reasons for this deviation from the theoretical are discussed.

4. A comparison between the distribution curve of a preparation of growth hormone and its electrophoresis pattern is presented.

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Transfer Reactions Catalysed by some Sucrase Preparations

By J. EDELMAN

Research Institute of Plant Physiology, Imperial College, London, S.W. 7

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In view of recent observations on the action of sucrase preparations from various sources (mainly yeasts and moulds, e.g. see Edelman, 1950; Bacon & Edelman, 1950; Blanchard & Albon, 1950; Fischer, Kohtès & Fellig, 1951; Bacon, 1952; Wallenfels & Bernt, 1952; White & Secor, 1952; Bealing & Bacon, 1953; Bealing, 1953) the existence of enzymes capable of catalysing solely the hydrolysis of sucrose to glucose and fructose is becoming increasingly doubtful. It appears that the fundamental action of sucraes may be to transfer fructose residues to suitable acceptors with consequent production of mono-, di- and oligo-saccharides. Some of the implications of fructose transfer have been discussed by Edelman & Bacon (1951) and Bealing & Bacon (1953).

This paper describes experiments with yeast- and mould-sucrase preparations, involving the use of radioactive sugars, which experiments further elucidate the fructose-transferring capacity of these enzymes. Some of the results described here are an extension and amplification of those communicated to the Biochemical Society on 24 October 1952 (Edelman & Bealing, 1953).

MATERIALS AND METHODS

Enzyme and yeast preparations

Dialysed mould sucrase. This was prepared from *Aspergillus oryzae* or *Penicillium spinulosum* using the Hughes' bacterial press by the method of Bealing & Bacon (1953). Alternatively, a 'Takadiastase' preparation, obtained from

Parke, Davis and Co. Ltd., London, was used. This was a powder prepared from *Aspergillus* sp. (probably *A. oryzae*) and standardized for use in analytical chemistry. Solutions were made in distilled water and dialysed for at least 12 hr. against distilled water before use.

Yeast sucrase. An invertase concentrate derived from yeast, and obtained from British Drug Houses Ltd., London, was used. It was dialysed against distilled water for at least 4 hr. before use.

***Candida krusei*.** A culture of this sucrase-free yeast was obtained from the Brewing Industry Research Foundation, Nutfield, Surrey. Subcultures were maintained on malt agar. A liquid medium containing 1% Difco yeast extract and 3% (w/v) glucose in tap water was inoculated and incubated in flat bottles at 25° for 4 days. The liquid was centrifuged, the cells washed three times by suspension in water and centrifuging, and packed hard by high-speed centrifuging to give a moist, crumbly cake. The weight of packed cells was about equal to that of the glucose originally present in the medium. Thick suspensions of this preparation rapidly fermented glucose and fructose, with copious production of CO₂ after several minutes. Sucrose was not attacked even on prolonged incubation (up to 30 hr.).

Enzyme and yeast preparations were stored at 3°.

Radioactive sugars

Uniformly labelled [¹⁴C]glucose was prepared in this laboratory by photosynthesis from ¹⁴CO₂, using tobacco leaves (Porter & Martin, 1953). Uniformly ¹⁴C-labelled fructose prepared by a similar method was purchased from the Radiochemical Centre, Amersham, Bucks. The radioactivities of these sugars were about 5.5 and 50 μC/mg., respectively, estimated at infinite thickness after conversion into BaCO₃. They were diluted with inactive glucose (A.R.) or fructose (microbiological reagent) to give suitable radioactivities.

Paper-partition chromatography

This was carried out by the descending method using Whatman no. 1 filter paper and butanol:acetic acid:water (Partridge, 1948).

Samples of enzyme digests were inactivated by previously adding 5 μl. 0.01 M-HgCl₂ to the position on to which they were to be delivered (Bealing & Bacon, 1953).

Sugars were hydrolysed on the dried paper by spraying with an aqueous solution (8–10%, v/v) of yeast sucrase and drying at room temp. This was sufficient to hydrolyse entirely, to glucose and fructose, as much sucrose as would run satisfactorily as a discrete spot (i.e. about 400 μg. at room temp.).

Sugars were detected by the benzidine/trichloroacetic acid or phloroglucinol spraying reagents (Bacon & Edelman, 1951; and Horrocks & Manning, 1949, respectively).

Estimation of sugars from the paper

Sugar solutions were applied using an Agla Micrometer Syringe (Burroughs Wellcome and Co., London). After development, the paper was extracted with water at 75–80° for 15 min., shaken, and the resultant suspension filtered. Total fructose in a sample of the filtrate was estimated by a resorcinol/HCl method (see Bacon & Edelman, 1951), and reducing sugar by the method of Nelson (Somogyi, 1937; Nelson, 1944).

