710. *A Proof of the Structure of Methyl β-D-Fructofuranoside prepared enzymically from Sucrose and Methanol.*

By J. S. D. Bacon and D. J. Bell.

A substance prepared by the action of a yeast invertase preparation on sucrose in the presence of methanol has been shown by oxidation with periodate and preparation of derivatives to be methyl β-D-fructofuranoside. Some evidence is given supporting the view that it is formed by transfructosylation from sucrose to alcohol, catalysed by the invertase.

Nelson and Schubert [1] found that addition of ethanol lowered the rate of hydrolysis of sucrose by yeast invertase, as measured by change in optical rotation; they attributed this to a decrease in the amount of available water. However, paper chromatography shows that in the presence of ethanol a new substance, probably ethyl β-D-fructofuranoside, is formed, and analogous products are obtained from a number of other alcohols.[2,3,4] Mould invertases behave in a similar manner.[5,6] There is evidence that homologous products are formed by the subsequent transfer of fructose residues to the simple fructosides.[5,7]

The substance formed in the presence of methanol was selected for closer examination, because the methyl fructosides have been studied by Purves and Hudson; they isolated

[1] Nelson and Schubert, *J. Amer. Chem. Soc.*, 1928, **50**, 2188.
[2] Bacon, *Biochem. J.*, 1952, **50**, xviii.
[3] Miwa, *Symposia on Enzyme Chemistry, Japan*, 1953, **8**, 57.
[4] Oparin and Bardinskaya, *Doklady Akad. Nauk S.S.S.R.*, 1953, **89**, 531.
[5] Bealing, *Biochem. J.*, 1953, **55**, 93.
[6] Kurasawa, Saito, Honma, and Yamamato, *Bull. Fac. Agric., Nigata Univ., Nigata, Japan*, 1955, **7**, 57.
[7] Breuer and Bacon, *Biochem. J.*, 1957, **66**, 462.

crystalline methyl α-fructofuranoside [8] and estimated the optical rotation of the β-anomer.[9]

Our product was at first separated by partition chromatography on cellulose powder, or by elution with water from charcoal–Celite,[2] but it was later found that a small gradient of ethanol in the water (0 ⟶ 10%) removed it from the charcoal after the monosaccharides, yet well in advance of the disaccharide fraction. In this way a single chromatographic separation yielded 5 g. from an incubation mixture containing initially 26 g. of sucrose.[7] The substance, which usually contained traces of fructose, was a syrup which has not yet crystallised. It was non-reducing, stable to alkali, but readily hydrolysed by dilute acid or by yeast invertase to yield fructose, and had the alkoxyl content (calculated as methoxyl) expected for a methyl hexoside. It reduced 1·01 moles of sodium metaperiodate per mole with the formation of less than 0·012 mole of acid. Subjected to Barry and Mitchell's phenylhydrazine–acetic acid degradation [10] the oxidised glycoside yielded only glycerosazone. Confirmation that the glycoside was indeed derived from D-fructose followed from its conversion [11] in high yield into 2 : 3-4 : 5-(" β ")-di-*O*-*iso*propylidene-D-fructopyranose.

These facts all agree with a fructofuranoside structure. The assignment of the β-configuration must perforce rest on the susceptibility of the substance to yeast invertase and on its negative rotation (cf. $[\alpha]_D^{20}$ +93° for methyl α-D-fructofuranoside).[8]

In 1939, Schlubach and Bartels [12] obtained the first authentic sample of methyl β-D-fructofuranoside, with $[\alpha]_D^{20}$ −49·95°, by a painstaking series of reactions starting with D-fructose 1 : 6-diphosphate, and concluding with enzymic dephosphorylation. If no ring enlargement occurred during the latter treatment, the structure of a furanoside must follow for their product; it was found, moreover, to be hydrolysed by invertase to the extent of 95%. More recently Augestad, Berner, and Weigner [13] have isolated the glycoside, by chromatography on cellulose powder, from the "γ-methyl fructoside" mixture; [14] their product (stated to be "97% pure" [15]) had $[\alpha]_D$ −46·9°.[13] Foster [16] has examined a sample of it ($[\alpha]_D$ −48°) by paper ionophoresis in the presence of sodium borate and finds it to have a mobility consistent with a β-fructofuranoside structure and quite different from that of the α-anomer, but as far as we know no chemical proof of its structure has yet been published. Purves and Hudson [9] calculated from the observed changes in reducing power and optical rotation when yeast invertase acted upon "γ-methyl fructoside" preparations that the $[\alpha]_D$ of methyl β-D-fructofuranoside should be −52° ± 2°. Our enzymically synthesised preparations had $[\alpha]_D$ between −50° and −52°. Similar independent preparations by Ishizawa and Miwa [17] had $[\alpha]_D$ −50·7°. If the figures for the enzymically synthesised products are taken as likely to be more nearly correct the agreement between them and the calculated value would seem to indicate that only one component of the γ-methyl fructoside mixture is attacked by yeast invertase preparations. (Wolfrom and Shafizadeh [18] have discussed the bearing of the figure of −46·9° on the accepted configuration of sucrose.)

