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Sucrose Formation by Taka-diastase: Action of the Enzyme on Methyl β-Fructofuranoside and Raffinose

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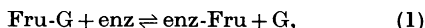
Invertase preparations from several sources (see Bacon, 1954, for summary; Allen & Bacon, 1956) have been shown to possess transfructosylase activity. Fischer, Kohtès & Fellig (1951) first suggested that this could be explained by a reaction mechanism involving a fructosyl–enzyme intermediate:



$$\text{Fru-G} + \text{enz} \rightleftharpoons \text{enz-Fru} + \text{G}, \qquad (1)$$

$$\text{enz-Fru} + \text{R}\cdot\text{OH} \rightleftharpoons \text{R}\cdot\text{O-Fru} + \text{enz}, \qquad (2)$$

$$\text{enz-Fru} + \text{H}_2\text{O} \rightleftharpoons \text{Fru} + \text{enz}, \qquad (3)$$

equation (2) representing 'transfer' and equation (3) 'hydrolysis'. Similar mechanisms have been proposed for other transglycosylases (see review by Edelman, 1956).

The fructosyl radical is usually transferred to a primary alcoholic group in the acceptor, the one notable exception being the transfer to the reducing group of glucose, which accounts for a large part of the total transferring activity of mould invertase in sucrose solution; by this action sucrose is resynthesized, as may be demonstrated by the use of radioactive glucose (Edelman & Bealing, 1953; Edelman, 1954). The failure of yeast invertase to bring about resynthesis of sucrose (Edelman, 1954) could be due to differences in the stereochemical specificity of the enzyme, or perhaps to the failure of the hypothetical fructosyl–enzyme compound to preserve the 'high-energy' fructoside bond of sucrose (cf. Hestrin, Feingold & Avigad, 1956).

Edelman (1954) mentioned, without giving details, that when sucrose was replaced by methyl β-fructoside as substrate for mould invertase, no sucrose or other oligosaccharides were formed in the presence of glucose. Since this observation raised doubts about the validity of the fructosyl–enzyme hypothesis (cf. Edelman, 1956), we thought it worth while to investigate it more fully, and also to see whether the enzyme would synthesize sucrose by fructose transfer to glucose when raffinose was the substrate. With methyl β-fructoside as substrate no sucrose formation was detected. With raffinose, sucrose was formed, and it was shown by the use of radioactive glucose that this arose by transfructosylation, not by the removal of the galactose residue from the trisaccharide.

In the following account we include details of the preparation of methyl β-fructoside by the action of yeast invertase on solutions of sucrose and methanol, a preliminary account of which has been published (Bacon, 1952).

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EXPERIMENTAL

Methods

The analytical methods used were essentially those described by Bacon (1954). The handling and counting of radioactive glucose followed that described by Edelman (1954); all the counts given are corrected for background. Uniformly labelled [^{14}C]glucose, from the Radiochemical Centre, Amersham, Bucks (1·3 mg. containing 0·1 mc), was dissolved in 0·5 ml. of 2% (w/v) glucose solution and was purified as described by Allen & Bacon (1956) before use.

In addition, the test of Raybin (1933) for sucrose and some of its derivatives was adapted for use on paper chromatograms: 15 mg. of diazouracil was dissolved in 10 ml. of 0·1 N-NaOH. The solution, which changed in colour from red to yellow-green within 30–40 sec., was sprayed at once on the chromatogram. This was then partly dried by heating in an oven at 60–80° for 3 min., dipped through 2% aqueous $MgCl_2$ and blotted between filter papers. Sucrose, raffinose,

stachyose and 6^G-β-fructosylsucrose [O-β-D-fructofuranosyl-(2→6)-O-α-D-glucopyranosyl-(1→2) β-D-fructofuranoside] gave a blue spot on a colourless background, stable for one day; about 100 µg. of material/spot was required. Glucose, fructose, maltose and melibiose gave no reaction.