Estimation of radioactivity

Before and after development of the chromatogram, radioactivity in the sugar spots was estimated by applying a thin end-window Geiger-Müller counter, type EHM2 (General Electric Co., London), directly to the paper; the counts/min. were obtained by using a scaler, type 1009A (Dynatron Radio Ltd., Maidenhead). Daily variations in the counter were corrected by using a factor calculated from the counts/min. of a ¹⁴C-labelled plastic block of activity

Table 1. *Estimation of radioactivity from developed chromatograms of ¹⁴C-labelled sugars*

Sugar applied (μg.)	Activity estimated as described under Materials and Methods.					
	Before development			After development		
	Counts/min.	Counts/min./μg.		Counts/min.	Counts/min./μg.	
A. Glucose (spots counted entire on one surface)						
8.25	3038	368		2780	337	
16.5	6089	369		5460	331	
B. Glucose (spots cut into four pieces. Sums of pieces counted on one surface)						
50	297	5.9		326	6.5	
100	629	6.3		623	6.2	
150	982	6.5		930	6.2	
200	1297	6.5		1230	6.2	
	Average	6.3		Average	6.3	
C. Sucrose (spots cut into four pieces. Sums of pieces counted on both surfaces)						
	Surface I (a)	Surface II (b)	From (a) and (b)	Surface I (c)	Surface II (d)	From (c) and (d)
15.2	301	340	21.1	328	318	21.3
30.2	611	634	20.5	592	618	19.9
45.6	910	937	20.3	1043	924	21.5
60.8	1166	1229	19.7	1250	1220	20.3
76.0	1505	1509	19.9	1672	1511	20.9
	Average		20.3	Average		20.8

1.0 $\mu\text{C/g}$. Unless otherwise stated, radioactivities are expressed as counts/min., corrected for lost counts due to counter paralysis time and for background, estimated directly from the paper. The weight of the paper was about 9 mg./cm.²; no corrections were made for differences in self-absorption of the radiation caused by variations in paper thickness: the weights of twenty 4 cm.² samples lay between 34.1 and 37.3 mg., average 35.4 ± 0.19 mg.

The diameter of the applied spot was never greater than about 1 cm. (equivalent to a single application of 5 μl .); its radioactivity was estimated by a single count (or the average of single counts on both paper surfaces).

At first only those developed spots of diameter > 2 cm. were cut into pieces, each small enough to be covered by the counter end-window (diameter 2.3 cm.). It was found, however, that recovery after development was low (80–90 % of the counts/min. applied) unless all the spots were cut into at least four pieces (see Table 1 *A, B*). This practice was subsequently adopted. It was also considered desirable to count both surfaces of each chromatogram, taking the average as the final value, as differences between surfaces sometimes exceeded 10 % of the mean (Table 1, *C*); this was done only when the most accurate values obtainable were necessary, as the increased tedium of counting many spots each cut into several pieces was otherwise considered unjustified.

Counting periods varied from 1 min. (>1000 counts/min. source) to 5 min. (<100 counts/min. source). Recoveries after development nearly always lay between 90 and 110 % of the counts/min. applied, and usually between 95 and 105 %. Table 1 shows some results illustrating this method of estimation.

The considerable advantage of the method is its relative rapidity; it eliminates the necessity for elution from the paper and consequent difficulties in handling and counting very small quantities of material. Inaccuracies are normally insignificant and can be attributed to (*a*) the closeness of the end-window to the source; this also increases errors caused by variation in sensitivity over the surface of the window (Putman & Boxall, 1947), (*b*) occasional significant variations in activity between the two surfaces of a chromatogram and (*c*) slight variations in paper thickness.

Gradient-elution chromatography

The method is described by Bacon & Bell (1953) and by Alm, Williams & Tiselius (1952). It consists of eluting charcoal columns with solutions of progressively increasing ethanol concentration. The apparatus was arranged so that aqueous ethanol dropped into a reservoir, initially of distilled water, at the same rate as the solution in the latter passed through a charcoal-Celite column.

Activated Charcoal (British Drug Houses Ltd., London) was mixed with an equal weight of Celite 535 (Johns Manville Co., Ltd., London) (Whistler & Durso, 1950). The mixture was suspended in distilled water, filtered on a Büchner funnel, and dried at 105°. Columns were packed with the powder either dry or as a slurry in water. They were washed with distilled water until the effluent, at first acid, became neutral. Sugar solutions were then applied and fractions collected by a Shandon Automatic Fraction Collector (Shandon Scientific Co., London).

Gradient-elution chromatography was found to have greater resolving power for the sugar mixtures used than did the stepwise method described by Whistler & Durso (1950).

RESULTS

The action of mould sucrase

The results of the action of mould sucrase on sucrose are illustrated in Fig. 1. Some aspects of this reaction have been discussed in detail by Bealing & Bacon (1953). Table 2 shows the effect of adding glucose at the start of the reaction; both the formation of oligosaccharides and the disappearance of sucrose as estimated by combined fructose analysis were depressed (cf. Bealing, 1953). Addition of ¹⁴C-labelled glucose to the reaction mixture (Table 3) caused radioactivity to appear in the sucrose spot. Boiled enzyme induced no detectable reaction in a mixture of sucrose and radioactive glucose.

Table 2 shows also the effect of adding free fructose; at the concentration investigated this did not greatly affect the formation of oligosaccharides, but appeared to retard sucrose disappearance. The use of labelled fructose showed that this sugar when added at the start of the reaction, also caused the sucrose spot to become radioactive (Table 4).

Fig. 1. The action of mould sucrase on sucrose. Chromatogram of 5 μl . samples of a reaction mixture at 20° containing 50 mg. sucrose, 0.9 ml. 0.05M sodium acetate buffer, pH 5.0, and 0.1 ml. 10 % (w/v) 'Takadiastase'. FGSR, reference sugar mixture containing 50 μg . fructose, 50 μg . glucose, 100 μg . sucrose and 150 μg . raffinose; F, fructose; G, glucose; S, sucrose; R, raffinose; x, probably a disaccharide containing glucose residues; α, a trisaccharide containing G and F, β, probably a tetrasaccharide containing G and F.

