CCLXVI. THE SPECIFIC ROTATION AND STABILITY OF (2, 5)-FRUCTOSE FROM A MATHEMATICAL STUDY OF THE HYDROLYSIS OF SUCROSE BY FRUCTOSACCHARASE.

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ALTHOUGH the sugars and polysaccharides have been successfully investigated by purely chemical methods in recent years, certain points in the complete elucidation of their structure remain obscure. For example, methods involving oxidation, reduction and methylation afford no clue to the type of linking between sugar molecules, but by utilising enzymes of appropriate specificity it has been possible to assign a specific linking to the hexose molecules composing some of the commoner di- and tri-saccharides.

The present communication concerns the use of yeast fructosaccharase as an agent in the elucidation of the specific rotation of (2, 5)-fructose as liberated from sucrose, and of the unimolecular velocity constant of the change of (2, 5)-fructose to equilibrium fructose.

From evidence derived by methylation procedure, sucrose appears to be 1-(1, 5)-glucosido-2-(2, 5)-fructoside [Avery *et al.*, 1927; Haworth *et al.*, 1927, 1, 2]. The configuration of the fructose 2-carbon atom is an open question. That of the glucose 1-carbon atom also is regarded by Haworth in the same light, but nevertheless there are the following reasons for assigning the α -form to the glucose component.

(a) Hudson [1908] found that after an almost instantaneous hydrolysis of sucrose by invertase, the mutarotatory fall continued long after the mutarotatory effects due to the fructose portion were presumed to have ceased. (The mutarotation of normal fructose is much more rapid than that of glucose, and in the present instance, as will be shown, one reaction involved is even faster, *viz.* the change of fructofuranose to fructopyranose.) Hudson concluded that the α -mutarotation at so late a period was due to α -glucose.

(b) Sucrose is not hydrolysed by emulsin (β -glucosidase).

(c) According to Weidenhagen [1929], yeast maltase (α -glucosidase) hydrolyses sucrose as rapidly as maltose at p_H 7, the optimum for the enzyme, but hydrolyses neither sucrose nor maltose at p_H 4.7, the optimum for fructosaccharase (β -*h*-fructosidase), so that the reaction at p_H 7 was not due to the latter enzyme. If this is confirmed, it affords evidence of the presence of α -glucose in the sucrose molecule.

Hudson [1909] could observe no striking change in rotation at the outset of his very rapid hydrolysis, and he concluded that the combined rotations of the products were equal to that of the sucrose. Assuming the one component to be α -glucose, he calculated the specific rotation of the fructose component as follows.

Since 1 g. of sucrose ($[\alpha]_D = +66^\circ$) yields 0.525 g. of glucose ($[\alpha]_D = +109^\circ$) and 0.525 g. of fructose ($[\alpha]_D = x$), then $0.525(109 + x) = 66$, *i.e.* $x = +17^\circ$.

Hudson's deduction from qualitative data is not entirely satisfactory, since nothing was known of the transformation of fructofuranose to equilibrium fructose; if this change were instantaneous or nearly so, it is evident that an inability to record an initial rise in rotation would be no foundation for the above calculation. The results obtained in the present investigation from strictly kinetic data, have, however, confirmed Hudson's calculation and have shown that the change from fructofuranose to equilibrium fructose is unimolecular, $k_{17^\circ}^{(\text{mins.}^{-1})} = 0.3$, between p_H 4.6 and 6.1.

With suitable concentrations of yeast fructosaccharase and sucrose, it is possible to obtain an accurately unimolecular inversion up to the stage of some 60–70 % hydrolysis, and it is with data furnished during this period that the present investigations are concerned. The rotational change of the enzyme sucrose mixture, suitably buffered, is followed polarimetrically. To aliquot portions of the same reaction mixture is added from time to time dilute ammonia in suitable volumes to arrest hydrolysis and to accelerate mutarotation. The final rotations of these portions, after correction for the slight rotation of the enzyme preparation and the dilution by ammonia, indicate the extent of inversion at the respective times. The results of such an experiment in which inversion was carried to the end of the strictly unimolecular stage are given in Table I (Exp. 1) and shown graphically in Fig. 1, in which observed rotations are plotted against time. Curve (b) represents the rotation of the mutarotating solution, curve (c) that of the mutarotated solution after applying the necessary correc-

Fig. 1. Inversion of sucrose by fructosaccharase. Temperature 17° . p_H 4.6.

