[CONTRIBUTION FROM THE BUREAU OF CHEMISTRY, U. S. DEPARTMENT OF AGRICULTURE.][1]

THE INVERSION OF CANE SUGAR BY INVERTASE. III.

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The present article is a continuation of Parts 1 and 2[2] of an investigation on the laws of action of the enzyme invertase which hydrolyzes cane sugar to glucose and fructose. In the second article of this series it was shown that the glucose which is liberated from cane sugar by the enzymic action is α-*d*-glucose, of specific rotation 100°. The experiments which will now be described were made in order to learn what form of fructose is simultaneously liberated.

1. *The Slow Rotatory Changes which Follow the Nearly Instantaneous Inversion of Cane Sugar at 0° by Invertase.*—The previous work has shown that the α-glucose which is liberated from cane sugar by invertase changes gradually and partially to β-glucose at the temperature of 30°, and the rate of this change is identical with the rate of mutarotation of glucose. Reasoning by analogy, it was expected that the similar change of the freshly liberated fructose would proceed with a velocity which is equal to that of the mutarotation of fructose, and since this change is about ten times as rapid as the mutarotation of glucose, it is obvious that it proceeds too fast to be accurately detected at 30°. The temperature was accordingly lowered to 0° for these experiments in anticipation that the mutarotation of fructose would be so much retarded at this low temperature that the slow change of the freshly liberated fructose to its stable condition could be clearly observed.

In the following two experiments the inversion of cane sugar at 0° has been accomplished almost instantaneously by using a considerable concentration of a very active invertase solution, which was prepared by the autolytic digestion of yeast at 25,° in the manner that has been previously described. This very rapid inversion has made the interpretation of the slow changes of rotation which go on for hours and hours after the mixing of the invertase with the cane sugar solution quite simple, because they may be considered as due in no wise to any gradually proceeding inversion but entirely to the change of the freshly liberated fructose and glucose to their stable state. In Table I are

[1] Published by permission of the Secretary of Agriculture.

[2] THIS JOURNAL, 30, 1160–6, 1564–83 (1908).

the values of the slowly changing rotatory powers of the two solutions of cane sugar which were inverted almost instantly at 0° by invertase. The rotations are for sodium light, the length of liquid observed was 400 mm., the rotation due to the invertase has been corrected for (+0.70° in the first experiment and 2.70° in the second), and the observed readings have been referred to 66° as the specific rotatory power of cane sugar. If it is ever desired to find the readings which were directly observed, this may be done for the first experiment by dividing the values of column 2 by 5.20 and adding the invertase correction, 0.70°, to the quotient, and for the second experiment by dividing by 4.65 and adding 2.70°. The solution used in the first experiment was prepared by mixing at the time marked zero, 1 liter of nearly 5 per cent. cane sugar solution with 100 cc. of invertase solution, both being previously cooled to 0° and filtered to permit clear polariscopic readings. The invertase solution had a dark red color when freshly pressed from month-old yeast, but this color was reduced to a very pale yellow by filtration through animal charcoal without there occurring any appreciable loss in its inverting power. This slight remaining color did not interfere at all with the polariscopic readings, which were particularly clear. The solution used in the second experiment was prepared by mixing one liter of 8 per cent. cane sugar solution with 500 cc. of the same invertase solution. In the first experiment a portion of the solution was removed four minutes after the mixing, and its rotatory power measured after the addition of sodium carbonate to alkaline reaction, in order to determine the real rate of inversion. It was found that 52 per cent. of the cane sugar had been inverted during the first four minutes, which shows that the real rate of inversion was so rapid that it may be regarded for the purposes here in view as instantaneous in comparison with the slow changes of rotation which are shown in the table. Especially is this conclusion accurate in the case of the second experiment, since five times as much invertase was there used, and the real rate of inversion was accordingly several times faster than in the first experiment, since the rate of inversion has been shown to be accurately proportional to the concentration of the invertase. The solutions under polariscopic study were kept at 0° in a brass tube immersed in a trough of melting ice[1] and in no case did the temperature of the solution differ more than 0.2° from zero.

