in frozen beef after storage which would distinguish it from fresh beef, a frozen beef knuckle six hundred and ten days old was boiled without seasoning and eaten. The flavor was identical with that of fresh beef nor was it possible for one not knowing the age or source of the meat to distinguish it from fresh meat. We may say that similar tests of frozen poultry have resulted similarly.

On the whole the results of the various lines of work reported in this paper, chemical, histological and bacteriological, indicate that cold storage, at temperatures below -0.9° C. at least, is an adequate and satisfactory method for the preservation of beef for a period of five hundred and fifty-four days and probably for a much longer time.

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

THE INVERSION OF CANE SUGAR BY INVERTASE, II.[1]

BY C. S. HUDSON.

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CONTENTS.

1. *Summary of Previous Work on the Laws of Action of Invertase.*—In most plants and animals there occurs a substance that changes cane sugar to invert sugar, which is a mixture of equal parts of dextrorotatory *d*-glucose and levorotatory *d*-fructose.[2]

This inverting substance, which plays such an important part in plant and animal life, has never been obtained in a pure condition because it does not crystallize from its solutions. Although there is some evidence indicating it to be an albuminoid its exact composition is unknown. Its synthesis takes place exclusively in the tissues of living organisms. Its presence in plants and animals is known only from the fact that cer-

[1] Published by permission of the Secretary of Agriculture. Read at the New Haven meeting of the American Chemical Society, June 30–July 2, 1908.

[2] For proof of its very wide if not universal distribution in the plant world see Kastle and Clark, *Amer Chem. J.*, 30, 422–427 (1903). It occurs in the intestinal walls of all mammalian animals that have been examined. For its occurrence in bees, locusts, butterflies, spiders and many other insects (excluding, however, the silk worm) see Axenfeld, *Zentralblatt für Physiologie*, 17, 268 (1903). O'Sullivan and Thompson state that "probably all organisms which have the power of assimilating cane sugar contain invertase."

tain of their juices invert cane sugar and that this action can be prevented by previously heating the liquid to boiling, which is the general process for destroying enzymes. Acids also invert cane sugar, but their activity is in no wise diminished by boiling. This enzyme, or possible mixture of enzymes, has been named invertase (called also invertin and sucrase), and although its composition is unknown the laws of its action can nevertheless be accurately studied. Several such investigations have been made during the last twenty years, but owing to an oversight the experimental method by which the subject was generally studied involved a source of large error and the conclusions of the various workers are generally erroneous and discordant. In the first paper of this series[1] a description of the error in question, namely, the neglect of the mutarotation of the invert sugar, was presented in detail and reference may accordingly be made to that article for further information on this point. Notwithstanding this error in the method of measurement of the inversion, some of the conclusions of previous observers remain valid when it is corrected. A summary of these conclusions will now be given, which is restricted, however, to the quantitative laws of the action of invertase. For a summary of the present state of knowledge on the occurrence, preparation, partial purification, and selective action of invertase, reference may be made to any of the several treatises on enzymes.

That the inversion by invertase is a catalytic reaction in the course of which the activity of the invertase is not altered or destroyed was first proved by the experiments of O'Sullivan and Tompson[2] on the unimolecular order of the inversion reaction, but is more directly shown by the experiments of Henri,[3] in which cane sugar was added after different intervals of time to portions of a cane sugar solution which was undergoing inversion by invertase; it was found that the added cane sugar in each case began to be inverted by the invertase at the same rate, irrespective of how much sugar the invertase had already inverted. That the inversion by invertase is a reaction of the first order was shown by O'Sullivan and Tompson from experiments in which the error above referred to was avoided. Their conclusion that the reaction is unimolecular was afterward disputed by Duclaux[4] and has since then been generally considered to be conclusively disproved by the later work of Henri.[5] As the writer pointed out, however, in the previous article, the experi-

[1] THIS JOURNAL, **30**, 1160–1166 (1908).

[2] *J. Chem. Soc.*, **57**, 834–931 (1890). The following frequent references to these authors all refer to this article.

[3] *Z. physik. Chem.*, **39**, 194–216 (1901).

[4] *Traité de Microbiologie*, Vol. 2, 129–169 (1899).

[5] Lois générales de l'action des diastases, Paris, 1905.

ments of Henri on the order of the inversion are erroneous on account of the neglect of the mutarotation and there is no longer any valid reason for not accepting the conclusion of O'Sullivan and Tompson that the inversion is a reaction of the first order. The rate of action of invertase is proportional to the quantity of this substance present; this was first shown by O'Sullivan and Tompson. And in dilute solutions the rate is proportional to the concentration of the sugar, but in stronger solutions (above 5 per cent.) it is not even approximately proportional but decreases and becomes practically zero in the strongest solutions; this behavior has been described by nearly all investigators and has been considered by Duclaux[1] as excluding the reaction from the unimolecular class. Against this objection it is to be said that proportionality between the rate and the concentration of sugar is not to be expected from the theory of unimolecular reactions except in dilute solutions and there such proportionality does hold. The rate is indeed proportional to the concentration of the sugar to about 0.1 molal and from evidence soon to be published the present writer regards as correct the hypothesis of O'Sullivan and Tompson that the decrease of the rate in concentrated sugar solutions is due principally to the high viscosity of the medium. Lastly, O'Sullivan and Tompson showed that the rate of inversion by invertase increases rapidly with the temperature but that above 50–60° the invertase is rapidly destroyed by the heat.