- (a) Rotation sucrose + mutarotating glucose.
- (b) Rotation sucrose + mutarotating glucose and fructose.
- (c) Rotation sucrose + mutarotated glucose and fructose.

Table I.

100 cc. 20 % sucrose, 10 cc. *N*/10 acetate buffer, p_H 4.6, total volume, 150 cc. Temperature 17°. Volumes of enzyme preparation: Experiment 1, 20 cc.; Experiment 2, 30 cc. of a more active preparation.

Polarimeter readings. Corrected for rotation of enzyme										Ratio $\frac{L.H.S.}{R.H.S.}$ (equation 5)							
Mutarotating portion				Mutarotated portion		Arbitrary times mins.	Sugars present				Rotation due to	Fructose (from graph, Fig. 1)	[α] _D of fructose				
Time mins. secs.	Angular degrees	Time mins.	Angular degrees	Time mins.	Angular degrees		Sucrose g.	Glucose or fruc- tose (g.)	Sucrose g.					Glucose	Sucrose + glucose		
k_s (mins. ⁻¹) k_s base 10																	
0 0	+8.88	0.0	+8.88	0.8	—	—	—	—	—	—	—	0.00	0.0	0.96			
0 25	8.81	1.5	7.71	2.0	0.0306	17.46	1.34	+7.75	+1.00	+8.75	-0.11	-12.3	0.97				
0 50	8.81			4.0	0.0293	15.20	2.53	+6.74	+1.88	+8.62	-0.52	-30.8	0.94				
1 15	8.74	3.0	6.74	6.0	0.0301	13.30	3.53	+5.91	+2.62	+8.53	-0.93	-39.5	0.98				
1 30	8.67			8.0	0.0298	11.54	4.45	+5.12	+3.26	+8.38	-1.31	-44.2	1.05				
2 0	8.61	5.0	5.46	10.0	0.0305	9.93	5.29	+4.41	+3.82	+8.23	-1.69	-47.9	1.07				
2 30	8.46			12.0	0.0305	8.51	6.04	+3.78	+4.28	+8.06	-2.02	-50.2	1.10				
3 0	8.32	7.0	4.43		0.0315								Mean				
3 30	8.11				Mean								1.01				
4 0	8.11	9.0	3.41		k_s ($\ln$)												
4 30	7.98				0.0698												
5 0	7.89	11.0	2.60		(base e)												
6 0	7.64																
7 0	7.37	13.5	1.59														
8 20	7.09																
9 30	6.73																
10 30	6.47																
11 30	6.17																
12 30	5.92																
13 15	5.68																
14 0	5.48																
15 0	5.26																
Experiment 2																	
0 0	+8.88	0.0	+8.88	1.0	—	17.93	1.10	+7.96	+0.80	+8.76	0.00	0.0	1.01				
0 25	8.72	1.66	6.97	2.0	0.0468	16.08	2.07	+7.14	+1.51	+8.65	-0.16	-11.6	0.99				
0 45	8.75			4.0	0.0477	13.15	3.60	+5.83	+2.71	+8.54	-0.66	-27.5	0.97				
1 2	8.65	2.83	5.76	6.0	0.0454	10.53	4.98	+4.68	+3.65	+8.33	-1.14	-34.3	1.04				
1 40	8.53			7.5	0.0463	8.82	5.88	+3.91	+4.24	+8.15	-1.46	-37.3	1.08				
2 20	8.38	4.50	4.50		0.0476								Mean				
3 5	8.22			9.0	0.0481								1.02				
3 30	7.98	6.08	3.30														
4 15	7.80																
5 0	7.53	7.58	2.29														
6 0	7.23	No longer unimolecular															
6 30	7.04	9.0	1.51														
7 0	6.82																
8 0	6.52	10.5	0.81														
8 30	6.33				0.0486												
9 0	6.18				Mean												
10 0	5.90				k_s ($\ln$)												
11 0	5.50				0.1080												
					(base e)												

tions, curve (a) that of unchanged sucrose *plus* mutarotating glucose, arrived at by calculation as indicated below. The difference between curves (a) and (b) at any time point therefore represents the rotation due to fructose at that instant. Curve (a) was arrived at from the following considerations—which apply only to the unimolecular period of the hydrolysis. Let a = initial amount of sucrose (g. in v cc.), $(a - 2q)$ = sucrose remaining after time t , q being glucose or fructose liberated in time t , uncorrected for water taken up on hydrolysis. (The necessary correction is applied later.)

$$\text{Then} \quad k_s = \frac{1}{t} \ln \frac{a}{a-2q} \quad \dots\dots(1),$$

where k_s is the unimolecular velocity constant of hydrolysis of sucrose.