For want of evidence to the contrary it has been assumed [19,20] that a single enzyme is responsible for both the fructose-transferring and the hydrolytic activity of yeast invertase preparations. If a sufficiently high concentration of methanol is chosen (5M), rather more fructoside than free fructose is formed during the breakdown of the first 20% of the sucrose.

[8] Purves and Hudson, *J. Amer. Chem. Soc.*, 1934, **56**, 708.
[9] *Idem, ibid.*, p. 702.
[10] Barry and Mitchell, *J.*, 1954, 4020.
[11] Bell, *J.*, 1947, 1461.
[12] Schlubach and Bartels, *Annalen*, 1939, **541**, 76; cf. Morgan and Robison, *Biochem. J.*, 1928, **22**, 1270.
[13] Augestad, Berner, and Weigner, *Chem. and Ind.*, 1953, 376.
[14] Menzies, *J.*, 1922, **121**, 2238.
[15] Augestad and Berner, *Acta Chem. Scand.*, 1954, **8**, 251.
[16] Foster, *J.*, 1957, 1395.
[17] Ishizawa and Miwa, *Symposia on Enzyme Chemistry, Japan*, 1954, **9**, 40.
[18] Wolfrom and Shafizadeh, *J. Org. Chem.*, 1956, **21**, 88.
[19] Bacon, *Ann. Reports*, 1954, **50**, 281.
[20] Edelman, *Adv. Enzymology*, 1956, **17**, 189.

The ratio of these two quantities may then be measured conveniently by quantitative paper chromatography,[21] and serves as a measure of the ratio of transferring to hydrolytic activity (T/H). As would be expected, the ratio is influenced by the initial concentrations of methanol and sucrose, but when these were kept constant it was found to vary hardly at all over a wide range of pH (3·5—8·9), the greatest divergence being a decrease of about 20% at alkaline pH. (The latter effect did not appear to be due to epimerisation of glucose under the conditions of incubation and analysis.) The T/H ratios differed little for samples of enzyme taken at various stages of the autolysis of baker's yeast. It was slightly increased in this and other enzyme preparations by dialysis, which reduces the total enzymic activity considerably.[22]

A small amount of fructoside was shown chromatographically to be produced enzymically from mixtures of methanol and fructose, but this was insignificant compared with the magnitude of fructoside synthesis when sucrose was present. The evidence thus strongly suggests that methyl β-D-fructofuranoside is formed by the transfer of fructose residues from sucrose to methanol and that the enzyme responsible is that which causes the simultaneous hydrolysis of the sucrose.

We found the rate of hydrolysis of β-D-fructofuranoside by yeast invertase, relative to that of sucrose, to be somewhat faster than that recorded for the labile component of γ-methyl fructoside; [9] this discrepancy might be due to the inhibitory effects of the other components of the mixture. The fructoside can serve as a donor in transfructosylation reactions with both yeast [23] and mould [7] invertase.

Experimental

All aqueous solutions were evaporated under reduced pressure at pH 6—7, below 50°. Specific rotations were determined in water, a 2 dm. tube being used. Enzyme incubations were at 18—22° in the presence of chloroform, and were stopped by heating quickly to 100° or by the addition of mercuric chloride.

Factors influencing Fructoside Formation.—The rate of fructoside formation (concurrent with sucrose breakdown from 0 to 20%), under a variety of conditions, was followed by quantitative paper chromatography with a colorimetric method for ketose determination.[21] The free fructose liberated during the experiments was measured by the same method. The molar ratio, fructoside formed : fructose set free by hydrolysis, was calculated from these measurements; this ratio is termed T/H (" transfer/hydrolysis ").

(a) *Methanol concentration.* With 0·25M-sucrose at pH 5·0 fructoside formation was seen with methanol concentrations ranging from 1 to 40% (v/v); concentrations greater than 20% (~6M) eventually inactivated the enzyme. For the majority of measurements of T/H, 5M-methanol was chosen; at this concentration, and pH 5·0, in the absence of sucrose the enzyme lost about 10% of its activity in 20 hr. The majority of the incubations were stopped before this time; nevertheless some decrease in the rates of sucrose breakdown was noticed. With 10M-methanol, at the early stages of the reaction T/H was 2·7, compared with 1·45 when 5M-methanol was used; 10M-methanol was therefore used in the preparative work.[7]

(b) *Sucrose concentration.* T/H increased slightly with increasing sucrose concentration, namely from 1·3 at 0·125M to 1·8 at 0·5M. With 0·25M-sucrose, oligosaccharide formation (fructosyl transfer to sucrose [22]) accounted for only about 10% of the total transfructosylation.