The specific rotatory power of inverted cane sugar at 0° is —28.2, or, in other words, this is the rotation which is shown at 0° by a solution of inverted cane sugar which before inversion rotated +66°. Since the measurement of the real rate of inversion in the two experiments of Table

[1] This excellent apparatus was used and described by Dr. H. W. Wiley in an investigation on the influence of temperature on the rotatory power of cane sugar. THIS JOURNAL, 21, 568.

I shows it to be very rapid for the first and practically instantaneous for the second, one would expect that the rotation would drop almost instantly on mixing the cane sugar and invertase solution from 66 to —28.2, if the first products of the inversion constitute normal or stable

TABLE I.—SLOW ROTATORY CHANGES FOLLOWING THE NEARLY INSTANTANEOUS TOTAL INVERSION OF CANE SUGAR AT 0° BY INVERTASE.

Experiment I.		Experiment II.	
Time after mixing. Min.	Specific rotation.	Time after mixing. Min.	Specific rotation.
0	66.0	0	66.0
5	56.1	1	57.6
7	51.5	3	50.2
10	45.2	4	43.7
		5	37.2
15	35.2	6	33.3
20	27.6	7	27.2
25	20.7	8	23.9
30	16.6	10	19.3
35	12.9	12	13.5
40	9.8	15	8.8
45	7.6	20	4.2
50	5.6	25	1.1
70	2.0	35	—1.4
80	1.0	45	—3.2
100	—1.2	65	—7.0
120	—1.6		
150	—4.4		
180	—5.7		

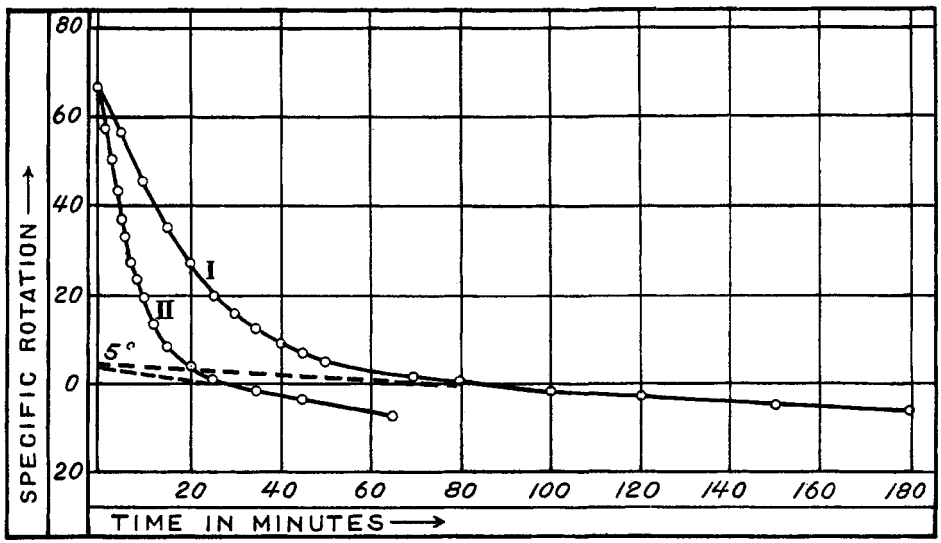

Fig. 1.—Slow rotatory changes following the nearly instantaneous inversion of cane sugar at 0° by invertase; (I) in faintly acid solution, (II) in stronger acid.

invert sugar. This conclusion is, however, wholly unfulfilled, for the rotation shows no instantaneous change at all, but instead a moderately rapid decrease for the first few minutes and then a very slow decrease which lasts through many hours. It is evident that the freshly formed invert sugar is not in its stable condition, and does not show the normal rotatory power of stable invert sugar solutions. The obvious interpretation of the irregular change in the rotation following instantaneous total inversion by invertase at zero is that the first rapid drop of rotation is due to the change of the freshly formed fructose to its stable state and the succeeding very slow change is due to the partial change of the freshly formed α-glucose to β-glucose. Following this interpretation, the rotatory powers of α-glucose and fresh fructose can be found from the data of these two experiments.

These data are also shown graphically in Fig. 1.