These are the fundamental facts that have been established by experiments in which the usual error of the measurement caused by neglecting the mutarotation of the invert sugar was avoided; namely, the purely catalytic action of the invertase, the proportionality of the rate of inversion to the concentration of the invertase and of the sugar in dilute solution, the lack of proportionality to the sugar concentration in strong solutions, the unimolecular character of the inversion, and the acceleration of the rate by increase of temperature. With exception of the peculiarly slow rate of inversion in concentrated sugar solutions the above facts are fully analogous to the laws of the common catalytic reactions of chemistry, a well-known type of which is the inversion of cane sugar by acids.

2. *Purpose of This Investigation.*—The present investigation was undertaken in order to learn the general laws of the action of invertase on cane sugar. Most of what are at present called the laws of action of invertase are only empirical formulas of no theoretical significance, and they are generally erroneous even as far as they go, because the measurements of the inversion by invertase have almost always been made by the polarimetric method without avoiding its serious error of mutarotation. It is necessary, therefore, to start again at the beginning, and by a correct

[1] *Loc. cit.*

method of measurement, to study the action of invertase. The author holds that some of the laws of its action have already been established (see previous section) particularly through the classical investigation of O'Sullivan and Tompson, but as their results have repeatedly been disputed by other investigators, who, however, neglected the error caused by the mutarotation, it is believed that a second investigation of them is required to decide conclusively between the contradictory views.

3. *Preparation of the Invertase.*—In nearly all of the experiments described in this article a solution of invertase was used which was prepared by the autolytic digestion of yeast according to the method of O'Sullivan and Tompson. Ten pounds of fresh brewers' yeast were washed and drained on a Buchner funnel until the mass became firm and it was then set away in a covered glass jar for two weeks at an average temperature of 22°. At the end of this time the mass of yeast cells had liquefied and on filtration gave a brown fluid which was then treated with animal charcoal and filtered through a layer of infusorial earth. This filtrate was perfectly clear, it was colored faintly yellow and possessed very strong inverting power. When one volume of it was added to two volumes of a 10 per cent. cane sugar solution at 30° it was found that within the first half minute 75 *per cent.* of the sugar was inverted. The invertase solution showed an optical dextrorotation of 0.25 circular degrees in a 40 cm. tube at 30° with sodium light. It was distinctly acid, a considerable quantity of acid, probably acetic, having been produced in the liquefaction of the yeast. The stock supply of it was saturated with toluene to prevent the growth of micro-organisms and kept in an ice box. After being preserved in this manner for a month, it was still perfectly clear and there was no perceptible change in its color or inverting power.

A second supply of invertase was prepared from brewers' yeast by mixing five pounds of the washed and pressed yeast with twice its bulk of a mixture of equal parts of coarse sand and ground glass, and allowing it to dry in the air for thirty-six hours. The mass was then finely ground in a mill, extracted two hours with a liter of cold water, filtered in a lever press, the extraction and filtration repeated and the filtrates combined and passed by suction through a layer of infusorial earth. The resulting pale yellow yeast extract was preserved in a bottle in an ice box, toluene having been added as an antiseptic. This extract was not so active as the first supply, one volume of it acting upon two volumes of a 5 per cent. cane sugar solution inverted 50 per cent. of the sugar in two minutes, but on the other hand it was free of acid and was therefore more suitable for some of the experiments.

4. *Method of Measuring the Rate of Inversion with the Polarimeter.*—In measuring the rate of inversion of cane sugar by acids the solution

which is undergoing inversion may be kept in the observation tube of the polarimeter, and its rotation read at successive instants; as the change of rotation in this case is proportional to the quantity of invert sugar formed, the equation of the unimolecular reaction may be written $\frac{1}{t} \log \frac{r_0 - r_\infty}{r - r_\infty} = k$, where t is the time at which the rotation is r, and r_0 and r_∞ are the rotations before any and after complete inversion, and k is the velocity coefficient.