(c) *Dependence on* pH. About 50 measurements of T/H were made with dialysed B.D.H. " Invertase Concentrate " acting on 0·25M-sucrose and 5M-methanol with acetate, barbiturate, and phosphate buffers to maintain the pH at values ranging from 3·5 to 8·9. In only three instances did T/H exceed 1·52 and in only one was it less than 1·13. These values were found over a range of sucrose-breakdown between 5 and 25%; over this range, at pH 5 T/H falls

[21] Bacon, in " Methods in Enzymology," Ed. Colowick and Kaplan, Academic Press, New York, 1955, Vol. I, p. 258.
[22] Bacon, *Biochem. J.*, 1954, **57**, 320.
[23] Whelan and Jones, *ibid.*, 1953, **54**, xxxiv.

slightly, from ~1·5 to ~1·4. A summary of the effect of pH at 17—23% sucrose-breakdown is given below; the fall in *T/H* at alkaline pH was noted in all experiments;

pH	3·6	4·6	5·0	5·6	6·3	7·0	8·0	9·0
T/H	1·39	1·40	1·46	1·44	1·44	1·52	1·23	1·25

That alkalinity alone had no effect on a mixture of fructose and glucose, in the absence of enzyme, was shown as follows. A solution of fructose (0·028M) and glucose (0·028M) in phosphate buffer (0·04M) at pH 9·0 was kept at 20°; samples were withdrawn at intervals, acidified with hydrochloric acid, and stored in the frozen state. Subsequent ketose measurements on the samples showed that no detectable change in the fructose content had occurred during 96 hr., a period which exceeded the duration of each of the enzyme experiments at alkaline pH.

Autolysis of Yeast.—A mixture of fresh baker's yeast (100 g.) with water (100 ml.) and toluene (40 ml.) was stirred intermittently and samples were removed at intervals. After centrifuging, the supernatant fluids were filtered by gravity in presence of Hyflo-Supercel and stored at 0° in presence of chloroform. Filtrates incubated at pH 5·0 with sucrose (0·25M) and methanol (0·5M) in acetate buffer (0·025M) gave the following results:

Time of removal of sample	Relative invertase activities	*T/H*
0	1	1·33
4 days	24	1·28
13 days	32	1·24

(Invertase activity was measured as release of reducing sugar, estimated by the method of Miller and Van Slyke.[24])

Effect of Dialysis on Invertase Preparations.—After dialysis against Sheffield tap-water for 20 hr. the 13-day autolysate had lost half of its original invertase activity and now gave *T/H* of 1·37. A sample of B.D.H. "Invertase Concentrate" which had lost 95% of its activity on dialysis against tap water, gave *T/H* 1·51 against 1·35 for the undialysed preparation (12% sucrose-breakdown). The latter was examined at a dilution of 1 : 2500 (v/v) so that the fructosyl-accepting effect of the glycerol which it contained might be expected to be negligible; this was confirmed by the addition to the incubation mixture with the dialysed B.D.H. invertase, of glycerol at 1% and 10% (v/v) levels, when *T/H* ratios of 1·51 and 1·50 respectively were found. Two different undialysed B.D.H. preparations gave indistinguishable *T/H* over the range 5—17% sucrose-breakdown, 1·38 and 1·42 at 5%; 1·31 and 1·31 at 17%.

Fructoside Formation from Free Fructose and Methanol.—Enzymic incubation of fructose (0·7M) with methanol (10M) at pH 6 yielded less than 1 mg./ml. of material showing the chromatographic behaviour of methyl β-fructofuranoside. A *T/H* ratio of 2·7 at 20% sucrose-breakdown corresponds to 13 mg./ml. of the glycoside being formed. When the action of the enzyme on sucrose–methanol systems was continued for 10—100 times the duration needed for complete breakdown of the sucrose the fructoside level fell to that formed in the free fructose–methanol system.

Isolation of the Fructoside.—The analytical methods used, and a typical procedure for the isolation of the fructoside, have already been described.[2,7] The substance had an R_F of 0·37—0·40 in butanol–acetic acid;[25] Miwa found R_F 0·40 for his product.[26]

125 mg. of a preparation purified by chromatography on cellulose powder and charcoal–Celite columns and containing no detectable monosaccharide were dissolved in 3·0 ml. of water. The solution had a rotation of −3·93° (at 22°), and estimations of ketose, and of reducing sugar after hydrolysis with 0·5% oxalic acid at 100° for 30 min., showed 36·2 and 36·5 mg./ml. respectively, corresponding to $[\alpha]_D^{22}$ −50·4° and −49·9°. Paper chromatograms of the hydrolysate showed a single spot, corresponding to fructose (Found : OMe, 16·0. Calc. for $C_7H_{14}O_6$: OMe, 16·0%).