2. The Rotatory Power of α-Glucose at 0°.—After the first rapid change of rotation caused by the passage of fresh fructose to its stable form is over, the curves of Fig. 1 show a slow linear change of rotation, due to the partial change of α-glucose to β-glucose. If these linear portions of the curves be extended to the time for the start of the experiment, as shown by the dotted lines, they agree in intersecting the axis of rotation at 5°. This value is accordingly the rotatory power of a solution containing pure α-glucose and stable fructose in equal quantities, but as the inversion of cane sugar binds one mol. of water per mol. of sugar, the specific rotation corresponding to the 5° is $(5)(342/360) = 4.75°$; since this correction is, however, less than the uncertainty of the linear extrapolation, its introduction is not important. The specific rotation of stable fructose solutions at 0° is —101°, and calling the specific rotation of α-glucose X we have, since 1 gram of cane sugar gives on inversion 0.525 gram fructose and glucose respectively, $(-101)(0.525) + (X)(0.525) = 4.75$, or $X = 110°$. This value for the specific rotatory power of pure α-glucose agrees almost exactly with the directly observed value of 109° at 20°–30°. As the extrapolation by which the value 5° or 4.75° is obtained is probably accurate within one degree, and as this error would change the calculated rotatory power of α-glucose only two degrees, this method of calculating the rotatory power from the nearly instantaneous inversion at 0° is quite accurate, and the results exactly confirm the conclusion that was drawn from the experiments at 30°, namely, that the glucose which is liberated from cane sugar by the action of invertase is α-glucose, of specific rotatory power 109°.

3. Proof that a New Form of Fructose, α-Fructose, is Liberated from Cane Sugar by the Action of Invertase.—The first rapidly changing portions of the curves of Fig. 1 are considered here to be due to the changing of fresh fructose to its stable form. If this interpretation is correct,

this portion of the curve should follow the law of unimolecular reactions and the velocity coefficient of this portion should be identical with that of the mutarotation of fructose. In order to test this conclusion there are given in Table II for each of the two experiments of Table I:

(I) The course of the initial portion of the curve considered as a unimolecular reaction whose end is at 5° rotation;

(II) The velocity coefficient (k) for this course, calculated from the usual formula $k = 1/t \log_{10} r_0 - r_\infty / r - r_\infty$, and

(III) The course and velocity coefficient of the mutarotation of pure crystalline fructose when dissolved in the solution.

TABLE II.—THE UNIMOLECULAR ORDER OF THE INITIAL REACTION AND ITS IDENTITY WITH THE MUTAROTATION OF FRUCTOSE.

Experiment I.

First change after inversion, from Table I.			Mutarotation of fructose dissolved in same solution.		
Time in min.	Rotation.	$k=1/t \log_{10} r_0 - r_\infty / r - r_\infty$.	t.	r.	k.
0	66.0(r_0)	...	0	−45.4(r_0)	...
5	56.1	0.015	3	−44.3	0.027
7	51.5	0.017	8	−43.2	0.023
10	45.2	0.018	13	−42.3	0.022
15	35.2	0.020	16	−41.7	0.023
20	27.6	0.022	26	−40.8	0.021
25	20.7	0.024	∞	−39.0(r_∞)	...
∞	5.0(r_∞)	...	..	...	...
	Average,	0.019		Average,	0.023

Experiment II.

0	66.0(r_0)	...	0	−30.7(r_0)	...
3	50.2	0.043	2	−29.7	0.062
4	43.7	0.049	5	−28.4	0.074
5	37.2	0.055	10	−27.6	0.065
6	33.3	0.056	17	−27.0	0.066
7	27.2	0.063	∞	−26.7(r_∞)	...
8	23.9	0.064			
10	19.3	0.063		Average,	0.067
∞	5.0(r_∞)	...			
	Average,	0.056			

The velocity coefficient k (see column three) for the first change after inversion is approximately constant,[1] which proves the change to be a unimolecular reaction and the nearness of the numerical value of this

[1] The slight increase in k during the reaction would be largely eliminated if the correction for the small quantity of β-glucose that is produced from α-glucose were introduced. But this refinement is hardly necessary for the present purpose. Also, the fact that the rate of the real inversion, which is here assumed to have been instantaneous, was not quite so, would largely account for the slight increase of k during the reaction.