Incubation with Taka-diastase. A preparation of Taka-diastase (Parke, Davis and Co. Ltd., London) was dissolved in water and dialysed for 48 hr. in the presence of $CHCl_3$ against running tap water. In all experiments, unless otherwise stated, a 20% (w/v) solution of the Taka-diastase preparation was made, and an equal volume of the dialysed solution was added to the buffered solution of sugars. (Because the Taka-diastase preparation contains large amounts of lactose the concentration of dry matter in the enzyme solution falls during dialysis to about 5% of its initial value; cf. Bealing & Bacon, 1953.) Incubations were performed at room temperature (16–20°) in glass-stoppered tubes. Chloroform was added if the time of incubation exceeded 10 hr. The initial concentration of all sugars was 10% (w/v) unless otherwise stated, and the pH was maintained at 5·0 by the presence of 0·05M-sodium acetate–acetic acid buffer. Samples for paper chromatography were inactivated by placing them on paper pretreated with $HgCl_2$; larger samples were heated for 15 min. in a boiling-water bath.

Materials

Sugars. Glucose, fructose, sucrose, maltose and raffinose were commercial samples. Stachyose was prepared by Dr F. J. Bealing (Bealing, 1953), and 6^G-β-fructosylsucrose and 6-β-fructosylglucose were isolated as described by Bacon (1954).

Methyl β-D-fructofuranoside. Preliminary investigations of the action of yeast invertase on solutions containing sucrose and methanol led to the selection of the following conditions for the large-scale preparation of methyl β-fructoside: 8·2 g. of sucrose (A.R.) was dissolved in 16 ml. of water and 80 ml. of 40% (w/v) aqueous methanol added. To the mixture was added 4·0 ml. of Invertase Concentrate (British Drug Houses Ltd.), which had been dialysed for 48 hr. against running tap water and diluted tenfold with 0·25M-sodium acetate–acetic acid buffer, and the reaction allowed to proceed at room temperature for a time determined by preliminary tests of the enzymic activity (usually about 5 hr.). Under these conditions about 2·5 times as much fructose is transferred to methanol as is liberated by hydrolysis. The reaction was stopped by adding 10 ml. of 0·1M-$HgCl_2$, and after neutralization with 0·1N-NaOH the solution was evaporated to dryness *in vacuo* with a bath temperature not exceeding 50°.

A typical separation of the methyl β-fructoside was as follows: the residue from the evaporation of the incubation mixture, corresponding to 26 g. of initial sucrose, was dissolved in 125 ml. of water and applied to a large Active Carbon no. 130–Celite column (300 g.:300 g.), which had been washed previously with 10 l. of water, 3 l. of 15% (v/v) ethanol and 10 l. of water in succession. The sugars were eluted with a gradient made by dropping 50% (v/v) ethanol into 10 l. of water. Fractions of about 100 ml. were collected and examined by paper chromatography. They showed fructose and glucose emerging after 1600 ml. and the methyl β-fructoside following immediately after, but completely separated from them, at 2500 ml. The methyl β-fructoside was contained in 615 ml. of effluent, and was separated by some 300 ml. from the next sugar to emerge (disaccharide). On evaporation to dryness 5·15 g. of a slightly mobile syrup was obtained, having $[\alpha]_D^{18} -51°$ (c, 5·15; 2 dm. tube). Paper chromatography showed a single component, and the material was used for enzyme experiments without any further purification. A preliminary characterization of the compound has already been given (Bacon, 1952); its complete characterization will be the subject of a separate communication (Bell & Bacon, 1957).

RESULTS

Action of Taka-diastase on methyl β-fructoside with and without glucose

When Taka-diastase was incubated with methyl β-fructoside alone, within a few hours a new compound could be detected on paper chromatograms. It ran in the glucose position, but the colour formed with benzidine–trichloroacetic acid was yellow, indicating a sugar comprising only fructose residues. When glucose was present there appeared two additional substances, one with the properties of 6-β-fructosylglucose (component I; Bacon, 1954), the other resembling isomaltose. The latter was also produced when Taka-diastase was incubated with glucose alone.

