the solution (20% (v/v) British Drug Houses Ltd. Invertase Concentrate) was pressed on the appropriate area. This treatment diminished the extent of diffusion but some material was lost by transference to the paper pad.

Incubation procedure. Mixtures of substrate, buffer and enzyme were incubated in the presence of $CHCl_3$ in glass-stoppered tubes at room temperature (18–24°). In many cases, successive samples were analysed by paper chromatography: inactivation of the small volumes used (twenty 5 μl. drops) was accomplished by placing 5 μl. quantities of 0·01 M-$HgCl_2$ on the starting line and allowing them to dry before application of the solution to be analysed. This procedure had no effect on the development of the chromatograms. Relatively large samples (0·5–4·0 ml.) were inactivated either by heating for 3 min. in tubes previously placed in a boiling-water bath, or by the addition of $HgCl_2$ solution to give a final concentration of 0·001–0·002 M-$HgCl_2$.

Quantitative estimations. (1) *Ketose.* A colorimetric method (Cole, unpublished; see Bacon & Bell, 1948) based on the Seliwanoff reaction was used. The values obtained for

extracts of blank areas of paper were usually negligible. (2) *Reducing substances.* As mentioned by Bacon & Edelman (1951), the method of Miller & Van Slyke (1936) was found to give relatively large values when applied to extracts of blanks from chromatograms: a colorimetric method (Nelson, 1944; Somogyi, 1937) was employed to estimate reducing sugars extracted from paper because with this the blank values were negligible. The presence of small amounts of $CHCl_3$, toluene or thymol interfered with the reaction, and solutions intended for estimation without a preliminary dilution were preserved by the addition of $HgCl_2$ solution, or by freezing. Solutions containing less than 3% (w/v) reducing sugar were estimated directly by delivering 5 or 10 μl. quantities into each tube from an Agla micrometer syringe (Burroughs Wellcome and Co., London).

RESULTS

Action of mould extracts on sucrose

An extract of crushed *Penicillium spinulosum* mycelium (10·0 ml.) was added to 40·0 ml. buffered sucrose solution to give a final concentration of 7·5% (w/v) sucrose in 0·007 M-acetate buffer at pH 5·0. The solution was incubated at room temperature and 4·0 ml. samples were removed at intervals. Each sample was inactivated by heating, cooled and left for 12 hr. to permit mutarotation, after which the optical rotation was measured in a 2 dm. polarimeter tube holding 3 ml. of solution. The reducing sugar content of a 5 or 10 μl. quantity of each solution was determined.

The amount of reducing sugar expected to be present was deduced from the polarimetric readings on the arbitrary assumption that glucose and fructose were the only products of sucrose breakdown. This assumption gave, for reducing sugar, results in good agreement with those obtained by direct estimation (Fig. 1). However, analysis of the solutions by quantitative paper chromatography showed that the initial rate of disappearance of sucrose from the reaction mixture was approximately three times as great as that inferred from the optical data on the above arbitrary assumption (see Fig. 1).

Despite the initially rapid disappearance of sucrose from the reaction mixture, complete hydrolysis was reached only after a prolonged incubation period (several weeks). The chromatograms showed that the disappearance of sucrose was accompanied by a simultaneous accumulation of oligosaccharides (described subsequently as the 'upper spots') having R_F values less than that of sucrose (Fig. 2).

Four upper spots appeared in relatively large amounts: they are referred to as α, β, γ and δ (R_F values in butanol-acetic acid: 0·07, 0·04, 0·02 and 0·01 respectively, compared with sucrose, R_F 0·11). These substances were not produced when mould extracts were incubated with glucose, fructose, galactose, mannose, trehalose (1-(α-D-glucopyranosyl)-α-D-glucose), turanose (3-(α-D-glucopyranosyl)-D-fructose), melezitose (2:3-di-(α-D-glucopyranosyl)-β-D-fructofuranose), raffinose (1-(β-D-fructofuranosyl)-6-(α-D-galactopyranosyl)-α-D-glucopyranose), or with a mixture of glucose and fructose, but were always present when sucrose was used as substrate. They were also produced by the action of Takadiastase (Parke, Davis and Co.) on sucrose,