$$\text{Differentiating} \quad dq = \frac{a}{2} \cdot k_s \cdot e^{-k_s t} dt \quad \dots\dots(1a).$$

Now consider the mutarotatory change of α -glucose to $\alpha\beta$ -glucose, which again is a unimolecular reaction. Let the unimolecular velocity constant of this change be k_G

$$k_G = \frac{1}{t} \ln \frac{P_0 - P_\infty}{P - P_\infty} = \frac{1}{t} \ln \frac{c}{P - P_\infty} \quad (\text{putting } P_0 - P_\infty = c),$$

where P_0 , P and P_∞ are the specific rotations of the glucose at times t_0 , t and t_∞ .

$$\text{From this,} \quad P = ce^{-k_G t} + P_\infty \quad \dots\dots(2).$$

Using P for specific rotations and ρ for actual rotations under observation, the rotation $d\rho$ of dq grams of α -glucose after an interval of time t , will be:

$$d\rho = \frac{dq}{v} (ce^{-k_G t} + P_\infty).$$

Substituting the value of dq from equation (1 a)

$$d\rho = \frac{a}{2v} \cdot k_s e^{-k_s t} (ce^{-k_G t} + P_\infty) dt.$$

The total rotation ρ , of Σdq grams of mutarotating α -glucose after time t will be,

$$\int d\rho = \int_0^t \frac{a}{2v} (k_s ce^{-(k_s+k_G)t} + k_s e^{-k_s t} P_\infty) dt, \\ \text{i.e.} \quad \rho = \frac{a}{2v} \left(P_\infty + \frac{k_s c}{k_s + k_G} - P_\infty e^{-k_s t} - \frac{k_s c}{k_s + k_G} \cdot e^{-(k_s+k_G)t} \right) \quad \dots\dots(3).$$

As an example, the values substituted in the case of Exp. 1 of Table I were $a = 20$, $v = 150$, $c = P_0 - P_\infty = 111.2 - 52.5 = 58.7$, $k_s = 0.07$, $k_G = 0.0115$.

(This value of k_G was taken from the data furnished by Nelson and Beegle [1919], who investigated the velocities of mutarotation of glucose and fructose at various temperatures and values of p_H .) The values of ρ so obtained were multiplied by $\frac{2}{3}$, since 19 g. of sucrose yield 20 g. of hexose. These values were added to the calculated rotations due to unchanged sucrose at the corresponding times respectively and plotted to give curve (a). The difference in reading between curves (a) and (b) at any time gives the rotation due to fructose at that time. Let this difference be F . Then the specific rotation of the fructose present, i.e. the mean specific rotation of Σdq , where each small increment is at a different stage of mutarotation, is $Fv/\frac{2}{3}q$.

These values are tabulated for each experiment (Exps. 1-5, Tables I-II) and plotted collectively against time in Fig. 2. These curves express the relation between specific rotation of fructose produced at known unimolecular rate and time. Extrapolation to the ordinate gives in all cases the value $+15^\circ$ to $+17^\circ$ for the specific rotation of the fructose as first liberated, i.e. for (2, 5)-fructose. This low positive rotation would suggest that the (2, 5)-fructose is in the β -form.

Table II.

Reaction mixtures as in Exps. 1 and 2. Temp. 17°.

Exp.	Volume of enzyme preparation	k_s (mins. ⁻¹) base e. (mean value)	Arbitrary times (mins.)	$[\alpha]_D$ of fructose	Ratio L.H.S. R.H.S. (equation 5)
3	30 cc.	0.0926	0.9	0.0	0.94
			2.0	-12.9	0.96
			3.0	-22.75	0.96
			4.0	-28.1	0.97
			5.0	-32.9	0.99
			6.0	-34.7	1.03
			8.0	-39.9	1.08
			Mean		0.99
4	40 cc.	0.1036	1.0	0.0	0.96
			2.0	-12.6	0.97
			4.0	-28.0	0.96
			6.0	-33.70	1.04
			8.0	-38.7	1.09
			Mean		1.00
5	40 cc. of a more active preparation	0.112	1.0	—	—
			2.0	-10.5	0.97
			3.0	-20.1	1.00
			4.0	-25.8	1.00
			6.0	-32.4	1.06
			7.5	-36.6	1.09
			Mean		1.02
6	As in Exp. 5, but at p_H 6.1	0.0857	1.0	0.0	0.97
			2.0	-12.8	1.04
			3.0	-20.9	0.96
			5.0	-33.1	0.98
			7.0	-40.9	1.00
			8.5	-44.2	1.01
			Mean		0.99

Exp. 6 (Table II, but not included in the graph) was similar to the others, but the reaction was allowed to proceed without added buffer, the p_H being 6.1. Except that the value of k_s was lower, the results, including the specific rotation of the fructofuranose, were similar to those of Exps. 1-5.