Table 2. *Some effects caused by the presence of added glucose or fructose on the action of mould sucrase on sucrose*

Each reaction mixture at 20° contained 500 mg. sucrose and 2.5 ml. 2% (w/v) 'Takadiastase' in a total volume of 5.0 ml. 0.05M sodium acetate buffer, pH 5.0. The appropriate mixtures also contained 250 mg. monosaccharide. Sugars were estimated from paper chromatograms. Results are expressed as mg. combined fructose/ml.

Substrates	Sugars estimated	Time of incubation (hr.)		
		0	5	20
Sucrose	Sucrose	53.2	31.4	10.2
	Oligosaccharides	—	15.7	28.3
Sucrose + glucose	Sucrose	54.2	37.8	17.3
	Oligosaccharides	—	9.9	21.0
Sucrose + fructose	Sucrose	53.2	36.6	19.9
	Oligosaccharides	—	13.4	26.7

Table 3. *The action of mould sucrase on sucrose and radioactive glucose*

A. Total activities. The reaction mixture contained 17.8 mg. sucrose, 9.8 mg. radioactive glucose with total activity about 5 μ c, 0.1 ml. 0.05M sodium acetate buffer, pH 5.0, and 0.1 ml. enzyme preparation from *A. oryzae*. Incubation was carried out in an Agla syringe at 20°. Samples (3 μ l.) were delivered on to filter-paper sheets. Results are expressed as counts/min. in the sugar spot.

	Time of incubation (hr.)						
	2 min.	0.5	1	2	4.25	18	28
Before development (total applied to paper)	5171	5468	5162	5196	5245	5400	5700
After development							
Glucose spot	4761	4921	5101	4448	4792	4950	5550
Sucrose spot	33	369	357	485	494	67	21
Spot α	7	5	4	30	48	52	38
Spot α	0	5	10	25	65	47	18
Spot β	—	—	—	—	6	6	8
Total	4801	5300	5472	4988	5405	5122	5635

B. Specific activities. Same reaction mixture as for A. Values deduced from paper chromatogram analyses combined with above table. Results are expressed as counts/min./ μ g. of free or combined glucose.

Glucose spot	39	36	34	30	26	21	20
Sucrose spot	0.3	2.3	3.5	6.5	10	5.6	3.2
Spot α	—	1.4	1.3	2.2	4.2	6.7	16

Table 4. *The action of mould sucrase on sucrose and radioactive fructose*

A. Total activities. The reaction mixture contained 18 mg. sucrose, and 11.6 mg. radioactive fructose with total activity about 6 μ c. Experiment performed as that described in Table 3. Results are expressed as counts/min. in the sugar spot.

	Time of incubation (hr.)					
	5 min.	0.5	1	2	4.25	18
Before development (total applied to paper)	5384	5738	6083	5459	5953	6000
After development						
Fructose spot	5760	5430	5705	5470	5330	5070
Sucrose spot	26	80	144	252	357	576
Spot α	—	—	—	—	—	28
Spot α	9	3	13	11	40	74
Spot β	—	—	7	8	11	10
Total	5795	5513	5869	5741	5738	5758

B. Specific activities. Same reaction mixture as for A. Values deduced from paper chromatogram analyses combined with above table. Results are expressed as counts/min./ μ g. of free or combined fructose.

Fructose spot	36	34	33	31	29	24
Sucrose spot	0.2	0.7	1.4	3.0	6.0	14
Spot α	—	—	—	—	1.0	2.5

Table 5. *The identity of the radioactive sugars incorporated into oligosaccharides synthesized by mould sucrase*

The reaction mixture contained 15 mg. sucrose, 10 mg. radioactive glucose or fructose with total activity about $5 \mu\text{C}$, 0.12 ml. 0.05M sodium acetate buffer, pH 5.0, and 0.08 ml. enzyme preparation. Incubation was carried out in an Agla syringe at 20°. Samples (4 μl .) were delivered on to a filter-paper sheet after 2 hr. for the purpose of two-dimensional chromatography. Sugars were hydrolysed after the first development. No attempt at accurate quantitative estimation was made. Results are expressed as counts/min. in the sugar spot.

	Spot β	Spot α	Sucrose	Glucose	Fructose
Enzyme preparation from <i>P. spinulosum</i>					
Added radioactive glucose					
1st development	83	500	450	4800	—
2nd development					
Derived glucose	202	582	484	4800	—
Derived fructose	—	4	15	—	11
Enzyme preparation from <i>A. oryzae</i>					
Added radioactive fructose					
1st development	7	27	321	—	5250
2nd development					
Derived glucose	2	7	4	—	—
Derived fructose	3	12	298	—	4991

Table 6. *Investigation of the components giving rise to the 'sucrose' spot after incubation of mould sucrase with a mixture of sucrose and radioactive fructose*

For description of the experiment see text. Components A and B were separated by gradient elution.

	Total fructose content (mg.) (a)	Total glucose content (mg.) (b)	Ratio of glucose: fructose from (a) and (b) (c)	Counts/min. estimated from filter paper		
				Per μg . hexose (d)	Total, from (a) + (b) and (c) (e)	Fraction of total in mixture, from (e) (%)
Component A	5.7	0.03	0.00	15.9	91×10^3	92
Component B	7.8	7.8	1.0	0.5	7.8×10^3	8

Two-dimensional chromatography, with complete hydrolysis of the saccharides after the first development, showed that the incorporated activity was in the expected glucose or fructose parts of the molecules corresponding to the radioactive sugar added (Table 5).