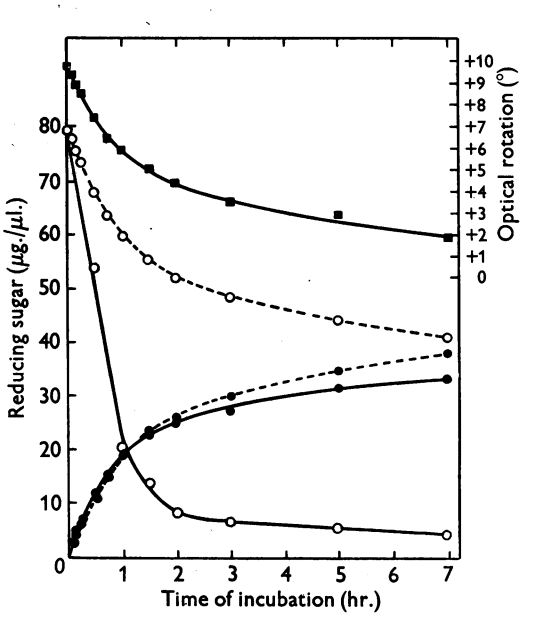

Fig. 1. Comparison of values for reducing sugars obtained by direct estimation with those deduced from the change in optical rotation of the reaction mixture. Extract of crushed *P. spinulosum* mycelium incubated with 7·5% (w/v) sucrose in 0·007 M-acetate buffer, pH 5·0. ●—●, reducing sugar by direct estimation; ○—○, residual sucrose (by quantitative paper chromatography, expressed as μg. reducing sugar/μl.); ●- - -●, reducing sugar, deduced from polarimetric data; ○- - -○, residual sucrose', deduced from polarimetric data; ■—■, optical rotation of reaction mixture.

and were present in sucrose culture media during the growth of all the fungi examined (*Aspergillus oryzae, A. niger, A. flavus, Penicillium spinulosum, P. chrysogenum, Eremascus fertilis* and *E. albus*). Growth of *Penicillium spinulosum* on a medium containing equal amounts of glucose and fructose (Czapek-Dox: sucrose replaced by 4% glucose, 4% fructose) did not result in the formation of upper spots. The presence of ketose in α, β, γ and δ was demonstrated by the reaction with a spraying reagent containing phloroglucinol (Horrocks & Manning, 1949). Treatment with silver nitrate (acetone solution) and ethanolic sodium hydroxide (Trevelyan, Procter & Harrison, 1950) failed to

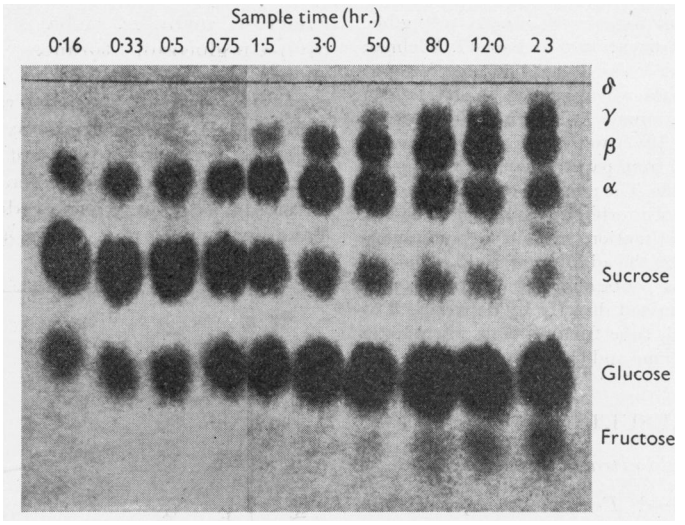

Fig. 2. Filter-paper chromatogram of successive samples of the reaction mixture during the incubation of an extract of autolysed *P. spinulosum* mycelium with 7·5% (w/v) sucrose in 0·007 M-acetate buffer at pH 5·0. Chromatogram developed for 2 days in butanol-acetic acid-water: sprayed with benzidine-trichloroacetic acid (Bacon & Edelman, 1951).