In measuring the rate of inversion of cane sugar by invertase the above method is not applicable on account of the fact that in this case the change in rotation is not proportional to the quantity of invert sugar formed. If the experiment be carried on for invertase as described above for the inversion by acids, the polarimetric readings will give a rate of inversion but this rate is the *apparent* rate of inversion only, not the real rate. To obtain the latter rate it is necessary to take into account the mutarotation of the invert sugar, a correction which is negligible in the case of the inversion by acids because these are powerful accelerators of the mutarotation, but which is large (20–50 per cent.) in the case of the inversion by invertase. The influence of this slow secondary reaction upon the rotation could be overcome and the inversion by invertase followed polarimetrically like that by acids, if some substance were known which would greatly accelerate the mutarotation without affecting the action of the invertase, but as acids and bases are the only substances known which powerfully accelerate the mutarotation and as both of these prevent the action of invertase, it seems impossible to measure the real rate directly by the polarimeter from a solution undergoing inversion by invertase. O'Sullivan and Tompson measured the real rate of inversion by invertase by adding a drop of caustic alkali solution at successive intervals to portions of a sugar solution that was undergoing inversion and then observing the reading in the polarimeter. They state that the alkali stops the action of the invertase and brings the mutarotation of the invert sugar to completion almost instantly, so that the rotation of the solution is then a correct measure of the extent of inversion at the time the alkali was added. During the first few of the present experiments, this method of O'Sullivan and Tompson was followed, but it was later given up for the reason that the excess of caustic alkali, which must be used to make certain that the invertase is destroyed, rapidly attacks the invert sugar and the polarimetric readings become quite erroneous. This is shown directly by the following experiment in which 5 cc. of 5 per cent. caustic soda solution were added to 100 cc. of a 20 per cent. solution of pure fructose at 30°. Immediately after the addition of the alkali, the polarimetric reading of the solution was —18.0°, after fifteen minutes —17.6°, after four hours —16.0°, after twenty-four hours

—10.0°, showing that the caustic alkali was rapidly destroying the fructose. On account of this objection to the use of caustic alkali, a 0.2 molal solution of sodium carbonate was tried in its place, as the alkaline carbonates are known to be much less destructive of the sugars. It is true that this strength of sodium carbonate will attack both glucose and fructose at 30°, but the action is so slow that no change in the rotation is noticeable within several hours after the addition of the carbonate. The following experiment shows this and also a point of equal importance, namely, that this strength of sodium carbonate stops the action of invertase instantly. A 10 per cent. solution of pure cane sugar being divided into three portions of 100 cc. each, to the first was added 10 cc. of the sodium carbonate solution, followed by 5 cc. of an invertase solution, to the second 15 cc. of water, and to the third 5 cc. of the same invertase solution, followed after fifteen minutes by 10 cc. of sodium carbonate solution. If the sodium carbonate instantly stops the action of the invertase the first portion should show no inversion, its rotation should be equal to that of the second portion, since the dilutions of the two portions were made the same; this was found to be the case, the rotations of both solutions being 37.75°. That the added invertase was active is shown by the fact that the third portion in which the invertase had acted during fifteen minutes gave a reading which showed that 10 per cent. of the sugar had been inverted during this time. Lastly the rotation of this third portion, after the addition of the sodium carbonate, was constant during at least two hours, showing that no appreciable destruction of the sugars takes place during this time. Other experiments were also made to find the rate of the destructive action of sodium carbonate in the above strength on solutions of cane sugar, glucose, and fructose, respectively. To 100 cc. of each of these three solutions were added 10 cc. of the sodium carbonate solution and the rotations observed during two days, the solutions being kept at 25–30°. The results are given in Table I, from which it is seen that no one of the sugars is appreciably changed within two hours, that cane sugar is unchanged after two days, but that during this time both glucose and fructose have been considerably altered.

TABLE I.—ACTION OF Na_2CO_3 ON CANE SUGAR, GLUCOSE, AND FRUCTOSE.

	Rotation.			
Substance.	Immediate.	2 hours.	24 hours.	2 days.
Cane sugar	50.65	50.65	50.70	50.65
Glucose	10.05	10.05	9.35	8.60
Fructose	—11.82	—11.82	—10.85	—9.95

The mutarotation of both glucose and fructose is strongly accelerated by sodium carbonate, but the strength of the carbonate solution that has been used to stop the action of the invertase is not sufficient to make

the mutarotation instantaneous. Direct experiments in which crystalline glucose and fructose were respectively dissolved in water containing 10 per cent. of the above carbonate solution showed that the rotation had not become quite constant five minutes after the dissolving of the sugar, though seven minutes were in both cases sufficient for the completion of the mutarotation.

The practical rule from these experiments for measuring the real rate of inversion by invertase is to add at a given time 10 cc. of a 0.2 molal sodium carbonate solution to each 100 cc. that is withdrawn from the solution undergoing inversion and to wait longer than seven minutes but not longer than two hours before reading the rotation in the polarimeter. Under these conditions the rotation is a correct indication of the amount of inversion that had taken place at the time of adding the alkali.