The glucose-labelled material represented by the sucrose spot arising from the products of the enzymic action on a mixture of sucrose and labelled glucose was shown to be sucrose as follows. Syrup (137 mg.), consisting mainly of radioactive glucose, but contaminated with a little inactive fructose, was dissolved with sucrose (100 mg.) in 0.5 ml. 0.05M sodium acetate buffer, pH 5.0; 0.5 ml. mould enzyme was added and the mixture was incubated at 22°. After 2.25 hr. the tube containing the digest was placed in a boiling-water bath and left for 5 min. The sugars were separated by the method of Whistler & Durso (1950) using a charcoal-Celite column (34 $\times$ 300 mm.) with aqueous ethanol as eluent. The fractions containing material giving rise to a spot in the sucrose position on a paper chromatogram were combined, evaporated to dryness under reduced pressure, and crystallized from aqueous ethanol. The crystals were washed with ethanol and dried. The first crop weighed 33.1 mg. X-ray powder photographs of this material and of

a known sample of sucrose, crystallized in a similar manner, were indistinguishable. The activity of the labelled sucrose after crystallization and recrystallization was 33.2 and 33.3 counts/min./ μg ., respectively.

When labelled fructose was added to an enzyme-sucrose mixture, radioactivity became incorporated into the sucrose spot less rapidly than when labelled glucose was added under similar conditions. This activity, however, eventually reached a higher level (Tables 3 and 4). The activity was not due to incorporation of labelled fructose into sucrose, but to the formation of a sugar with similar R_f . This sugar, which was shown to consist only of fructose residues, was isolated and identified as follows.

A solution containing 50 mg. sucrose, 50 mg. radioactive fructose with total activity about 20 μC , 0.25 ml. 0.05M sodium acetate buffer, pH 5.0, and 0.25 ml. 2% (w/v) 'Takadiastase' was incubated at 20°. After 14 hr. 3 drops of 0.01M-HgCl₂ were added and the sugars separated on a charcoal-Celite column (34 $\times$ 300 mm.) using gradient elution with 75% (v/v) aqueous ethanol dropping into water (21.). Fractions were analysed both for sugar content and radioactivity. Appropriate fractions were combined and evaporated at reduced pressure to syrups of small volume.

Of the emergent sugars, two were investigated; component *A* which followed the monosaccharides, and component *B* which followed *A*. The syrups were made up to 2.6 ml. with water, and samples measured with an Agla syringe were chromatographed and analysed. Both sugars had the same *R_f* as sucrose on paper chromatograms. The results of the analyses are given in Table 6.

The action of yeast sucrase

The results of the action of yeast sucrase on sucrose are shown in Fig. 2. The effect of adding free glucose or fructose at the start of the reaction is shown in Table 7. Results of an experiment involving the use of labelled glucose are shown in Table 8; the activity values for sucrose in this table, although positive, showed no trend during the course of the reaction and were almost certainly due to slight trailing of the labelled glucose. There was significant incorporation of activity into spot I.

Similar experiments using labelled fructose demonstrated the incorporation of activity into the sucrose spot, but into no other oligosaccharides (Table 9).

Two-dimensional chromatography, with complete hydrolysis of the saccharides after the first development, showed that the activity was in the expected glucose or fructose parts of the molecules corresponding to the radioactive sugar added (Table 10).

That the formation of the material represented by spot I was entirely dependent on the presence of free hexose as well as sucrose was shown by eliminating hexose from the reaction mixture immediately it was liberated, by adding *C. krusei*, a yeast which lacks sucrase and thus will not ferment sucrose. Under these circumstances the action of yeast sucrase on sucrose did not give rise to free glucose or fructose (except in traces), or to spot I; spots II and III were formed in the usual manner. When *C. krusei* was added to products of the action of the enzyme on

sucrose, namely glucose, fructose, sucrose, and I, II and III, only the monosaccharides disappeared; I was not attacked. These results are shown diagrammatically in Fig. 3.

As in the case of mould sucrase, the activity incorporated into the sucrose spot in the presence of radioactive fructose (Table 9) could be ascribed to the formation of a sugar (component *C*, Table 11) containing no glucose residues. This and a second sugar (component *D*) were separated, in a manner

Fig. 2. The action of yeast sucrase on sucrose. Chromatogram of 2 μ l. samples of a reaction mixture at 20° containing 400 mg. sucrose, 2.0 ml. 0.05 M sodium acetate buffer, pH 5.0, and 0.4 ml. 1% (v/v) yeast sucrase. I, probably a disaccharide; II and III, trisaccharides. I, II and III contain both glucose and fructose. Other symbols as in Fig. 1.

Table 7. *Some effects caused by the presence of added glucose or fructose on the action of yeast sucrase on sucrose*

The experiment was performed as described in Table 2, except that the enzyme solution used was 1% (v/v) yeast sucrase. Results are expressed as mg. combined fructose/ml.

Substrates	Sugars estimated	Time of incubation (min.)			
		0	20	40	90
Sucrose	Sucrose	49.5	24.8	10.3	1.4
	Oligosaccharides I	—	0.6	0.6	0.3
	II + III	—	3.6	2.8	0.8
Sucrose + glucose	Sucrose	51.0	28.0	15.1	3.0
	Oligosaccharides I	—	1.3	1.8	1.5
	II + III	—	2.6	3.4	1.7
Sucrose + fructose	Sucrose	50.5	29.8	16.6	5.0
	Oligosaccharides I	—	0.6	0.6	0.6
	II + III	—	2.8	3.1	1.5

Table 8. *The action of yeast sucrase on sucrose and radioactive glucose*

A. Total activities. The reaction mixture contained 40 mg. sucrose, 19.9 mg. radioactive glucose with total activity about 10 μ c, 0.175 ml. 0.04M sodium acetate buffer, pH 5.0, and 0.025 ml. yeast sucrase. Incubation was carried out in an Agla syringe at 20°. Samples (2 μ l.) were delivered on to filter-paper sheets. Results are expressed as counts/min. in the sugar spot.