The readings of the mutarotating sugars (curve (b), Fig. 1) lie, save for an initial lag, on a straight line, which on extrapolation, cuts the ordinate at a point higher than the theoretical initial reading (checked by controls) of the sucrose before commencement of the hydrolysis. However, in some 40 experiments in which the rotation of the enzyme-sucrose mixture was read 20-30 seconds after mixing, no initial rise in rotation was ever observed. Each curve (b) when plotted from the data furnished in Tables I and II exhibits an initial lag which is not exhibited by the corresponding curve (c), and is not due therefore to a tardiness in the production of glucose and fructose. The curve (b) does not pass below the curve (a) until this lag is over. At the point of separation, the specific rotation of the fructose is exactly zero, and is subsequently negative. Before this point, it must have been positive. This accounts for the lag in curve (b). If the curve (b) fell below curve (a) directly from the theoretical initial reading, it is evident that the fructose would always exhibit a negative rotation.

It will be seen that the fall in specific rotation is less rapid for a rapid hydrolysis than for a slow one. If the change from (2, 5)-fructose to equilibrium fructose is unimolecular, the velocity constant k_F can be calculated. To deduce the values of k_F at various points along each curve in Fig. 2, and to show

that k_F is not only constant for each individual curve, but the same for all the curves (*i.e.* independent of k_S), would furnish striking confirmation of the validity of the deductions by which the curves themselves were derived.

Fig. 2. The specific rotation of fructose produced unimolecularly in the hydrolysis of sucrose by fructosaccharase, at 17° , p_H 4.6.

Calculation of k_F .

By means of equation 3, the actual rotation of the glucose present could be evaluated. If the change from (2, 5)-fructose to equilibrium fructose is unimolecular, we have a reaction comparable in its manifestation with the mutarotation of the glucose. By substituting fructose constants in equation 3, and multiplying each side by $v/\frac{20}{19}q$ since we are now dealing with specific rotation, an equation is obtained which represents the course of the curves in Fig. 2,

$$\rho \cdot \frac{v}{20} = \frac{av}{2 \cdot 20 \cdot vq} \left(P_\infty + \frac{k_{Sc}}{k_S + k_F} - P_\infty e^{-k_S t} - \frac{k_{Sc}}{k_S + k_F} \cdot e^{-(k_S + k_F)t} \right),$$

$$i.e. \quad P = \frac{19}{40} \cdot \frac{a}{q} \left(P_\infty + \frac{k_{Sc}}{k_S + k_F} - P_\infty e^{-k_S t} - \frac{k_{Sc}}{k_S + k_F} \cdot e^{-(k_S + k_F)t} \right) \quad \dots (4).$$

This equation has no formal solution. By rearranging,

$$e^{-(k_S + k_F)t} = 1 + \left[P_\infty - P_\infty e^{-k_S t} - P \cdot \frac{40q}{19a} \right] \left[\frac{k_S + k_F}{k_{Sc}} \right],$$

and equating y with each side of this equation and solving graphically, the value of k_F can be found. The values substituted were, P_∞ at $17^\circ = -92.3^\circ$, $P_0 = +17^\circ$, $c = +17 - (-92.3) = +109.3^\circ$, and P at any arbitrary time t , was read from

Fig. 2. Graphical solution was effected in this way for one or two values of t in each curve of Fig. 2. It was found to vary only between the values 0.28 and 0.35. It was now necessary to see whether the substitution of the mean value of k_F , viz. 0.31, in equation 4 at every arbitrary time point of the curves of Fig. 2 would give an accurate identity in all cases.

Rewriting equation 4 as

$$\underbrace{\frac{40 Pq}{19 a} + P_{\infty} e^{-k_S t} - P_{\infty}}_{\text{L.H.S.}} = \underbrace{\frac{k_S c}{k_S + k_F} (1 - e^{-(k_S + k_F) t})}_{\text{R.H.S.}} \quad \dots\dots(5),$$

and substituting $k_F = 0.31$, and finding the ratio L.H.S./R.H.S. we obtain an idea of the extent of variation from the unimolecular ideal. It is obvious from the values of this ratio given in Tables I and II that there is only variation within reasonable limits and therefore that the assumption that the fructofuranose transformation is unimolecular is correct.