The action of invertase has been found by O'Sullivan and Tompson to be very sensitive to small amounts of acid; a trace of alkali will stop its action entirely but acids in small quantities accelerate it. In larger amounts they retard its action and in still greater concentration they again accelerate the inversion, though this last effect is not related to the invertase but is due to the inverting power of the acids themselves (Kjeldahl). The following experiments were made at 30° on the rate of inversion by a constant quantity of invertase of 5 per cent. solutions of cane sugar in pure water and in hydrochloric acid solutions of different strengths. After the invertase had acted eight and fifteen minutes respectively portions of the solutions were removed, sodium carbonate solution added (see above) to stop the action of the invertase and to complete the mutarotation, and the quantity of cane sugar inverted was then estimated from the polarimetric reading of the solution. The data are given in Table II.

TABLE II.—INFLUENCE OF WEAK HCl ON THE ACTION OF INVERTASE.

Experiment No.	1.	2.	3.	4.	5.	6.
HCl concentration mol/liter	Pure water	0.00062	0.0025	0.0050	0.0075	0.010
Rotation after 8 min.	5.20	—0.25	—0.25	—0.40	—0.13	0.40
Rotation after 15 min.	2.36	—2.25	—2.25	—2.25	—2.20	—2.07
$k = \frac{1}{t} \log \frac{r_0 - r_\infty}{r - r_\infty}$ (average)	0.026	0.082	0.082	0.083	0.079	0.073

The rotation at the start (r_0) was the same for all the solutions, 10.20°, and likewise the rotation after complete inversion, (r_∞), —3.11°. In the last line of the table are shown the average velocity coefficients calculated by the usual formula. From Fig. 1, which shows the relation between the velocity coefficient and the concentration of hydrochloric

acid, it is seen that the rate is enormously influenced by small additions of acid to the neutral solution but that for a considerable range of acidity it is practically constant. On account of the extreme sensitiveness of the rate in neutral solutions to small additions of acid it is important

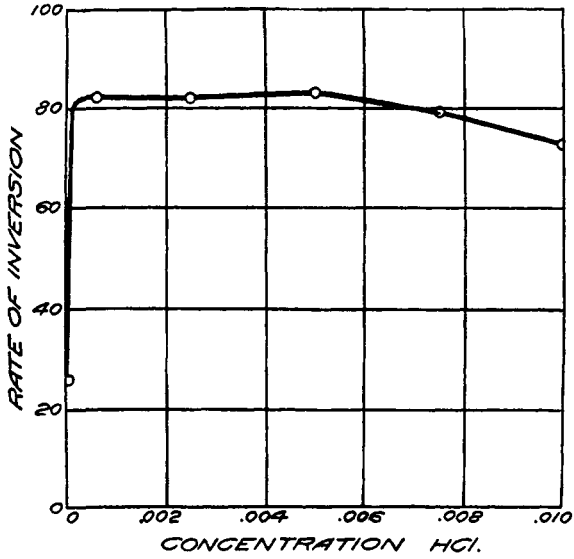

Fig. 1.—Effect of dilute HCl on the activity of invertase.

in studying the influence of other factors upon the action of invertase that the solutions be always very slightly acid; accordingly in the experiments hereafter to be described five drops of glacial acetic acid were added to each liter of the sugar solutions. This acidity is not enough to cause appreciable inversion by its own action, but it serves to make the rate of action of the invertase independent of the very small amounts of acid or alkaline impurities that frequently get into the solutions by accident.

The estimation of the extent of the inversions was in all cases made by the polarimetric method. The polarimeter that was used is a triple field nicol prism instrument of the Landolt-Lippich type made by Schmidt and Haensch.[1] Its scale is graduated in tenths of circular degrees and was read to hundredths. Sodium light was used. The observation tubes which contained the solutions under examination were 40 and 50 cm. long respectively and their considerable length allowed an accurate measurement of the rate of inversion in dilute sugar solutions, the total change of rotation, for instance, of a 5 per cent. sugar solution during inversion

[1] The instrument is the one that was used by Dr. H. W. Wiley in an investigation on the influence of temperature on the rotation of cane sugar, THIS JOURNAL, 21, 568–596 (1899).

in the 40 cm. tube is 17.3° and is thus large enough to allow its course to be accurately followed. In some of the glucose and fructose mutarotation measurements in which more concentrated solutions were used, the polarimetric readings were made with a 5 cm. observation tube. The polarimeter tubes were surrounded by metal jackets through which water from a thermostat flowed, keeping the solutions at constant temperature. The pumping of the water from the thermostat through the jacket was accomplished by a simple device which can be strongly recommended for experimental work of this kind. The stirrer of the thermostat is a hollow brass T tube and by means of a mercury seal at its top an air-tight and almost frictionless connection is made to a rubber hose which leads to a glass tube containing a thermometer and thence to the inlet of the jacketed observation tube. From the outlet of the same a hose passes to the thermostat.

To start the circulation of the water in the combined stirrer and pump the passage is first filled by removing the end *A* (Fig. 2) from the bath and applying suction to it; when all the air has been drawn out, *A* is again sunk in the thermostat. If leaks in the circuit occur, they may generally be stopped by painting with shellac or flexible collodion. After the circuit is completely filled with water and absence of all leaks is ascertained the electric motor which drives the stirrer is started. The revolution of the T stirrer (80 r. p. m.) throws water by centrifugal force from its hollow ends and a suction is thereby made at the end *A*, causing a rapid stream of water to flow through the circuit. A small laboratory motor has been found quite sufficient to drive the combined stirrer and pump.