coefficient to that for the mutarotation of fructose dissolved in the same solution, the corresponding values being 0.019 and 0.023 for the first experiment and 0.056 and 0.067 for the second, proves that the first change after inversion is indeed the mutarotation of the fresh fructose which is initially liberated from cane sugar by the action of invertase. That the coefficient k has different values for the two inversion experiments is due to the fact that the invertase contained some acetic acid, and since five times as much invertase liquor was used in the second experiment as in the first, its acidity was greater and consequently the rate of mutarotation of fructose in it was greater than in the first. This fact strengthens the above proof of the identity of the first change after inversion with the mutarotation of fructose, for it shows that the rates of these reactions are almost equal not purely by chance, for they remain almost equal when their absolute values are largely changed by the presence of increasing amounts of a catalyzer. For the mutarotation of pure fructose dissolved in distilled water at 0° I find the velocity coefficient to be 0.057; we therefore have the following changes in this coefficient at 0° with increasing acidity, 0.057 for pure water, 0.023 for very weak acetic acid, 0.067 for stronger acetic acid. These results show that the rate of mutarotation is decreased by small acidity and increased by greater, which is exactly what was found to be the case at 30°. Though this influence of acids on the mutarotation of fructose is thus quite peculiar, the velocity of the first change after inversion conforms to it exactly, proving in a very clear manner that the two reactions have the same cause, namely, the change of fresh fructose to its stable form. The specific rotatory power of the fresh fructose which is liberated from cane sugar by the action of invertase can be found as follows: Since there is no nearly instantaneous change in the rotatory power of cane sugar solutions when they are nearly instantaneously inverted by strong invertase at 0° (see Table I) the initial products of inversion must have rotatory powers whose average just equals that for cane sugar. It is true that there is a moderately rapid drop in dextrorotation during the first half hour after the inversion, but the facts given above show that this drop is due to the mutarotation of the freshly liberated fructose. The specific rotation of one of the products of the inversion of cane sugar, α-glucose, has been shown to be 109°; the specific rotation of cane sugar is 66°, and consequently if the specific rotation of the freshly formed fructose is X, we have $(109)(0.525) + (X)(0.525) = 66°$, since one gram of sugar produces by inversion 0.525 gram of glucose and of fructose, and hence $X = 17°$. Only one form of fructose has ever been isolated in the crystalline condition, β-fructose of specific rotation -140 at 30° changing on standing to $-90°$. This new form of fructose of specific rotation 17° is, therefore, the α-fructose whose

existence has been considered probable from the fact that fructose shows the phenomenon of mutarotation and is structurally closely related to glucose, arabinose, lactose, and galactose, for all four of which the existence of α- and β-forms has been established. In a previous article I gave a calculation of the specific rotatory power of this α-fructose, which showed the value $-77°$. This calculation seems thus to be quite incorrect, though I am unable to say which of the assumptions made in it has caused the error. At a later time I hope to return to this discrepancy.

4. The Bearing of These Results on the Relations among the Sugars Glucose, Fructose, Cane Sugar, Raffinose and Melibiose.—The experiments described above show that cane sugar has a specific rotation, 66°, which is additively related to those of its components, 109° for α-glucose and 17° for α-fructose. This conclusion can be tested in an independent manner as follows: The sugar raffinose is hydrolyzed by invertase to melibiose and fructose, and melibiose is hydrolyzed by acids to galactose and glucose. On the other hand, raffinose is hydrolyzed by the enzyme of almonds, emulsin, to galactose and cane sugar, and acids hydrolyze raffinose to galactose, glucose and fructose. These facts all show that raffinose has the structure