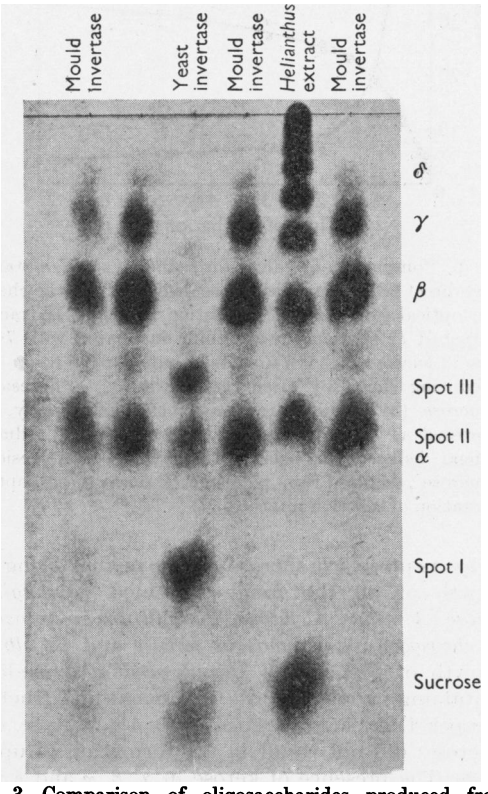

Fig. 3. Comparison of oligosaccharides produced from sucrose by a *P. spinulosum* extract with those produced by yeast invertase, and with the oligosaccharides present in extracts of *Helianthus tuberosus* L. (Jerusalem artichoke) tubers. Chromatogram run 4 days in butanol-acetic acid: sprayed with benzidine-trichloroacetic acid.

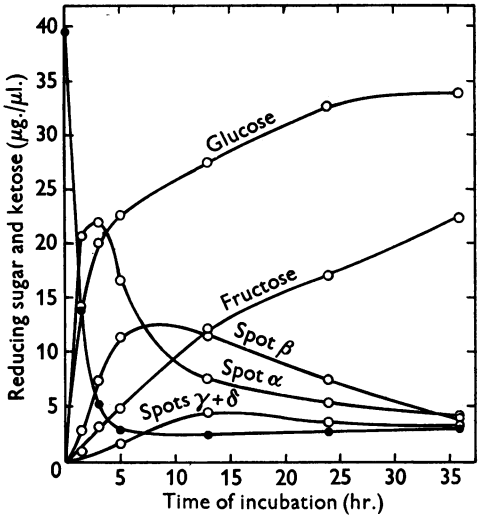

Fig. 4. Action of an extract of crushed *P. spinulosum* mycelium on 7·5% (w/v) sucrose in 0·007 M-acetate buffer, pH 5·0. Fructose and oligosaccharides estimated and expressed in terms of ketose contents of eluates from filter-paper chromatograms. Values for glucose derived from difference between estimates of total reducing substances and free fructose. ●—●, sucrose.

demonstrate the presence of reducing properties in these substances, although the spots eventually became visible at about the same time as a control sucrose spot.

Yeast invertase hydrolysed α, β, γ and δ; the presence of both glucose and fructose in each spot was shown by two-dimensional chromatography after treatment with this enzyme.

Fructose-containing oligosaccharides are also produced by the action of yeast invertase on sucrose; one of these substances (spot II; see Bacon & Edelman, 1950) possessed an R_F value corresponding with that of spot α (Fig. 3). This chromatogram also shows that four of the oligosaccharides present in an extract of *Helianthus tuberosus* (artichoke) tubers possessed R_F values similar to, but probably not identical with, those of α, β, γ and δ.