In order to obtain more information about the sugars formed 1·5 g. each of methyl β-fructoside and glucose were incubated with Taka-diastase for 100 hr. and the resulting mixture was analysed by gradient elution from a charcoal–Celite column. After glucose, fructose and methyl β-fructoside there emerged, incompletely separated, four other sugars (*A*–*D*). *A* and *B* appeared on paper chromatograms near the glucose position; *C* corresponded with 6-β-fructosylglucose and *D* with isomaltose.

A and *B* were non-reducing, and each yielded fructose as the only sugar on hydrolysis with yeast invertase, or with 0·5% oxalic acid at 100° for 25 min. Substance *C* yielded fructose and glucose in approximately equal amounts on hydrolysis with yeast invertase or oxalic acid, but was found to have associated with it a small proportion of a sugar of the same R_F, resistant to oxalic acid and containing no ketose. A total of 13 mg. of *C* was obtained after removal of small amounts of contaminating substances by a second elution from charcoal–Celite.

To gather more evidence for the transferring action of Taka-diastase, experiments with radioactive glucose were carried out. A solution (total volume 0·25 ml.) containing 0·1 ml. of 20% (w/v) Taka-diastase, 10 mg. of methyl β-fructoside and 10·5 mg. (5 µc) of radioactive glucose was incubated for 240 hr. at room temperature. The results of this experiment are shown in Table 1. They demonstrate the progressive incorporation of labelled glucose into the fructosylglucose spot during the first 90 hr. Radioautographs indicated that the amount of

radioactive fructosylglucose remained more or less constant between 100 and 240 hr. of incubation. After 90 hr. radioactivity was detected in the isomaltose position, and increased during the following 100 hr. No radioactivity was detected in the sucrose position at any stage.

Action of Taka-diastase on raffinose with and without glucose

When incubated with raffinose alone Taka-diastase formed fructose, melibiose, another disaccharide (probably a difructose; cf. Bealing, 1953) in the sucrose position, and a substance with R_F less than that of raffinose. When glucose was present the chromatograms showed two disaccharide spots other than melibiose. That in the sucrose position now seemed to contain glucose and was considered to be non-reducing on the basis of its reaction with the alkaline silver nitrate spray of Trevelyan, Procter & Harrison (1950).

In order to obtain larger amounts of these substances Taka-diastase was incubated with 2 g. each of raffinose and glucose for 100 hr. and the resulting mixture fractionated by gradient elution from charcoal–Celite. The order of sugars as they emerged was: (1) glucose, (2) fructose, (3) melibiose, (4, 5) two disaccharides E and F, incompletely separated, (6) raffinose and (7) probably a tetrasaccharide (cf. Pazur, 1952; Bealing, 1953). The fractions containing E and F were bulked (21·6 mg. of dry residue) and an unsuccessful attempt was made to separate them by a further elution from charcoal–Celite. Complete separation was achieved on a cellulose column, with aqueous butanol as solvent. Each yielded glucose and fructose on hydrolysis with yeast invertase or dilute oxalic acid; one corresponded chromatographically with sucrose, the other with 6-β-fructosylglucose. Although the latter could have been formed only by the transfer of the fructose residue from raffinose to free glucose, the sucrose might not have arisen in this way, but simply by the removal of the galactose residue from raffinose. An attempt was therefore made to distinguish between these alternatives by the use of radioactive glucose.