$$galactose < \alpha\text{-}glucose < > \alpha\text{-}fructose,$$

in which galactose and glucose are united in the same manner as in melibiose, which is $galactose < glucose <$, and glucose and fructose in the same manner as in cane sugar, the symbol $<$ or $>$ denoting the carbonyl group of the monose. If the above conclusion that the rotatory power of cane sugar is the sum of the rotations of its components is correct, then in similar manner the rotatory power of raffinose should be equal to those of its similar components, melibiose and fructose, since the union between fructose and the glucose residue of melibiose is the same as the union between the two hexoses in cane sugar. As there are two forms of melibiose, the α- and β-modifications, which obviously correspond to the α- and β-forms of the glucose residue in melibiose, theory predicts that the rotatory power of raffinose is the sum of those of α-melibiose (corresponding to α-glucose) and α-fructose. The specific rotation of β-melibiose has been observed by Loiseau to be 124°, from which I have calculated in a recent article that that of α-melibiose is 171°. The specific rotation of α-fructose is shown in what precedes to be 17°. The molecular weight of melibiose is 342, of fructose 180, of anhydrous raffinose 504, hence the specific rotation of anhydrous raffinose is calculated to be $((171)(342) + (17)(180)) \div 504 = 122°$. The specific rotation of anhydrous raffinose has been found to be 124° by direct observation.[1] This close agreement is an entirely independent proof that the union of α-glu-

[1] Lippmann, *Z. Ver. d. Zuckerind.*, **35**, 257.

cose and α-fructose to give cane sugar does not appreciably alter the rotatory powers of these sugars.

Although the specific rotation of cane sugar is additively related to those of its components, α-glucose and α-fructose, this simple relationship does not hold in general for the polysaccharides; thus maltose, which is composed of two molecules of glucose, has such a high specific rotation, 166° for α-maltose and 119° for β-maltose, that no additive combination of the specific rotations of the α- and β-glucoses, 109° and 20°, respectively, can give these higher numbers. Similarly, the specific rotations of the α- and β-melibioses are so large that they cannot possibly be additively related to those of the constituent sugars, galactose and glucose. But it appears to hold in general that the union of α-glucose to α-fructose, whether this union give cane sugar or raffinose, carries with it an additive relation between the rotatory powers of the constituents and their compound. It is also a fact, probably a closely related one, that α-glucose and α-fructose always combine to give a non-reducing, non-mutarotating sugar, for cane sugar, raffinose and stachyose, the only polysaccharides which contain glucose and fructose, do not reduce Fehling's solution nor show mutarotation nor combine with phenylhydrazine. The converse is also true, for these three are the only sugars which do not possess these three properties. The data on stachyose are not sufficient to permit calculations similar to those for raffinose.

5. *The Relative Rates of Fermentation of the α- and β-Forms of Glucose by Yeast.*—The antipodal stereoisomer of *d*-glucose, *l*-glucose is not fermented at all by yeast; since the fermentation is thus influenced so greatly by any difference in structure, it was decided to investigate the relative rates at which yeast attacks the α- and β-modifications of *d*-glucose. The following simple experiment was made with this purpose in view: A half liter of a ten per cent. *d*-glucose solution was heated to boiling and then kept at 25° for two hours, in order to establish equilibrium between the α- and β-forms of the sugar. One hundred grams of washed brewers' yeast were then added to the glucose solution and the fermentation proceeded rapidly at 20°–25°. Immediately after the mixing of the yeast and solution, a portion was filtered off and its rotation in a 200 mm. tube was found to be 24.6° V, and this value remained unchanged after the filtered solution was made alkaline with sodium carbonate, which proves that the original solution contained the α- and β-forms of glucose in the proportions in which they exist in equilibrium. After fermentation had gone on during two hours a filtered portion showed the rotation 16.8° V, which became 17.5° when sodium carbonate was added; after fermenting two hours longer, the rotation of a filtered sample was 13.3° V, becoming 13.8° V with sodium carbonate; after fermenting one more hour, the reading was 11.2, becoming 11.6