A fifth upper spot, having an R_F value of 0·09, appeared when mould extracts were incubated with glucose, or when sucrose or maltose was used as the initial substrate. This substance did not react with the phloroglucinol reagent and was the only upper spot capable of the rapid reduction of alkaline silver nitrate solutions. It represented only a small fraction of the total carbohydrate in the solutions analysed (see last sample, Fig. 3, between sucrose and spot α), and the factors influencing its formation were not investigated.

The formation and disappearance of the fructose-containing components of the reaction mixture, and the liberation of glucose, were followed by quantitative paper chromatography.

An extract of autolysed *Penicillium spinulosum* mycelium (0·4 ml.) was added to 1·6 ml. of a buffered sucrose solution (final concentration, 7·5% (w/v) sucrose in 0·007 M-acetate buffer, pH 5·0). Samples taken during the reaction were placed on quantitative chromatograms pre-treated with mercuric chloride solution. The amount of each component of the chromatogram was determined in terms of ketose content.

Disappearance of sucrose in the early stages of the reaction was accompanied by the liberation of glucose and the formation of upper spots (Fig. 4). These changes occurred rapidly; after incubation for 2 hr. spot α contained 55% of the total fructose, 15·5% being in spots β, γ and δ, and 25% in the sucrose spot. The monosaccharide fraction of the same sample contained 40% of the total glucose but only 4·5% of the fructose. After 5 hr. the ketose content of the upper spots reached a maximum value, equivalent to 81% of the total fructose. Subsequently, a constant rate of decrease in upper spot fructose was accompanied by a corresponding increase in free fructose, there being no significant change in the ketose content of the sucrose spot before the experiment ended (36 hr.).

Effect of sucrose concentration

One volume of a dialysed extract of *Aspergillus oryzae* mycelium was diluted to 12 vol. with water, and 1·0 ml. samples of the dilute solution were added to each of a series of buffered sucrose solutions (3·0 ml.) to give final concentrations of 0·6–60% (w/v) sucrose in 0·005 M-acetate buffer (pH 5·0). The mixtures were incubated for 1·5 hr., after which the enzyme was inactivated by heating. A sample (1·0 ml.) of each solution was diluted to 10 ml. for the estimation of reducing sugar.

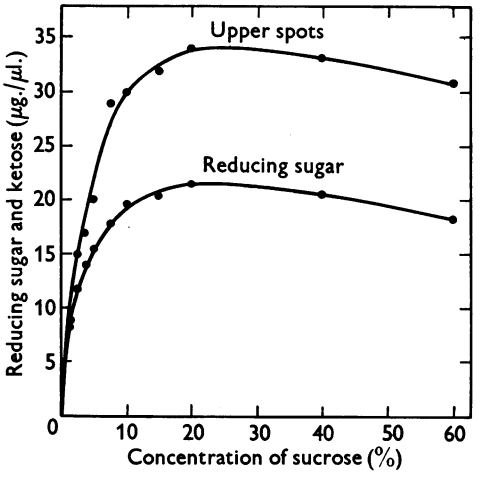

Fig. 5. Effect of sucrose concentration on liberation of reducing sugar and formation of upper spots. Extract of autolysed *A. oryzae* mycelium incubated for 1·5 hr. with sucrose solutions buffered with 0·005 M-acetate buffer, pH 5·0. Upper spots expressed as ketose.

Under the conditions of this experiment the amounts of reducing sugar liberated were small (never greater than 10% of the sucrose originally present) so that the reaction rates measured could be regarded as initial velocities. The rate of liberation of reducing sugar increased with substrate concentration, reaching a maximum value at about 40% (w/v) sucrose and decreasing slightly thereafter (Fig. 5). Values for the Michaelis constants calculated from the results of this and other experiments varied from 0·06 to 0·08 M for extracts of *A. oryzae*, but the values obtained for Takadiastase, presumably from the same species, were as low as 0·02–0·03 M.