Between p_H 4.6 and 6.1, $k_F = 0.31$. The change of fructofuranose to equilibrium fructose is therefore by no means instantaneous. It is 27 times as fast as the mutarotation of α - or β -glucose under the same conditions, and 3 times as fast as that of β -(2, 6)-fructose.

Assuming, as seems most probable, that the liberated fructofuranose is in the β -form, its subsequent transformation may conceivably proceed in either or both of two directions, (1) to α -(2, 5)-fructose, (2) to (2, 6)-fructose. The latter transformation, which must involve the opening of the ring and temporary formation of the keto-form (or a hydrate of this) would be relatively rapid owing to the instability of β -(2, 5)-fructose. The former change, even if proceeding independently, would not affect the latter since, presumably, both (2, 5) forms would tend to pass through the keto-form to (2, 6)-fructose. Finally the keto-form would yield α - and β -(2, 6)-fructose directly in normal equilibrium proportions so that the relatively slow reaction velocity of mutarotation of (2, 6)-fructose would not limit the rate of change of fructofuranose to equilibrium.

There are certain reasons, into which it is not necessary to go, for believing that equilibrium fructose contains a small proportion of one or both of the (2, 5)-forms. If this is so, the change from β -(2, 5)-fructose through the keto-form to $\alpha\beta$ -(2, 6)-fructose would cease at an equilibrium point, with a relatively small residual quantity of (2, 5)-fructose which would finish presumably as $\alpha\beta$ -(2, 5)-fructose, the β -(2, 5)-form having changed to α -(2, 5)-form either through the keto-form or some other intermediate form.

The hydrolysis of sucrose may then be represented thus:

EXPERIMENTAL.

The sucrose employed was pure; $[\alpha]_D + 66.5^\circ$. The fructosaccharase was prepared from brewer's top yeast by the method of Nelson and Born [1914]. It was a clear colourless liquid, freed from inorganic salts by dialysis, rotation

+1.24° (1 dm.), contained 1.56 g. of solid matter per 100 cc. and possessed no maltase activity. It was stored at -5° with no loss in activity.

Preliminary experiments showed that:

(1) the fructosaccharase preparation had no influence on the rotation of fructose;

(2) ammonia in the concentration employed in the main experiments, completely arrested hydrolysis and had no effect on the rotation of the enzyme preparation or of sucrose, glucose or fructose;

(3) the fructosaccharase *plus* ammonia, in the same concentrations as used in the main experiments, had no influence on the rotation of sucrose.

100 cc. of 20 % sucrose (*i.e.* 107.7 g.) were mixed with 10 cc. of *N*/10 acetate buffer (p_H 4.6) and allowed to attain room temperature (17°) by standing in a bath of water for 2 hours. The enzyme preparation was similarly brought to room temperature. At time zero, 40 cc. of the enzyme solution (the exact weight was observed and the volume calculated) were added rapidly from a cylinder, and thorough mixing was achieved after 5 seconds. A polarimeter-tube with water jacket at room temperature was quickly filled and the rotation taken 20–25 seconds after mixing and at subsequent intervals. Meanwhile 15 cc. portions were withdrawn from the main portion of the reaction mixture and run into a series of small flasks contained in a water-bath at room temperature. To these at intervals 1 cc. of *N* ammonia was added with shaking, the exact time being noted after 0.5 cc. had been run in. The final rotations of the mixtures were read in a 2 dm. tube at 17° about 30 minutes later.

The success of the experiment depends entirely on temperature control, that of the main reaction mixture must not differ from that of the portion in the polarimeter-tube by more than 0.1–0.2°. The jacket of the tube was fed with water circulating through the bath and all pipettes were fitted with rubber shields.

It will be seen that the individual experiments differ only in regard to the amount of enzyme used. It was found to be of no advantage to use less than 20 cc. of enzyme (diluted to 40 cc.) ($k_s=0.07$) or more than 40 cc. ($k_s=0.11$) since in the latter case the hydrolysis was too fast to allow of accurate polarimetry.

SUMMARY.

1. The specific rotation (17°, p_H 4.6–6.1) of (2, 5)-fructose is found to be between +17° and +15°.

2. The change of fructofuranose to equilibrated fructose is shown to conform to a unimolecular law. The unimolecular velocity constant $k_{F\ 17^\circ}^{(mins.^{-1})}=0.3$.

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