After development	Time of incubation (min.)				
	2	6	11	15	60
Glucose spot	5900	5820	5470	5720	6060
Sucrose spot	37	29	34	16	23
Spot I	183	227	152	113	68
Spot II	0	8	2	4	1
Spot III	12	11	17	8	5
Total	6132	6095	5675	5861	6157

B. Specific activities. Same reaction mixture as for A. Values deduced from paper chromatogram analyses combined with above table. Results are expressed as counts/min./ μ g. of free or combined glucose.

Glucose spot (initial specific activity 36)	—	23.5	20.5	—	18
Spot I*	—	25.4	25.3	—	—

* The glucose content of this material was deduced from its fructose analysis, assuming it to be a fructosyl-glucose disaccharide.

Table 9. *The action of yeast sucrase on sucrose and radioactive fructose*

A. Total activities. The reaction mixture contained 42 mg. sucrose, and 20 mg. radioactive fructose with total activity about 10 μ c. Experiment performed as described in Table 8. Results are expressed as counts/min. in the sugar spot.

	Time of incubation (min.)				
	2	6	11	15	60
Before development (total applied to paper)	6757	6494	6888	6154	6910
After development					
Fructose spot	6228	5673	5882	5796	6555
Sucrose spot	76	134	147	119	57
Spot I	6	7	15	11	5
Spot II	8	14	12	11	5
Spot III	13	17	16	10	12
Total	6331	5845	6072	5947	6634

B. Specific activities. Same reaction mixture as for A. Values deduced from paper chromatogram analyses, combined with above table. Results are expressed as counts/min./ μ g. of free or combined fructose.

Fructose spot (initial specific activity 36)	—	23.3	21.4	—	18
Sucrose spot	—	2.0	5.4	—	—

Table 10. *The identity of the radioactive sugars incorporated into oligosaccharides synthesized by yeast sucrase*

Two-dimensional chromatograms were developed using samples from the experiments described in Tables 8 and 9. Sugars were hydrolysed after first development. No attempt at accurate quantitative estimation was made. Results are expressed as counts/min. in the sugar spot.

Added radioactive glucose (10 min. sample)	Spots II and III	Spot I	Sucrose	Glucose	Fructose
1st development	6	170	20	4920	-2
2nd development					
Derived glucose	2	156	14	4255	—
Derived fructose	-3	0	-3	5	-5
Added radioactive fructose (5 min. sample)					
1st development	2	16	94	—	4326
2nd development					
Derived glucose	1	8	-2	—	—
Derived fructose	6	14	72	—	3847

Table 11. Investigation of the components giving rise to the 'sucrose' spot after incubation of yeast sucrase with a mixture of sucrose and radioactive fructose

For description of the experiment see text. Components C and D were separated by gradient elution.

	Total fructose content (mg.) (a)	Total glucose content (mg.) (b)	Ratio of glucose: fructose, from (a) and (b) (c)	Counts/min. estimated from filter paper		
				Per μ g. hexose (d)	Total, from (a) + (b) and (d) (e)	Fraction of total in mixture, from (e) (%)
Component C	2.6	0.01	0.00	16.7	43.5×10^3	89
Component D	3.8	3.4	0.9	0.8	5.7×10^3	11

Fig. 3. The effect of *C. krusei* on the action of yeast sucrase on sucrose. Diagram of chromatogram. A, solution containing 2.0 ml. 3% (w/v) sucrose, 1.0 ml. H_2O , and 0.1 ml. 0.5% (v/v) yeast sucrase. B, as A but 1.0 ml. of a boiled suspension of *C. krusei* substituted for 1.0 ml. water. C, as B but with living *C. krusei* substituted for the boiled yeast. D, solution A boiled after 12 hr. incubation, cooled, and 1.0 ml. added to 1.0 ml. suspension of living *C. krusei*. Solutions incubated 12 hr. at 30°. Other symbols as in Figs. 1 and 2.

similar to that already described for the mould enzyme reaction, from a 1 hr. reaction mixture at 20° containing 50 mg. sucrose, 50 mg. radioactive fructose with total activity about $20 \mu C$, 0.2 ml. 0.05M sodium acetate buffer, pH 5.0, and 0.1 ml. 4% (v/v) yeast sucrase; the isolated sugars had R_f similar to sucrose. The results of the analyses are given in Table 11.

DISCUSSION

The experiments described in the previous section confirm the findings, first noted by Edelman (1950), Bacon & Edelman (1950), Blanchard & Albon

(1950) and Bacon (1952), that sucrase preparations from both yeasts and moulds are capable of synthesizing oligo- and hetero-saccharides, apparently by the transfer of fructose residues from sucrose to suitable acceptors. Bealing & Bacon (1953) have suggested that all previously described invertase preparations from moulds have had similar transferring ability and Fischer *et al.* (1951), using electrophoretically purified yeast sucrase, found it capable of transferring fructose residues. Therefore, although little attempt was made to purify the enzyme preparations, it is considered justifiable to consider the results described here as of general application to mould and yeast sucrases. The term 'invertase' has come to be associated with a simple hydrolysis of sucrose to glucose and fructose; it is now suggested that the term 'sucrase' is more appropriate in view of the many types of reaction which these preparations are capable of catalysing using sucrose as substrate.