Taka-diastase, raffinose, glucose and radioactive glucose (5 μc) were incubated for 120 hr. The course of the reaction was followed by spotting 3 μl. samples on paper chromatograms at suitable intervals. In a second experiment, Taka-diastase was incubated with the same amounts of raffinose and radioactive glucose, but without adding inactive glucose. The results of both experiments are shown in Table 2. At very low glucose concentrations (0·3 % in Expt. 2), a rapid and distinct incorporation of radioactivity into the sucrose spot was observed which reached its maximum at 27 hr. In the presence of 5 % glucose, however, the incorporation of labelled glucose into the sucrose position appeared to be less rapid, and the amount of radioactivity was comparatively small. On the other hand, the examination of the fructosylglucose spots revealed

Table 1. *Action of Taka-diastase on methyl β-fructoside in the presence of radioactive glucose*

The incubation mixture described in the text was sampled at intervals, 3 μl. samples being placed on chromatograms. After development the radioactivity of each spot was measured by direct counting from the paper. The glucose spot gave about 5100 counts/min. in this experiment.

	Time of incubation (hr.)								
	0·1	2	19	28	44	51	68	75	90
	Counts/min./spot								
Fructosylglucose spot	3	3	13	18	18	21	22	27	34
Isomaltose spot	0	0	0	0	0	—	—	0	7

Table 2. *Action of Taka-diastase on raffinose in the presence of radioactive glucose*

In each experiment 1 drop of purified [^{14}C]glucose solution (5 μc, 0·6 mg.) was incubated with 0·1 ml. of 20 % (w/v) Taka-diastase (dialysed), 0·02 ml. of buffer and 0·05 ml. of 20 % (w/v) raffinose. In Expt. 1, 0·05 ml. of 20 % (w/v) glucose was added; in Expt. 2, 0·05 ml. of water. After development of the chromatogram the radioactivity of the spots was measured directly, and is expressed as counts/min./3 μl. spot. The glucose position counted about 5000 in both experiments.

		Time of incubation (hr.)								
		0·2	1	6	19	27	46·5	68	95	121·5
	Expt.	Counts/min./spot								
Sucrose spot	1	2	—	13	—	37	40	38	35	30
	2	20	41	—	68	69	37	30	—	23
Fructosylglucose spot	1	3	—	13	—	20	24	35	32	20
	2	0	4	—	5	11	16	17	—	10

Table 3. *Isolation of radioactive sucrose from incubation of Taka-diastase with raffinose and radioactive glucose*

The sugars in the reaction mixture (see text) were mixed with 25 mg. of sucrose and successively fractionated on charcoal (1), cellulose (2) and charcoal (3) columns, to isolate sucrose. This was crystallized once (4) and the crystals obtained were recrystallized twice, (5), (6), after a further addition of sucrose (20·4 mg.). The radioactivity of the sucrose spot on a paper chromatogram run in butanol–acetic acid–water was counted directly and is given for the whole fraction, per mg. dry wt., and per mg. of sucrose calculated from the ketose content. In some counts in the 'whole fraction' column only round numbers are given. The total counts measured on individual spots ranged from 700 to 2400, hence the accuracy nowhere exceeds ±2 %.

Fraction	Counts/min. In whole fraction	Per mg. dry wt.	Per mg. of sucrose
Untreated mixture	6000	—	—
Sucrose from (2)	3150	—	—
Sucrose from (3)	3100	—	—
Crystals from (4)	2400	200	210
Mother liquor from (4)	1200	110	210
Crystals from (4) + 20·3 mg. of sucrose	1900	55	62
Crystals from (5)	1300	56	63
Crystals from (6)	620	52	53
Mother liquor from (6)	400	55	58

that, while at a very low glucose concentration only small amounts of radioactivity appeared in the fructosylglucose position, in the presence of a higher glucose concentration the rate of incorporation of radioactivity was greater.