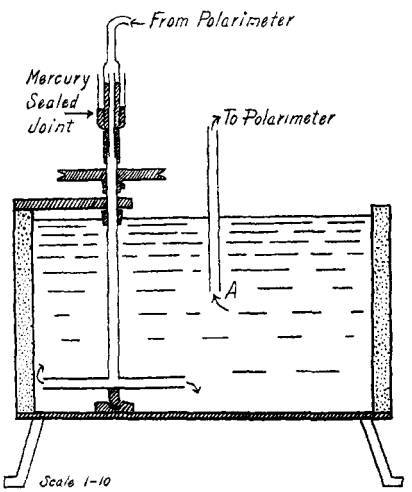

Fig. 2.—Constant temperature bath with stirrer and pump combined.

5. *The Unimolecular Order of the Inversion.*—In the first paper of this series an experiment was described which shows the considerable difference between the real and apparent rates of inversion by invertase, and attention was there called to the fact that while the unimolecular velocity coefficient of the apparent rate increases regularly during the inversion the similar coefficient for the real rate is satisfactorily constant throughout the reaction. In order to emphasize this point further and also to present data on it which will be used in another connection later in this

article a second experiment similar to the previous one is here described. A solution of cane sugar was prepared containing 4.6 grams sugar per 100 cc. solution and to one liter of it five drops of glacial acetic acid were added and a few cc. of a strongly active invertase preparation. A portion of the solution was observed at intervals in the polarimeter in a tube kept accurately at 30° and these readings gave the apparent rate of inversion. From time to time 50 cc. of the stock solution, which was kept in the thermostat at 30°, were removed, 5 cc. of two per cent. sodium carbonate added to stop the action of the invertase and complete the mutarotation, and the rotation then read in the polarimeter. These readings show the real inversion at the time the alkali was added and they are given in the third column of Table III, the corresponding apparent inversions being in column two. In columns four and five are given the velocity coefficients of the apparent and real rates of inversion as calculated from the unimolecular reaction formula. The constancy of the coefficients for the real rate proves that the reaction follows the mass action law, and the regular increase in the coefficient of the apparent rate which approaches that of the real rate as a limit is quite similar to the increases of Henri's experiments and proves beyond question that unless the mutarotation of the invert sugar is overcome the polarimetric readings give wholly incorrect values for the rate of inversion.

TABLE III.—REAL AND APPARENT RATES OF INVERSION BY INVERTASE.

Time. Min.	Rotation. Acid solution. (Apparent inversion).	Rotation. Alkaline solution. (Real inversion).	$k = \frac{1}{t} \log_{10} \frac{r_0 - r_\infty}{r - r_\infty}$ Apparent.	Real.
0	12.20	12.20	.....	.....
5	10.75	9.57	0.0083	0.0157
15	8.07	5.89	0.0087	0.0146
25	5.45	2.97	0.0096	0.0151
35	3.25	0.83	0.0103	0.0155
50	0.80	—1.17	0.0109	0.0159
65	—0.85	—2.24	0.0114	0.0159
90	—2.17	—2.87	0.0112	0.0141
∞	—3.72	—3.72	.....	.....
		Average		0.0153

In Fig. 3 are given the two curves of inversion, the real and the apparent. It may be noticed from it that the apparent curve is almost exactly linear during the first third or nearly half of the inversion, after which it slopes off to the logarithmic form. This characteristic has been emphasized particularly by several investigators, and two of them, Barendrecht[1] and Armstrong,[2] have devised hypotheses to explain it, but these workers have mistaken the apparent inversion for the real inversion and

[1] *Z. physik. Chem.*, **49**, 456–482 (1904).

[2] *Proc. Royal Soc.*, **74**, 195 (1904).

their hypotheses to explain the deviation of the inversion from the mass action law are accordingly unnecessary.

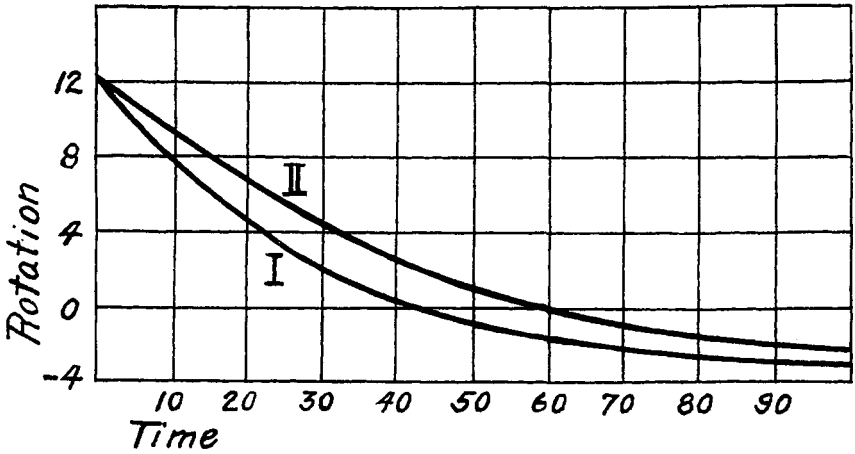

Fig. 3.—Real (I) and apparent (II) curves of inversion by invertase.