Sucrases have been classified into two major groups, viz. glucosaccharases (α -glucosidases, attacking sucrose at the glucose moiety) and fructosaccharases (β -fructofuranosidases, attacking at the fructose moiety). An account of the development of this classification has been given by Bealing & Bacon (1953); it is sufficient to say here that a major factor was the observation that some sucrases were inhibited by glucose and some by fructose. It has been generally agreed that the former group contained, among others, sucrases derived from moulds, and the latter group those derived from yeasts (e.g. see Neuberger & Mandl, 1950).

Inhibition by added glucose and fructose of sucrose breakdown certainly seems to occur (Tables 2 and 7), but the experiments described in this paper show that the effect of adding these sugars is more complex than was formerly believed and that, apart from any physical effect they may exert upon the enzyme, they enter into the reactions in characteristic ways. Although these experiments have indicated major differences between mould and yeast sucrases, they have further provided evidence that the previous classification of these into glucosaccharase and fructosaccharase is not

justified (cf. Bealing & Bacon, 1953; Bealing, 1953). They have also elucidated the hexose-transfer mechanisms involved in the formation of some of the oligosaccharides.

Radioactive-glucose experiments

A major difference between mould and yeast sucrases is demonstrated by the respective actions of these enzymes on mixtures of sucrose and radioactive glucose. Radioactivity was rapidly incorporated as combined glucose into oligosaccharides in each case.

With mould enzyme, sucrose was the first oligosaccharide to become labelled, indicating that the action responsible was a transfructosylation which may be considered to occur thus:

Combining the equations we have

where $[G \rightarrow \leftarrow F]$ denotes sucrose, and $[G \rightarrow H]$ glucose, the arrow indicating the reducing group and '*' indicating radioactivity. In these equations the fructosyl and not the fructoside group is represented as being transferred: there is some evidence that this alternative is correct (Koshland & Stein, 1953). Later in the reaction the saccharides of higher molecular weight (compounds α and β , Fig. 1) which are formed become labelled in their glucose components. This supports the theory that these compounds, known to have fructose:glucose ratios greater than unity and to be composed entirely of these two sugar residues, are formed by further fructose transfer, e.g.

a hydrogen atom on the fructoside moiety of sucrose being replaced by a fructosyl group (Fischer *et al.* 1951; Edelman & Bacon, 1951; Wallenfels & Bernt, 1952; Pazur, 1952; Bealing & Bacon, 1953). Table 3 shows this effect to some extent, but it is better demonstrated in Table 5. In the latter an enzyme preparation from *P. spinulosum* was used; this was known to have higher transferring capacity to sugar (as evidenced by oligosaccharide formation) relative to water (hydrolysis) than have preparations from *A. oryzae* (Bealing & Bacon, 1953). The structure of the trisaccharide α has recently been established as *O*- β -D-fructofuranosyl-(2 $\rightarrow$ 1)-*O*- β -D-fructofuranosyl-(2 $\rightarrow$ 1)- α -D-glucopyranoside (Bacon & Bell, 1953). The fructose-transferring capacity of mould sucrose has been used in this laboratory to synthesize glucose-labelled sucrose on a preparative scale from inactive sucrose and radio-

active glucose (L. H. May, unpublished results). Spot α (Fig. 1, Tables 3 and 4) was possibly a disaccharide formed by the condensation of two molecules of free glucose; it was formed only in very small amount.

Yeast sucrose did not catalyse the incorporation of free glucose into sucrose, but into a reducing oligosaccharide designated I (Table 8A). This was almost certainly a disaccharide in which a hydrogen atom from one of the non-reducing hydroxyl groups of a glucose molecule was exchanged for a fructosyl group. This indicates that the transfer in this case proceeds thus:

The position of attachment of fructose to the glucose molecule is not known but, in view of the findings of Whelan & Jones (1953) who isolated a similar disaccharide from the products of the action of yeast sucrose on a mixture of *O*- β -D-methyl fructofuranoside and glucose, it is probably at $C_{(6)}$. The overall reaction was thus:

The absence of radioactivity in the reducing disaccharide when labelled fructose was added under comparable conditions (Table 9), shows that a fructose and not a glucose radical was transferred to give I. This explains the characteristically later appearance of this spot than spots II and III during the normal reaction of yeast sucrose with sucrose, and its earlier appearance and enhanced increase in the presence of added glucose at the start of the reaction (Table 7). The specific activity values given in section B of Table 8 provide further evidence supporting the suggested mechanism of formation of I. Thus the specific activity of the glucose moiety of I at any time should have been not less than that of the free glucose at the same instant. It could be expected to have been rather higher as the specific activity of the free glucose progressively decreased owing to the liberation of free glucose from sucrose.

Radioactive-fructose experiments

The addition of fructose to sucrose-sucrase mixtures had less effect upon the production of higher oligosaccharides than did that of glucose under similar conditions (Tables 2 and 7). It was noticeable, however, that there was considerable inhibition of sucrose disappearance as estimated by fructose content of the sucrose spot. Addition of labelled fructose showed that activity became incorporated into the sucrose spot under the influence of both types of enzyme (Tables 4 and 9). This active material was entirely hydrolysed by sucrose to give only inactive glucose and labelled fructose.