Chromatographic analysis showed that the rate of formation of upper spot material (at this stage of the reaction consisting almost entirely of spot α) was dependent on substrate concentration in the same way as the liberation of reducing sugar. The amounts of free fructose present were small, and since the more concentrated solutions had to be diluted before analysis, it was not possible to obtain

reliable values for the rate of liberation of this sugar.

In another experiment samples of dialysed 10% (w/v) Takadiastase (1·0 ml.) were added to 4·0 ml. volumes of buffered sucrose solutions, giving final concentrations of 2–39% (w/v) sucrose: the course

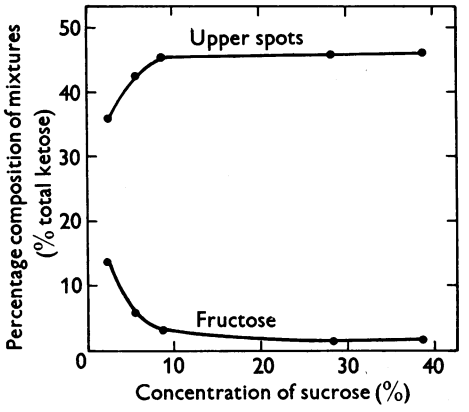

Fig. 6. Effect of sucrose concentration on liberation of fructose and formation of upper spots. Takadiastase (2%, w/v) incubated with sucrose solutions: composition of mixtures determined at time when 50% of the sucrose had disappeared. Amounts of fructose and upper spots estimated as ketose and expressed as percentages of total ketose in the mixture.

of the reaction was followed by the analysis of 0·5 ml. samples which were removed at intervals and inactivated by the addition of 0·1 ml. 0·01M-mercuric chloride solution. A preliminary dilution (by the addition of 2·0 ml. water) was made of those solutions which contained more than 10% (w/v) sucrose.

The greater extent of substrate breakdown in these mixtures permitted a more precise estimation of fructose liberation. Whereas the concentration of sucrose influenced the rates of formation of glucose and upper spots in the same way as in the previous experiment, it produced no significant change in the rate of fructose liberation, in the range 4–39% sucrose.

A comparison was made between different reaction mixtures, based on the composition of the solution (deduced from time curves) when the ketose content of the sucrose spot had decreased to 50% of its initial value. The amounts of fructose present as the free sugar and combined in the upper spots are shown as percentages of the total fructose in Fig. 6. These values were independent of substrate concentration except in solutions containing less than 10% (w/v) sucrose when relatively, although not absolutely, more fructose was liberated.

Comparison of different mould extracts

Several extracts of *Penicillium spinulosum* material were incubated with 7·5% (w/v) sucrose in 0·007M-acetate buffer at pH 5·0. Time curves of the reactions showed that the amounts of oligosaccharide formed at the stage at which 50% of the sucrose had disappeared, were approximately the same (Table 1). Analyses 2 and 3 of this table refer to the action of extracts of mycelium inoculated at the same time, grown under the same conditions and harvested after 3 and 11 days, respectively: analysis 6 refers to the action of an extract of crushed spores.

Extracts of *Aspergillus oryzae* and *A. niger* liberated greater and more variable amounts of fructose under the same conditions.

Table 1. *Comparison of oligosaccharide-forming activities of different extracts*

(Dialysed extracts incubated with 7·5% (w/v) sucrose in 0·007M-acetate buffer, pH 5·0. Samples taken at intervals and analysed by quantitative paper chromatography; composition of solutions compared at the point (deduced from time curves) when the sucrose spot contained 20 μg. ketose/μl., equivalent to 49·3% substrate breakdown. All extracts made from mycelium, except 6 and 14.)