6. *The Effect of Concentration of Invertase on the Rate of Inversion.*—It is generally agreed among investigators that the rate of the inversion is proportional to the concentration of the invertase. The quantitative data of O'Sullivan and Tompson are the most valuable because they appreciated and avoided the error of mutarotation in their measurements. They have found that the time required for the inversion of 74 per cent. of the cane sugar of a certain solution at 15.5° by varying amounts of invertase was as follows: 0.15 g. invertase preparation, 283 minutes; 0.45 g., 94.8 min.; 1.5 g., 30.7 min. These times are inversely proportional to the amounts of the invertase as the respective products are 424, 427, and 460, and it is therefore to be concluded that the rate of inversion is directly proportional to the concentration of the invertase.

In the present work the influence of the concentration of invertase on the rate of inversion has been carefully studied and the results show conclusively that the above proportionality holds in both dilute and concentrated solutions of cane sugar. In the experiments a small quantity of invertase solution was diluted to various strengths and equal portions (10 cc.) of these dilutions were then added to 100 cc. portions of the stock solutions of cane sugar, the concentrations of the resulting solutions being 45.5, 90.9, and 273 grams cane sugar per liter respectively. To obtain a measure of the rate of inversion these solutions were allowed to proceed in reaction at 30° during an interval of time which was proportional to the dilution of the invertase. If the rate is proportional to the concentration of the invertase, these solutions of the same strength of cane sugar but of different invertase concentrations should all show

the same extent of inversion after these proportionate intervals of time. The data are condensed into Table IV, from which it may be seen that this conclusion holds. The rate of inversion is therefore proportional to the quantity of the invertase.

TABLE IV.—INFLUENCE OF CONCENTRATION OF INVERTASE ON THE RATE OF INVERSION AT 30°.

Concentration of invertase.[1]	Time of action.	Concentration × time.	Per cent. inversion.		
			45.5 g./l.	90.9.	273.
2.00	15 min.	30	73.2	45.3	11.2
2.00	30	60	93.0	74.2	22.0
1.50	20	30	73.2	44.8	11.2
1.50	40	60	92.8	74.5	22.7
1.00	30	30	72.9	45.3	11.5
1.00	60	60	93.0	74.7	22.3
0.50	60	30	72.9	45.2	11.4
0.50	120	60	92.7	74.5	22.6
0.25	120	30	73.1	45.2	10.9
0.25	240	60	92.7	74.7	21.9

7. *Theory of the Inversion of Cane Sugar by Invertase.*—Since the invert sugar which is formed in the inversion of cane sugar by invertase shows mutarotation it is to be inferred that one or both of its constituent sugars, glucose and fructose, are liberated from the cane sugar in a form which afterwards changes partially to a second form of the sugar. O'Sullivan and Tompson in their work recognized this initial liberation of the invert sugar in a form which shows mutarotation but as at that time it was not known that fructose shows mutarotation they considered the phenomenon to be due entirely to the glucose and concluded that it is set free as the form of glucose that is now known as α-glucose. This conclusion cannot, however, be retained without further evidence because it is now known that fructose as well as glucose shows mutarotation and it is quite possible that the mutarotation of the invert sugar is due largely or entirely to the fructose. Quantitative evidence is needed, but before the experiments on this point are described it is necessary to enter into certain theoretical considerations.

Let it be assumed that the first products of the inversion of cane sugar by invertase are glucose 1 and fructose 1, these names being given provisionally until the later experiments which are based upon this theory shall show exactly what forms of these sugars are the initial products.

The mutarotation of the invert sugar is to be considered as due to the establishment in solution of equilibria between glucose 1 and its second form, glucose 2, and fructose 1 and its second form fructose 2. The inversion is an irreversible reaction but the establishment of the equilibria

[1] As invertase is not known in the pure state, its concentration can only be expressed relative to some arbitrary solution of it. The concentrations here given were obtained by the successive dilution of the solution marked 2.

of the 1 and 2 forms of glucose and fructose respectively is due to two reversible or balanced reactions. The inversion of cane sugar by invertase may therefore be provisionally represented by the formula:

$$\text{cane sugar} \begin{cases} \nearrow \text{glucose } 1 \rightleftarrows \text{glucose } 2 \\ \searrow \text{fructose } 1 \rightleftarrows \text{fructose } 2 \end{cases}$$

In studying the rates of these dependent and therefore complicated reactions it simplifies matters considerably to regard the balanced reactions as irreversible, according to the following provisional plan:

$$\text{cane sugar} \begin{cases} \nearrow \text{fresh glucose} \longrightarrow \text{stable glucose} \\ \searrow \text{fresh fructose} \longrightarrow \text{stable fructose.} \end{cases}$$

This simplification will introduce no error if it is remembered that by *stable glucose* and *stable fructose* are meant the mixtures of the 1 and 2 forms of these sugars in their respective equilibrium proportions, and by *fresh glucose* and *fresh fructose* the amounts of the 1 forms of the two sugars that are present in solution in excess of the amounts that are in equilibrium with their respective 2 forms.