In view of these findings, it seemed desirable to set up an experiment on a larger scale, with the aim of isolating radioactive sucrose. A solution (total volume 1·48 ml.) containing 50 mg. of raffinose, 2·8 mg. (25 μc) of glucose, 0·10 ml. of 0·05 M-sodium acetate buffer, pH 5·0, and 0·50 ml. of 20 % (w/v) Taka-diastase was incubated for 20 hr. After inactivation of the enzyme, 25 mg. of sucrose was added and the mixture transferred to a charcoal–Celite (20 g.: 20 g.) column. Gradient elution was applied [50 % (v/v) ethanol into 500 ml. of water] and the fractions consisting mainly of sucrose were bulked, evaporated and chromatographed on cellulose powder to remove trace amounts of melibiose and fructosylglucose. After this procedure, fractions were obtained which showed on paper chromatograms a single spot, Raybin-positive, with the R_F value of sucrose; these were subjected to chromatography on a small charcoal–Celite column, to remove cellulose degradation products. The purified sucrose fraction [sucrose from (3); Table 3], weighing 22·1 mg., was then crystallized. This and subsequent recrystallizations were carried out by dissolving the material in a small volume (x ml.; about 0·3 ml. for 20 mg. of sucrose) of 50 % (w/v) ethanol, and then adding over a period of several days portions of absolute ethanol to a total of about $8x$ ml. The crystals grew firmly attached to the walls of the flask so that the mother liquor could be removed with a Pasteur pipette, and the crystals washed without transference to a filter funnel. The recrystallization was so arranged that an appreciable fraction (30–50 % of the material) was at each stage left in the mother liquor.

At each stage of the isolation procedure the radioactivity of the suspected sucrose fraction was measured by applying suitable small samples (3 or 5 μl.) to paper chromatograms and counting the unsprayed spots before and after running (Table 3). It was found that the radioactivity measured in this way (about 10–20 counts/min./spot) decreased consistently by about 25 % when the chromatogram was developed with butanol–acetic acid–water for 2 days. With one exception [crystals from (4): Table 3], this loss was not due to the separation from the spots of substances other than sucrose; no radioactivity could be detected elsewhere by direct counting or by radioautography. In the crystals from (4) the radioautograph showed that a little fructosylglucose was also present.

After the first crystallization [(4); Table 3] of the purified sucrose fraction a further addition of inactive sucrose (20·4 mg.) was made to facilitate the recrystallization. The results of analyses on the various fractions are given in Table 3.

From the proportion of the radioactivity of the sucrose fractions (6000 counts/min.) to the radioactivity of the glucose present (1 616 000 counts/min.) it could be calculated that only about 20 μg. $\left(\frac{6\,000}{1\,616\,000} \times 2{\cdot}8 \times \frac{342}{180}\ \text{mg.}\right)$ of radioactive sucrose had been formed in this experiment, and it therefore seemed possible that its formation was due to transfer of fructose residues not from raffinose, but from small amounts of sucrose produced by melibiase action on the raffinose. This possibility was therefore investigated more closely.

By the use of the alkaline silver-nitrate spray of Trevelyan *et al.* (1950), which is more sensitive than

benzidine–trichloroacetic acid, it was found that significant amounts of glucose or galactose, or both, were produced when dialysed Taka-diastase solutions were incubated with raffinose. However, after its initial appearance during the first 24 hr. of incubation, the amount of sugar in the glucose–galactose spot did not seem to increase further. At least two contributory causes, not so far considered, were found for this behaviour: one was the hydrolysis of small amounts of carbohydrate (probably lactose) still remaining in the dialysed enzyme preparation; the other was the presence in the raffinose sample of small amounts of sucrose, which were detectable with benzidine–trichloroacetic acid when a 5 μl. spot of 20 % (w/v) raffinose was applied to the chromatogram. This sucrose disappeared quickly when the raffinose was incubated with the enzyme preparation, and probably accounted for the initial, relatively rapid glucose liberation.

The raffinose was purified by gradient elution from an Active Carbon no. 130–Celite column, and a dialysed enzyme preparation was incubated at pH 5 for several days and again dialysed, after which it no longer showed any glucose–galactose liberation when incubated alone. When these were incubated together for 48 hr., chromatograms of 3–5 μl. samples sprayed with the silver reagent had barely detectable glucose–galactose spots. A sample of another Taka-diastase preparation, twice dialysed against running tap water for 2-day periods, yielded nothing detectable with the silver reagent in the glucose–galactose position during 48 hr. incubation with the purified raffinose, most of which was hydrolysed in this time to melibiose and fructose. Expt. 2 of Table 2 was repeated with the purified raffinose and Taka-diastase and gave essentially the same result, the radioactivity of the sucrose spot (5 μl.) reaching 99 out of a total of 6340 counts/min.