Species	Extract	Fructose (μg./μl.)	Upper spots (μg. ketose/μl.)	Fructose recovery (%)
1. *P. spinulosum*	Autolysed	1·0	19·2	102
2. *P. spinulosum*	Autolysed	0·7	18·0	99
3. *P. spinulosum*	Autolysed	0·8	18·1	99
4. *P. spinulosum*	Autolysed	0·8	18·3	99
5. *P. spinulosum*	Crushed	1·4	18·3	100
6. *P. spinulosum*	Crushed spores	0·7	17·7	98
7. *A. oryzae*	Autolysed	2·6	16·2	98
8. *A. oryzae*	Autolysed	2·4	18·2	103
9. *A. oryzae*	Crushed	3·8	15·3	100
10. *A. oryzae*	Crushed	4·0	14·5	97
11. *A. oryzae*	Crushed	5·8	12·2	96
12. *A. niger*	Autolysed	8·0	9·8	96
13. *A. niger*	Autolysed	4·8	13·8	98
14. *A. niger*	Growth medium	1·8	15·5	95

Table 2. *Effect of partial inactivation by heat*

(Dialysed extract of autolysed *P. spinulosum* mycelium incubated with 7·5% (w/v) sucrose in 0·007M-acetate buffer, pH 5·0. Part of same extract heated to 55° for 10 min., cooled and incubated under the same conditions. Composition of sample compared with that of unheated control at same stage of sucrose breakdown (sucrose spot containing 8·0 μg. ketose/μl.).)

Extract	Time of incubation (hr.)	Fructose (μg. ketose/μl.)	Upper spots (μg. ketose/μl.)	Recovery of fructose (%)
Untreated	2·2	1·6	28·7	97
Heated	7·0	1·8	29·1	99

All of the mould extracts examined liberated reducing sugars from maltose, lactose, trehalose, turanose, melibiose, melezitose and raffinose.

Effect of partial inactivation by high temperature

An extract of autolysed *Penicillium spinulosum* mycelium was incubated with 7·5% (w/v) sucrose in 0·006M-acetate buffer at pH 5·0; samples taken at intervals up to 3 hr. were analysed by paper chromatography.

Part of the same mycelial extract was heated to 55° for 10 min. in a water bath. After cooling, it was incubated under the same conditions, and a sample was analysed after 7 hr.

The heat treatment was found to have decreased the rate of sucrose breakdown to 30%, but the composition of the sample analysed was found to be very similar to that of the control solution (deduced from a time curve) when the concentration of ketose in the sucrose spot had fallen to the same level (Table 2).

Effect of different pH *values*

Equal volumes of an extract of autolysed *Aspergillus oryzae* mycelium were incubated with sucrose in a series of solutions containing 7·5% (w/v) sucrose and 0·008M-veronal buffer adjusted to different pH values. Samples taken at intervals from each solution were analysed by paper chromatography.

The rate of formation of upper spots reached a maximum value at about pH 6·0, but the rate of liberation of free fructose was greatest under slightly more acid conditions (pH 4·5) (Fig. 7). As a result of this, there was a greater proportion of free fructose in the solutions at pH 4–5 (examined at 50% sucrose breakdown) than in those at pH 6·5.

Growth of fungi on sucrose media

The course of sucrose breakdown during the growth of *A. niger* on a sucrose medium was followed by paper chromatography, in one instance, quantitatively.

Czapek-Dox medium (100 ml.) containing 7·5% (w/v) sucrose was sterilized by heating for 10 min. on a steam bath on each of three consecutive days, inoculated with 0·1 g. wet *A. niger* spores and incubated at 30°. Mycelium became apparent within 10 hr. of inoculation and sporulation began at 73 hr. Samples were taken at intervals up to 31 hr. when more than 50% of the sucrose had disappeared: 1·0 ml. samples of the solution were inactivated by the addition of 0·1 ml. 0·01M-mercuric chloride solution to each. The course of sucrose breakdown during this period was qualitatively similar to that