Start with A mols. cane sugar in unit volume of solution at constant temperature, and let there be present in the solution at the time t, w mols. fresh glucose, x mols. fresh fructose, y mols. stable glucose, z mols. stable fructose. Then the rates of formation of these four substances at the time t are:

(1) Fresh glucose, $dw/dt = k_1 (A - (w + y)) - k_2 w$.
(2) Fresh fructose, $dx/dt = k_1 (A - (w + y)) - k_3 x$.
(3) Stable glucose $dy/dt = k_2 w$.
(4) Stable fructose $dz/dt = k_3 x$.

In these expressions, the k's are the velocity-coefficients of the respective reactions. The solution of these four equations under the conditions which obtain that at the time zero, w, x, y, z, dy/dt and dz/dt are all zero, is

$$(5)\quad w = A \frac{k_1}{k_2 - k_1} \left[e^{-k_1 t} - e^{-k_2 t} \right]$$

$$(6)\quad x = A \frac{k_1}{k_3 - k_1} \left[e^{-k_1 t} - e^{-k_3 t} \right]$$

$$(7)\quad y = A \left[1 + \frac{k_1}{k_2 - k_1} e^{-k_2 t} - \frac{k_2}{k_2 - k_1} e^{-k_1 t} \right]$$

$$(8)\quad z = A \left[1 + \frac{k_1}{k_3 - k_1} e^{-k_3 t} - \frac{k_3}{k_3 - k_1} e^{-k_1 t} \right]$$

During the first part of the progress of the reaction, the quantities of the fresh sugars in the solution, w and x, increase, they then reach a maximum at a certain time t *max.*, and finally become zero when all the

reactions are completely ended. The times at which the maximum amounts of the *fresh* forms are present in the solution are obtained by writing the first differentials of equations (5) and (6) equal to zero and solving, giving the respective values,

$$\text{for fresh glucose, } t_{max} = \frac{2 \log k_2/k_1}{k_2 - k_1}, \qquad (9)$$

$$\text{and for fresh fructose, } t_{max} = \frac{2 \log k_3/k_1}{k_3 - k_1}. \qquad (10)$$

The maximum quantity of either of the fresh forms present during the reaction is best found by obtaining from (9) or (10) the time at which this maximum quantity is present and substituting the resulting value for t in equations (5) or (6).

It will be seen from these equations that an accurate knowledge of the rates of the mutarotation of glucose and fructose, giving values for the coefficients k_2 and k_3, is necessary to the quantitative study of the inversion reaction as a whole. These measurements are given in the next section; after their presentation the work will return to a study of the details of the inversion as outlined in the above theory.

8. *The Rates of Mutarotation of Glucose and Fructose.*—It is well known that the mutarotations of glucose and fructose are unimolecular reactions. The velocity coefficients at 30° have here been measured in pure water, in weakly acid solutions, and in solutions containing invertase, the method being the same as that previously described by the writer.[1] The data are given in Tables V and VI.

TABLE V.—RATE OF MUTAROTATION OF GLUCOSE AT 30°.

No.	Conc. HCl mol/liter.	Rotation at time (minutes). 0.	5.	7.	10.	13.	16.	22.	∞	$k_2 = \frac{1}{t}\log_{10}\frac{r_0 - r_\infty}{r - r_\infty}$.	
1	Dis. water	50.90	46.21	44.57	42.25	40.22	38.45	35.75	24.65	0.0166	0.0167
2	" "	50.90	46.20	44.50	42.25	40.35	38.55	35.80	24.65	0.0168	
3	Inver. sol.	48.80	44.70	42.90	40.80	38.31	36.56	33.84	23.60	0.0170	0.0167
4	" "	47.70	43.78	42.37	39.48	38.06	36.20	33.80	23.10	0.0163	
5	0.05	50.90	41.60	38.91	35.49	33.05	30.90	28.25	24.65	0.0380	0.0383
6	0.05	50.90	41.45	38.85	35.40	32.62	30.85	28.30	24.65	0.0385	
	Time	0	4	5	7	9	11	13	∞		
7	0.10	50.90	39.45	37.46	34.50	32.19	30.15	28.99	24.65	0.060	0.0620
8	0.10	50.90	38.81	37.07	34.12	31.61	29.71	28.36	24.65	0.064	
9	0.20	50.90	34.47	32.50	29.37	27.65	26.27	25.95	24.65	0.105	0.105
10	0.20	50.90	34.99	32.17	29.25	27.58	26.61	25.81	24.65	0.105	