DISCUSSION

Although no attempt has been made to characterize all the substances produced by Taka-diastase from methyl β-fructoside and raffinose, in both cases there is evidence for more products than would be expected to arise from the action of a hydrolytic fructosidase. Its action on methyl β-fructoside yields, in addition to fructose, non-reducing substances whose only sugar component is fructose. When glucose is present a reducing glucose-containing substance is also formed. From their R_F values one may conjecture that these substances are fructosyl methyl β-fructosides, and a fructosylglucose (possibly 6-β-fructosylglucose). [Pazur (1953) has already shown that a mould-enzyme preparation will transfer fructose residues from one inulobiose (1-β-fructosylfructose) molecule to another, thus forming inulotriose.] From glucose alone the preparation forms a substance with the chromatographic properties of isomaltose, perhaps the result of the action of mould maltase (Wallenfels & Bernt, 1952), or transglucosidase (Pazur & French, 1952).

From raffinose the enzyme preparation also forms more substances than the melibiose and fructose expected from simple hydrolysis. In the presence of glucose the most interesting of these is a substance which has the properties of sucrose, though here also a substance resembling 6-β-fructosylglucose is formed.

The absence of sucrose from the products of action on mixtures of methyl β-fructoside and glucose has here been demonstrated both by a careful examination of the products by chromatography on paper and on charcoal, and also by the addition of radioactive glucose to the reaction mixture. The failure of the latter method to induce the appearance of radioactivity in the sucrose position is particularly significant, because by this means the conversion of only 0·1 % of the glucose into sucrose could readily be detected.

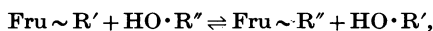
With a mixture of raffinose and glucose the same techniques showed that sucrose was formed, and that it was the result of the transfer of fructose to free glucose. Although 'carrier' sucrose had to be added, the constancy of the specific activity of the sucrose through two recrystallizations, at each of which an appreciable proportion was left in the mother liquor, indicates that the radioactivity was present in sucrose itself and not in some contaminating material. (With methyl β-fructoside, and with raffinose, radioactive fructosylglucose was formed, confirming its origin from free glucose. Both this and radioactive isomaltose may readily be distinguished from sucrose, but do not necessarily comprise the only contaminants.)

The experiments with raffinose were undertaken chiefly as a control for those with methyl β-fructoside; they demonstrate that sucrose can be detected and isolated under conditions similar to those used for the incubations with mixtures of the fructoside and glucose. However, in themselves they are not entirely satisfactory. In particular it cannot be altogether excluded that radioactive sucrose is a secondary product; that sucrose is first formed by an α-galactosidase (melibiase) and then acts as the substrate for transfructosylation to free glucose. Such quantitative data as are available seem to argue against this possibility. The amount of glucose–galactose seen on chromatograms of incubations with raffinose alone represents the upper limit of melibiase action, and the sucrose formed must represent a balance between its formation and breakdown, the latter being rapid compared with that of raffinose. In Expt. (1) of Table 2 it may be

calculated that a minimum of 84 μg. of free glucose had been incorporated into sucrose after 46·5 hr. Let us assume that under these conditions one-half of the sucrose formed by (hypothetical) melibiase action is converted into radioactive sucrose (cf. Edelman, 1954). Without added glucose melibiase action might be expected to proceed at the same rate, but virtually all the sucrose would be hydrolysed, yielding 168 μg. of glucose which, together with the galactose, gives a total of 336 μg. of glucose–galactose in 250 μl., or about 7 μg./5 μl., a quantity easily detectable by the benzidine–trichloroacetic acid spray. In practice nothing was seen with the reagent under these conditions. Taking the much more unfavourable conditions for sucrose synthesis represented by Expt. 2 of Table 2, or the larger-scale isolation experiment (Table 3), the corresponding figures are 0·8 μg. and 0·14 μg./5 μl. respectively. These amounts would not be seen with benzidine–trichloroacetic acid, and would be barely detectable with the silver reagent.