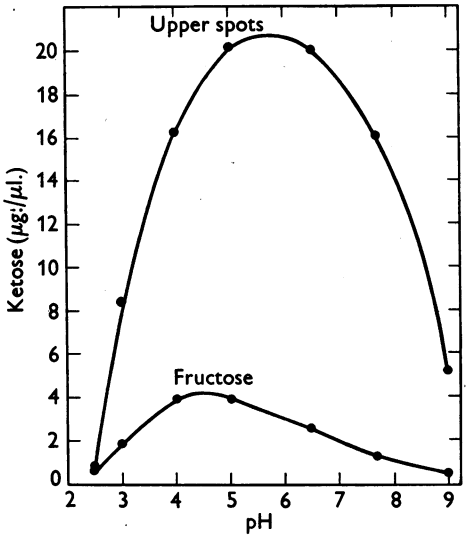

Fig. 7. Effect of pH on liberation of fructose and formation of upper spots. Extract of autolysed *A. oryzae* mycelium incubated for 2 hr. with 7·5% (w/v) sucrose in 0·008M-veronal buffers. Upper spots expressed as ketose.

resulting from the action of mould extracts. Quantitatively, slightly more upper-spot material was produced (50% substrate breakdown) than by extracts of the same species (see Table 1, analysis 14); this difference may not be significant.

DISCUSSION

The breakdown of sucrose by mould extracts results in the formation of a number of oligosaccharides (upper spots). Four of these substances contain fructose and appear in relatively large amounts: their formation could be explained in terms of a transference of fructose residues to

acceptor molecules, a reaction which would involve the simultaneous liberation of glucose:

Fruf2β-α1Gp	+	X	→	Fruf2β-X	+	G
α-D-Gluco-pyranosyl-β-D-fructo-furanoside (sucrose)		acceptor		β-fructo-furanoside		glu-cose

A formulation of this type, with sucrose acting as acceptor, has been suggested by Fischer, Kohtés & Fellig (1951) and Edelman & Bacon (1951): more general equations presented by Wallenfels & Bernt (1952) refer to the group-transferring activities of mould lactase, maltase and invertase preparations.

Of a number of substances known to act as acceptors for fructose residues, only sucrose, oligosaccharides derived from this sugar, and water are considered in the present paper. It should be noted, however, that the inhibitory effect of added glucose, fructose and alcohols might also be attributable to their action as acceptors.

Transference to sucrose would lead to the formation of trisaccharides containing two fructose residues and the initiation of a series of upper spots, each substance formed acting as an acceptor for additional fructose units.

The liberation of free fructose in the reaction mixture could result from the transference of fructose to water. This reaction, involving the disappearance of a glycosidic link, would not be expected to be reversed to any appreciable extent and would thus result in a continuous loss of substrates. The oligosaccharides produced by transfer reactions are susceptible to enzymic attack; breakdown of these substances, accompanied by the transference of fructose units to water, would lead eventually to complete hydrolysis of sucrose.

It is convenient, until evidence is produced to the contrary, to attribute both the formation of oligosaccharides and the hydrolytic liberation of fructose to the action of a single enzyme. This view is supported by observations that the ratio of these activities remained constant under controlled conditions in all of the *Penicillium spinulosum* extracts examined, even when the age and morphological nature (mycelium or spores) of the material differed, and when extracts were subjected to partial heat inactivation (Tables 1 and 2). A similar constancy was found when the substrate concentration was varied between wide limits; the relatively greater fructose liberation at low substrate concentration could be attributed to an insufficiency of carbohydrate acceptor molecules.

In the equation presented above, the product of fructose transfer is considered to be a β-fructofuranoside. Support for this idea is provided by the fact that yeast invertase (β-fructofuranosidase) hydrolyses the upper spots produced by mould extracts.

It is probable that an enzyme catalysing the transfer of fructose residues from sucrose will be a β-fructofuranosidase rather than an α-glucosidase. Observations which indicate the presence of a fructosidase in mould extracts have been reported in the introduction: further evidence, referring to fructose-transference when β-fructofuranosides other than sucrose are used as substrates, will be presented in a later paper.