Fructose-labelled sucrose could not be formed by fructose transfer from sucrose under these conditions, and the possibilities remained that glucose transfer from sucrose to free fructose had occurred or that a compound other than sucrose was present in the sucrose spot. The following evidence indicates the latter alternative to be correct.

It was noticeable in the absence of added glucose or fructose that with either type of enzyme the sucrose spot appeared more yellow in colour (as shown by the benzidine/trichloroacetic acid spraying reagent) towards the end of the reaction than at the start. When free fructose was added to the reaction mixture this effect was greatly increased. These observations indicated that the fructose:glucose ratio became greater than unity as the reaction proceeded, and this was supported, in the case of the mould enzyme, by analytical results. It appeared, therefore, that compounds with a greater fructose content than sucrose but with the same R_f were being formed. By the method of gradient elution it was eventually found possible to separate two sugars with R_f similar to sucrose from the products of both the mould and yeast enzyme reactions on mixtures of sucrose and labelled fructose (see Tables 6 and 11). The two sugars emerged from the chromatographic column very close together and were possibly not completely separated. One of the sugars of each pair (component *A*, mould enzyme; component *C*, yeast enzyme) contained only fructose units, while the other (component *B*, mould enzyme; component *D*, yeast enzyme) contained equal proportions of fructose and glucose and was almost certainly sucrose. In both cases the glucose-free component contained about 90 % of the total activity. Both components were hydrolysed by yeast sucrase entirely to monosaccharide.

From these results it seems very probable that the activity incorporated into the 'sucrose' spot in the presence of labelled fructose was due in each case to the formation of a fructose disaccharide with the same R_f (under the chromatographic conditions used) as sucrose. As neither mould nor yeast enzyme had any relevant effect upon a mixture of glucose and fructose, difructose formation is considered to take place by transfer of fructose from sucrose to free fructose (cf. Bealing, 1953).

Further support for this hypothesis was provided by the results of experiments with raffinose and free fructose, possible confusion of sucrose with other sugars of similar R_f thus being eliminated. Of the many compounds formed when using a preparation from *P. spinulosum*, one was produced in relatively small amount and was shown by two-dimensional chromatography to contain only fructose residues. Where labelled fructose was added this compound became radioactive. Since raffinose is known to act as fructose donor under the conditions of these

experiments, this compound is considered to have been a difructose.

The specific activity values shown in Tables 3, 4 and 9 can be explained on the basis of difructose formation. As the quantity of material in the 'sucrose' spot was deduced from fructose analysis assuming a glucose:fructose ratio of unity, weight values were increasingly overestimated during the reaction owing to the progressive formation of difructose. Thus the specific activities attributed to sucrose in Table 3 (labelled-glucose experiment) decreased towards the end of the reaction, and were probably underestimated at all stages. The true value for glucose combined in sucrose or other saccharides would be expected to approach that of the free glucose and would eventually be in equilibrium with it. Compound α did reach a high value, as a true estimate of the glucose content was probably obtained here (glucose:fructose ratio = 0.5).

Fructose turnover

The true capacity of a sucrase for the turnover of fructose radicals can be estimated only when the rates of all the individual reactions which constitute its action on sucrose are fully known. These include transfer from sucrose to the elements of water (hydrolysis), to liberated glucose and fructose, to sucrose, and to oligosaccharides so formed, some or all of which may act as acceptors; in addition, these oligosaccharides may act as fructose donors. It is virtually certain that the rates of these reactions vary widely. An estimate of fructose transfer can be obtained, however, from experiments of the type already described using radioactive sugars. In the experiment described in Table 3, in which the reaction mixture contained about 0.5M sucrose and 0.5M glucose, total transfer per ml. of the reaction mixture in the first 30 min. (during which time the sucrose concentration fell only by about 8 % of its initial value) was made up of transfer to (a) the elements of water, 2.5 mg. by direct fructose estimation, (b) free glucose, 2.4 mg. from total radioactivity incorporated into sucrose divided by the mean specific activity of free glucose during this period and (c) sucrose, 0.7 mg. by estimation of fructose in spot α (glucose:fructose ratio = 0.5). All these values are necessarily approximate. Assuming that transfer to free fructose, and from difructose, compound α , and labelled sucrose was negligible during this period, the total transfer was about 5.6 mg. fructose/ml.; of this, about 45 % appeared free and 13 % was incorporated into oligosaccharide of greater molecular weight than sucrose.

In the case of added radioactive fructose (experiment described in Table 4, about 0.5M sucrose and 0.5M fructose) the corresponding fructose-transfer values per ml. for the first 30 min. (sucrose concentration reduced by about 12 % of its initial value)

were (a) to the elements of water, 3.0 mg., (b) to free fructose, 0.7 mg. and (c) to sucrose, 2.7 mg. The total transfer was thus about 6.4 mg./ml., of which 47 % appeared as free fructose and 42 % formed oligosaccharide. There may be a larger error here as it was not possible to estimate transfer to the free glucose liberated during the reaction. Although the concentration of glucose was low (about 0.03M at 30 min.), its affinity for the enzyme is unknown.

Although the total transfer values in both cases are of roughly the same order, it does appear that the presence of free glucose may cause some inhibition as compared to the presence of free fructose. There is very marked competition for transferred fructose between glucose and sucrose, this being for very different groups on the respective molecules, as the glucose accepts on its reducing group, whereas sucrose accepts on position 1 of the fructose moiety. It is interesting to note that the formation of levan from sucrose by levan sucrase is also competitively inhibited by free glucose (Hestrin & Avineri-Shapiro, 1944).