If it is conceded that sucrose is formed by fructose transfer from raffinose, this reaction has a very different order of magnitude from that with sucrose as donor. It would be interesting to know whether the strong inhibitory action of α-glucose, which led to the characterization of mould invertase as a glucosaccharase (see Bealing, 1953), is also exerted against raffinose hydrolysis, since in this case the rate of re-formation of the sucrose linkage, which may equal the rate of hydrolysis when sucrose is substrate (Edelman, 1954), is much smaller.

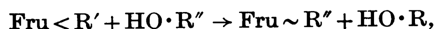
Hestrin, Feingold & Avigad (1955) have shown that levansucrase (from *Aerobacter levanicum*) is capable of synthesizing sucrose from mixtures of glucose and raffinose; that their preparations contained no melibiase was shown both by their failure to produce galactose or glucose from raffinose, and also the failure of mixtures of the enzyme with dextransucrase to produce dextran from raffinose. In a further publication these authors (Hestrin *et al.* 1956) have suggested that levansucrase acts only on a terminal β-fructofuranosidic group attached to the anomeric carbon of an aldose, and not on the same group attached to a carbinol carbon; they symbolize the former as a high-energy fructoside ($\mathrm{Fru} \sim \mathrm{R}$) and the latter as a low-energy one ($\mathrm{Fru} < \mathrm{R}$). The invertases are apparently capable of attacking both types. Their transferring action is chiefly to form from $\mathrm{Fru} \sim \mathrm{R}$ compounds $\mathrm{Fru} < \mathrm{R}$ linkages to primary alcoholic groups, as occurs with levan formation from sucrose, but, like levansucrase, mould and higher-plant invertases are able to catalyse the reversible reaction



$$\mathrm{Fru} \sim \mathrm{R}' + \mathrm{HO}\cdot\mathrm{R}'' \rightleftharpoons \mathrm{Fru} \sim \mathrm{R}'' + \mathrm{HO}\cdot\mathrm{R}',$$

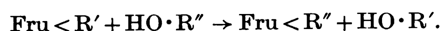
at least in the case where both $\mathrm{Fru} \sim \mathrm{R}'$ and $\mathrm{Fru} \sim \mathrm{R}''$ represent sucrose.

The present experiments with methyl β-fructoside as substrate would seem to show that mould invertase is not capable of bringing about the reaction



$$\mathrm{Fru} < \mathrm{R}' + \mathrm{HO}\cdot\mathrm{R}'' \rightarrow \mathrm{Fru} \sim \mathrm{R}'' + \mathrm{HO}\cdot\mathrm{R},$$

a reaction which may in any case, as Edelman (1954) points out, be expected to be reduced to insignificant proportions by thermodynamic requirements. Whether levansucrase can catalyse this type of reaction has been investigated in several Laboratories. Working with levansucrase from *Bacillus subtilis* Doudoroff & O'Neal (1945) found indirect evidence for the formation of sucrose by a reversal of the normal action of the enzyme, and Péaud-Lenoël & Dedonder (1955) have been able to isolate crystalline sucrose formed by its action on a mixture of degraded bacterial levan and glucose. On the other hand, Hestrin and his colleagues, working with the levansucrase from *Aerobacter levanicum*, and using a somewhat different test system, have not been able to prove that sucrose is formed in this way. Edelman (1954, 1956) has failed to find any sucrose synthesis by mould invertase acting upon mixtures of glucose with inulin, or with its short-chain hydrolysis products. Nevertheless, mould invertase (?unlike levansucrase; cf. Feingold, Avigad & Hestrin, 1956) is capable of bringing about the hydrolysis of the $\mathrm{Fru} < \mathrm{R}$ linkage and of catalysing the reaction



$$\mathrm{Fru} < \mathrm{R}' + \mathrm{HO}\cdot\mathrm{R}'' \rightarrow \mathrm{Fru} < \mathrm{R}'' + \mathrm{HO}\cdot\mathrm{R}'.$$