when the alkali was added. After fermenting twenty-four hours in total, the solution gave the rotation 1.4° V, and this was not changed when sodium carbonate was added. Now, if yeast ferments α-glucose more rapidly than it does β-glucose, the former will be removed more rapidly than the latter and the equilibrium which existed in the original solution before fermentation started will be disturbed; if a portion of the fermenting solution in which the equilibrium is disturbed is treated with sodium carbonate in order to reestablish the equilibrium, there will be an increase of dextrorotation resulting from the addition of the alkali, since α-glucose is more strongly dextrorotatory than is β-glucose. On the other hand, if yeast ferments β-glucose more rapidly than it does α-glucose, there will be a decrease of dextrorotation on the addition of alkali to reestablish equilibrium between the two forms. The experiment above described shows that there is a very slight increase of rotation on adding sodium carbonate, which might be taken to indicate that yeast attacks α-glucose a trifle faster than it does β-glucose. It must be remembered, however, that alcohol is produced in the fermenting solution and it must be considered whether the presence of this alcohol can cause a slight change in the equilibrium proportions for the α- and β-forms, and thus give rise to the increases of rotation which were observed. To test this question a 6.7 per cent. solution of glucose was heated to boiling and then kept at 25° three hours to establish equilibrium, and absolute alcohol was then added to it to the strength of 1.7 per cent., making its composition closely that of the above-described solution which had fermented during two hours. Immediately after adding the alcohol the rotation was 14.8°, and when sodium carbonate was added to alkalinity it remained exactly at this value, which proves that the alcohol produced during the fermentation is not the cause of the slight changes of rotation noted above. The changes cannot be due to any neutralization of optically active acids by the sodium carbonate, since no change was observed on the solution after complete fermentation, the rotation being 1.4 both before and after adding sodium carbonate. It thus remains that the changes of rotation observed when the fermenting solution is neutralized must be due to a displacement of the equilibrium between the α- and β-forms of glucose caused by a more rapid action of the yeast on α-glucose than on the β-form. Yeast appears therefore to ferment α-glucose a trifle faster than it does the β-form.

Summary.

The contents of this article, which is a continuation of two former ones bearing the same title, may be summarized as follows:

By the use of a concentrated and strongly active invertase solution cane sugar has been inverted almost instantaneously at 0°; the rotation of the cane sugar solution did not change immediately after inversion

to the usual rotatory value for invert sugar, but a peculiar series of rotation changes lasting through many hours was observed, though the final rotation was that of invert sugar. The quantitative interpretation of these rotatory changes shows that the substances which are initially liberated from cane sugar by invertase are α-glucose, specific rotation 109°, and a new form of fructose, α-fructose, specific rotation 17°. The inversion of cane sugar by invertase thus follows the order

$$\begin{array}{ccc} & & \alpha\text{-fructose} \rightleftarrows \beta\text{-fructose} \\ & \uparrow & \\ \text{Cane sugar} & & \\ & \downarrow & \\ & \alpha\text{-glucose} \rightleftarrows \beta\text{-glucose} & \end{array}$$

The experiments show that the rotatory power of cane sugar (66°) is equal to the sum of those of its constituent sugars, α-glucose (109°) and α-fructose (17°); in similar manner the rotation of raffinose (124°) is equal to the sum of those of its constituents, α-melibiose (171°) and α-fructose (17°). If the symbol $<$ denote the free carbonyl group of the aldehyde and ketone sugars the constitution of cane sugar and of raffinose can be written, *cane sugar* = *α-glucose* $<>$ *α-fructose*, and *raffinose* = *galactose* $<$ *α-glucose* $<>$ *α-fructose*. It is not known yet whether the first member of raffinose is α- or β-galactose, though it is probable from the fact that the enzyme emulsin splits raffinose to give galactose and cane sugar, that the member is β-galactose.

Lastly, it is shown that brewers' yeast attacks the α- and β-forms of glucose at very nearly the same rate, though there is an indication that the α-form is fermented slightly more rapidly.

SOME ORGANIC TUNGSTATES.

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Freshly prepared tungstic acid, H_2WO_4, dissolves readily in aqueous solutions of most aliphatic amines, with the formation of substituted ammonium tungstates. From the resulting solutions, the salts crystallize out on evaporation, except in some cases where it is first necessary to evaporate to dryness, drive off the excess of amine at about 105°, and then crystallize. Of the salts described in this paper, all but two are readily soluble in water. Ethylenediammonium tungstate is difficultly soluble after it has once crystallized out. Diamylammonium tungstate dissolves with difficulty in water, but readily in methyl alcohol.

If any of these salts are heated slowly, the amine is driven off and at the same time the tungstic acid is partially reduced to the blue oxide, which, on further heating, glows and quickly changes to the yellow WO_3.