Characterisation of the Fructoside.—(a) *Products of periodate oxidation.* After drying to constant weight over phosphoric oxide at 15 mm., the glycoside (0·7205 g., 3·714 millimoles) was dissolved in water (20 ml.), and 0·3M-sodium metaperiodate solution (25 ml.) added. A control with water and periodate was set up at the same time. Samples of both solutions were

[24] Miller and Van Slyke, *J. Biol. Chem.*, 1936, **114**, 583.
[25] Partridge, *Biochem. J.*, 1948, **42**, 238.
[26] Miwa, personal communication.

titrated with 0·05N-sodium arsenite solution in the usual way. At 17 hr. the glycoside (1·00 mol.) reduced 1·01 mols. of IO_4^-; at 24 hr., 1·00 mol. Titration of the solution with 0·01N-potassium hydroxide, after destruction of periodate by purified ethylene glycol, showed that less than 0·012 mol. of acid had been formed.

Treated with phenylhydrazine and dilute acetic acid according to Barry and Mitchell [10] with addition of 0·2 ml. of saturated potassium metabisulphite solution to the reaction medium,[27] the oxidation product (from 485 mg. of glycoside) yielded 370 mg. of glycerosazone (stout needles from benzene), m. p. and mixed m. p. 131—132°. Chromatography [10] both by column and paper gave no evidence of the presence of glyoxalosazone or of higher osazones among the products of this reaction. A repetition of the whole experiment gave similar results.

(b) *Treatment with acetone–5% sulphuric acid.*[11] The glycoside (580 mg.) was shaken with 30 ml. of dry ($CaSO_4$) acetone containing 1 ml. of sulphuric acid. Dissolution was complete in 30 min. After 6 hr. at room temperature the product was isolated as a hard crystalline mass (715 mg., 92%), m. p. 95—96°. Recrystallised from ligroin (b. p. 60—80°), needles (620 mg.) were obtained with m. p. and mixed m. p. 95—96°, $[\alpha]_D^{18}$ −24·8°.[11]

Hydrolysis of the Fructoside by Yeast Invertase.—The action of undialysed Invertase Concentrate on 0·12M-methyl β-fructoside, and on 0·13M-sucrose at pH 5·0 was compared. On the assumption that fructose is half of the total reducing sugar liberated from sucrose, the mg. of fructose liberated per ml.[24] were:

Time (min.)	15	30	60	90	120	180	300
Sucrose	4·40 *	8·55	14·9	—	—	—	—
Fructoside	—	1·25	2·47	3·05	3·95*	5·87	10·0

From the figures marked (*) the ratio of the rates of breakdown (on a molar basis) may be calculated as 8·7 : 1. Purves and Hudson [9] found a value of 13·5 : 1 in their experiments with γ-methyl fructoside mixtures.

Comparison with γ-Methyl Fructoside Mixture.—Preparations of γ-methyl fructoside [9] showed, in addition to fructose, two faster-running spots on butanol–acetic acid chromatograms (R_F 0·39, 0·46). Both disappeared when the preparation was treated with 0·5% oxalic acid at 100° for 30 min. Yeast invertase was observed qualitatively to act upon that with R_F 0·39, *i.e.*, the one corresponding to methyl β-fructofuranoside. However, prolonged incubation under conditions in which the enzyme was proved to retain its activity failed to cause the complete disappearance of the spot (in the presence of a final concentration of about 0·2M-fructose). No change could be seen in the other spot during the enzyme treatment. Chromatograms of sucrose or of fructose which had been treated with 0·5% oxalic acid at 100° in the presence of 25% methanol showed two spots with R_F 0·39 and 0·45. The amounts of these substances were small; they were comparable with the yield of methyl β-fructofuranoside from enzyme action on mixtures of fructose and methanol.

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THE DEPARTMENTS OF BIOCHEMISTRY,
UNIVERSITIES OF SHEFFIELD AND CAMBRIDGE.
[Present Addresses:
(J. S. D. B.) MACAULAY INSTITUTE, CRAIGIEBUCKLER, ABERDEEN.
(D. J. B.) A.R.C. POULTRY RESEARCH CENTRE,
EDINBURGH, 9.]
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[27] Hamilton, *J. Amer. Chem. Soc.*, 1934, **56**, 487.