In terms of the fructosyl–enzyme hypothesis this would seem to imply that either $\mathrm{Fru} \sim \mathrm{enz}$ or $\mathrm{Fru} < \mathrm{enz}$ may be formed by mould invertase. If the different energy levels are to be given a structural basis two different fructosyl–enzyme compounds would be required. There is, perhaps, an indication of this in the relatively greater rate of incorporation of glucose into fructosylglucose than into sucrose when the glucose concentration was increased (Table 2). An alternative explanation might be sought in a mechanism involving the binding of the acceptor molecule to the enzyme simultaneously with the cleavage of the substrate. Edelman (1956) has already discussed this in more detail, and it does not seem profitable to pursue the discussion further with the data available. The use of a mould-invertase preparation freed completely from other carbohydrases, and a comparison of the structure of the active centres of it and of yeast invertase, would seem to be indispensable preliminaries to further speculation. In fact, the elucidation of the reactions at the active centre of any carbohydrase might help us considerably to visualize phenomena which at present are accessible only in terms of specificity and of the inhibitory effects of sugars.

SUMMARY

1. Methyl-O-β-D-fructofuranoside has been prepared by the action of yeast invertase on sucrose in the presence of methanol.

2. The fructoside has been used as a substrate for a mould-enzyme preparation (Taka-diastase). An examination of the products showed that both hydrolysis and transfructosylation occurred.

3. Sucrose could not be detected among the products of action upon mixtures of methyl β-fructoside and glucose, either by chromatographic fractionation or by the use of ^{14}C-labelled glucose.

4. Taka-diastase acting on mixtures of raffinose and glucose formed sucrose. The incorporation of radioactive glucose into the molecule demonstrated that sucrose formation was the result of fructose transfer and not of melibiase (α-galactosidase) action.

5. The implications of these findings are discussed in relation to the hypothesis that invertases form fructosyl–enzyme compounds as common intermediates in hydrolysis and transfructosylation.

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The Amino Acid Sequence in a Fraction of the Fibroin of *Bombyx mori*

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The silk fibroin of *Bombyx mori* was the first protein to which the methods of partial hydrolysis were applied in an attempt to elucidate its structure. In 1902, Fischer first isolated from fibroin a dipeptide thought to be glycylalanine, and since that date much work has been done to identify the products of the partial hydrolysis of fibroin, and thus to gain information about its molecular architecture. The considerable literature of this work up to 1943 has been summarized by Synge (1943), and from that date to the end of 1951 by Sanger (1952).

Since 1951, Levy & Slobodian (1952), Slobodian & Levy (1953), Kay & Schroeder (1954) and Joffe (1954) have extended our knowledge of the structure of fibroin by studying the products of its partial hydrolysis, but deductions from such studies of a minimum repeating sequence throughout the fibroin molecule make assumptions about its regularity that have been shown to be incorrect. The work of Abderhalden & Bahn (1933), Meyer, Fuld & Klemm (1940) and of Zahn and co-workers (1952, 1954*a*, *b*, 1956) has clearly demonstrated that sections of the fibroin molecule differ in composition from other sections. The latter two authors interpreted these differences by suggesting that fibroin contained crystalline and amorphous phases of differing compositions.

The isolation and identification of the crystalline fraction of fibroin was achieved in these laboratories by the action of chymotrypsin on an aqueous solution of fibroin (Drucker, Hainsworth & Smith, 1953). This fraction, which appeared as a pre-