The characterization of the *Penicillium* invertase as a fructosidase implies a similarity between this enzyme and yeast invertase, considered to be a typical β-fructofuranosidase (Neuberg & Mandl, 1950). Yeast invertase preparations also produce fructose-containing oligosaccharides from sucrose (Blanchard & Albon, 1950; Bacon & Edelman, 1950), even when highly purified (Fischer *et al.* 1951). Of the substances formed, one (spot II; see Bacon & Edelman, 1950) may be identical with spot α: the others possess R_F values differing from β, γ and δ, and may represent a second series of upper spots. This difference is probably not of fundamental importance and could be explained on the basis of differences in enzyme specificity determining whether both or only one of the trisaccharide types Fru – (Fru – G) and Fru – (G – Fru) would be formed with sucrose acting as an acceptor, or, alternatively, whether one of these substances would be hydrolysed preferentially.

Repeated transference of fructose to the same oligosaccharide molecule would result in the formation of a fructosan, the formulation of the reaction then approximating to that given for levansucrase (Hestrin & Avineri-Shapiro, 1944):

$$(n+m)\ \text{Ald-Fru}f + n\text{H}_2\text{O} \rightarrow (n+m)\ \text{Ald} + n\text{Fru} + (\text{levan}).$$

(Levan) represents a polymer derived from *m* fructose residues. The extent to which this process occurs, i.e. whether relatively few large molecules or large numbers of smaller molecules are produced, must depend on the affinity of the enzyme for large and small acceptor molecules. The formation of more than four fructose-containing upper spots by mould extracts acting on sucrose has not been observed under any conditions.

Extracts of *Aspergillus* mycelium possessed a greater hydrolytic tendency than the *Penicillium* extracts (see Table 1): the magnitude of this difference was variable and influenced by the method of extraction so that here more than one enzyme was probably involved in sucrose breakdown. The greater hydrolytic tendency at pH 5·0 than at pH 7·0 may indicate differences in pH optima of the enzymes.

The complexity of the action of mould extracts is in contrast to the previously accepted view, which assumed glucose and fructose to be the only substances formed. Earlier workers regarded the change in optical rotation of the reaction mixture as a convenient means of following invertase action, calculating their results on the basis of this assumption. There was no indication that results obtained by this procedure differed significantly from those obtained by direct estimation of reducing sugar, or that the initial assumption was invalid. However, in spite of the formation of upper spots, similar agreement was found in the present investigation when mould extracts were incubated with sucrose, which indicates that there is no reason to suppose that the reaction described in this paper is more complex than that studied by other authors using similar preparations.

While it is clear that a similar quantitative relationship between certain aspects of two quite different paths of sucrose breakdown cannot be fortuitous, we have been unable to produce an explanation for it on theoretical grounds; nor does it seem likely that any exact formulation will be possible for what is at best only an approximate agreement.

SUMMARY

1. Extracts were prepared from *Penicillium spinulosum*, *Aspergillus oryzae*, and *A. niger* by autolysis under toluene and by crushing frozen mycelium and spores. The action of these extracts on sucrose was followed by paper chromatography.

2. The breakdown of sucrose by mould extracts, and by these and other fungi growing on sucrose media, is not a simple hydrolysis. An initially rapid disappearance of substrate from the solution is accompanied by the formation of four non-reducing fructose-containing oligosaccharides and the liberation of glucose. Hydrolysis of these oligosaccharides proceeds slowly, fructose being liberated at an almost constant rate throughout the reaction.

3. The formation of the oligosaccharides was not observed when mould extracts were incubated with other sugars singly, or with a mixture of glucose and fructose.

4. The effects of pH, substrate concentration, and partial heat inactivation were investigated.

5. The ratio of oligosaccharide formation to fructose liberation was the same with all *Penicillium spinulosum* extracts examined, but was smaller and more variable with extracts of *Aspergillus* spp.

6. The breakdown of sucrose by mould extracts is discussed and attributed to the transference of fructose residues to suitable acceptors by a β-fructofuranosidase, considered to be the only enzyme responsible for the hydrolysis of sucrose by *Penicillium spinulosum* extracts.

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