It is obvious from considerations mentioned above that liberation of free reducing sugar may give no indication of the total capacity of a sucrase to transfer fructose residues.

General considerations

The transfer of fructose radicals which has been discussed above is presumably effected by means of an intermediary fructose-enzyme complex; from the evidence it appears that the enzymes are incapable of catalysing glucose transfer (contrast the findings of White & Maher (1953), who used honey sucrase). Both types of enzyme may therefore be considered to be β -fructofuranosidases in that the fructofuranose group is more intimately involved with the enzyme during the cleavage of sucrose. In common with other workers it is shown that several sugars can act as fructose acceptors; the compounds formed, however, may differ according to the enzyme used.

It was considered possible that a particular case of the general reaction might be the enzymic net synthesis of sucrose by transfer of fructosyl radicals from donors other than sucrose to glucose under the influence of mould sucrase. However, when 'Takadiastase' was allowed to act upon mixtures of glucose and inulin or *O*- β -D-methyl fructofuranoside (prepared by the method of Bacon, 1952), although free fructose was liberated, there was no indication of sucrose or oligosaccharide synthesis. This failure could be attributed to substrate specificity (implying that fructose liberation and transfer are due to different enzymes under these conditions), or to a high breakdown:synthesis ratio of oligosaccharides, or to thermodynamic considerations. Should the standard free energy of hydrolysis

of sucrose be considerably greater than that of the fructoside, the number of sucrose linkages formed would be very small; under the conditions used this could be increased only by effective coupling between hydrolysis and fructose transfer to glucose.

The significance of fructose transfer in metabolism is as yet unknown. There appears to be correlation between the mechanisms described here and the wide occurrence in the plant kingdom of fructose polymers ranging from sucrose, through oligosaccharides, to polyfructoses (Bacon & Edelman, 1951; Porter & Edelman, 1952). Fructose-transferring enzymes do exist in plants, e.g. Jerusalem artichoke (Edelman & Bacon, 1951), and barley (Edelman, unpublished experiments). Similar glycosidic linkages are present in the naturally occurring plant fructosans and in products of mould and yeast sucraes; thus the 2:6-fructofuranoside linkage is found in kestose (spot III of the yeast reaction, see Albon, Bell, Blanchard, Gross & Rundell, 1953) and in the levan series of fructose polymers (Challinor, Haworth & Hirst, 1934; Laidlaw & Reid, 1951), while the 2:1 linkage occurs in spot α of the mould reaction (Bacon & Bell, 1953) and in the inulin series (Hirst, McGilvray & Percival, 1950; Bacon & Edelman, 1951).

SUMMARY

1. Free glucose and fructose enter into the action of mould and yeast sucraes on sucrose in characteristic ways. Previous reports of inhibition of these enzymes by free hexose may be partly or wholly explained on this basis.
2. The use of radioactive sugars showed that, under the influence of mould sucraes, free glucose and the glucose combined in sucrose are exchangeable by means of the transfer of fructose residues. In this way glucose-labelled sucrose has been obtained. Further fructose transfer gives rise to glucose-labelled saccharides of greater molecular weight.
3. Similar experiments have shown that yeast sucrase will transfer fructose residues from sucrose to free glucose to form a reducing compound, probably a fructosyl-glucose disaccharide.
4. Both types of enzyme transfer fructose residues from sucrose to free fructose to give a glucose-free sugar, probably a difructose.
5. Mould and yeast sucraes are both β -fructofuranosidases but differ in their specificity towards fructose acceptors.
6. The capacity of sucraes for fructose turnover cannot be deduced from hexose liberation.
7. Some correlation between fructose transfer and naturally occurring fructose polymers has been drawn.

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Chromatographic Determination of Cystine as Cysteic Acid

BY E. SCHRAM, S. MOORE* AND E. J. BIGWOOD

Laboratory of Biochemistry, Faculty of Medicine, University of Brussels

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The determination of the cystine content of proteins is frequently complicated by the instability of cystine and cysteine during protein hydrolysis, particularly in the presence of carbohydrate (cf. Martin & Synge, 1945; Block & Bolling, 1951). Oxidation of the cystine and cysteine residues in the intact protein can convert the amino acids into cysteic acid, which is stable under the conditions of acid hydrolysis. The present method is based upon this step, followed by the quantitative determination of cysteic acid by ion-exchange chromatography on columns of Dowex-2. The chromatographic identification of cysteic acid as the end product of the oxidation contributes to the specificity of the method. The procedure does not distinguish between cystine and cysteine residues in the protein.

* Visiting Professor (Francoqui Chair) 1950-51. Member of the Rockefeller Institute for Medical Research, New York.

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The conditions of Toennies & Homiller (1942) for the oxidation of the sulphur-containing amino acids by performic acid have been applied to proteins in several investigations. Toennies (1942) oxidized casein to convert methionine into the sulphone and observed the disappearance of part of the cystine and all of the tryptophan. Sanger (1949) used the reaction to split the —S—S— bridges of insulin as a first step in the investigation of the structure of the hormone. The reaction has been applied to oxytocin by Mueller, Pierce, Davoll & du Vigneaud (1951). The oxidation of wool by performic acid has been studied by Blackburn & Lowther (1951).

A quantitative study of the yield from the reaction requires an accurate method for the determination of cysteic acid in protein hydrolysates. Paper chromatograms have been used qualitatively to identify cysteic acid after its formation from