Invertase does not accelerate or retard the mutarotation of glucose (see Table V, experiments 3 and 4), and in this respect differs remarkably from the acids, which accelerate both the mutarotation and the inversion. Indeed, it is on account of the absence of any accelerative action by invertase on the mutarotation that the successive steps of

[1] THIS JOURNAL, **29**, 1572 (1907).

the inversion can be studied. In the case of the similar inversion by acids the mutarotation is so strongly catalyzed by the inverting agent that the apparent and real curves of inversion coincide and it is therefore impossible to study the successive stages of the inversion by acids. On account of this lack of the possibility of experimental evidence on this subject the recently published hypothesis of Julius Meyer[1] that α-glucose and a hypothetical substance which he calls α-fructose are the first products of the inversion of cane sugar by acids cannot be considered as other than a hypothesis which is not subject to experimental test, because there is no evidence whatever nor is there in the nature of the case likely to be any to show the successive stages in the inversion of cane sugar by acids. Any inference on this subject must at present be drawn exclusively from the behavior of cane sugar to the other inverting agent, invertase, as the successive stages in the inversion of cane sugar by acids cannot be realized experimentally on account of the fact that acids are far more powerful catalysts of the mutarotations than they are of the inversion.

TABLE VI.—RATE OF MUTAROTATION OF FRUCTOSE AT 30°.

No.	Conc. HCl mol/liter.	Rotation at time (minutes). 0	2.5	3	4	5	7	9	∞	$k_3 = \frac{1}{t}\log_{10}\frac{r_0-r_\infty}{r-r_\infty}$	
1	Dis. water	62.30	.....	45.59	44.00	42.36	40.91	.....	39.75	0.187	0.186
2	" "	62.30	.....	.....	43.77	42.55	40.91	.....	39.75	0.184	
3	0.0005	62.30	.....	47.82	45.97	44.55	42.05	41.35	39.75	0.146	0.140
4	0.0005	62.98	50.65	49.02	47.10	45.44	42.42	41.69	40.18	0.134	
5	0.0010	62.30	50.35	48.85	46.40	45.40	43.00	41.95	39.75	0.126	0.128
6	0.0010	62.30	50.08	48.79	46.34	44.90	42.59	41.50	39.75	0.130	
7	0.0040	62.30	50.18	49.11	46.92	44.53	42.87	41.53	39.75	0.128	0.130
8	0.0040	62.30	49.24	49.03	46.75	44.93	42.41	41.49	39.75	0.132	
9	0.0100	62.30	.....	45.77	43.75	41.45	.....	40.10	39.75	0.201	0.196
10	0.0100	62.30	46.25	.....	44.09	42.54	40.80	.....	39.75	0.191	

The rate of mutarotation of glucose in pure water and hydrochloric acid solutions can be represented by a linear relation between it and the hydrogen ion concentration, $k_2 = 0.0167 + 0.44\ (H^+)$, but in the case of fructose the relation is more complicated, a minimum rate being observed in dilute acid solutions similar to the less pronounced minimum which has been found in the case of glucose at 25°.[2] The measurement of the rate in alkaline solutions will probably throw light on the existence of this minimum in weakly acid solutions and the discussion is therefore deferred until these data are obtained.

9. *The Form of Glucose that is Liberated from Cane Sugar by the Action of Invertase.*—From the data of the previous section it is seen that at 30° the mutarotation of fructose ($k_3 = 0.186$) is eleven times faster in

[1] *Z. physik. Chem.*, **62**, 59–88 (1908).

[2] THIS JOURNAL, **29**, 1571–1576 (1907).

water than that of glucose ($k_2 = 0.0167$); on this account there can never be much fructose in the solution undergoing inversion by invertase in comparison with the amounts of fresh glucose present, and the drop in rotation between the apparent and the real curves of inversion at this temperature must be due almost entirely to the mutarotation of the fresh glucose. It would appear therefore that if the inversion of cane sugar could be accomplished almost instantaneously by a very active invertase solution any subsequent change of rotation of the inverted sugar would be due almost entirely to the mutarotation of the freshly formed glucose and that the velocity coefficient of the subsequent change of rotation would be identical in value with that for the mutarotation of glucose. The following experiment to test this conclusion was performed, in which 50 cc. of an approximately 0.54 normal cane sugar solution were mixed with 25 cc. of yeast liquor (sample one of section 3) at the time indicated as zero in the accompanying Table VII, and the polarimetric rotation read after successive intervals, no alkali of course being added as the apparent rate of inversion was being measured. The initial rotation was found by measuring that of a separate mixture of 50 cc. of the 0.54 cane sugar solution with 25 cc. water, as it was observed that the yeast liquor was very nearly optically inactive. The final constant rotation was obtained by adding three drops of strong caustic soda to the solution in the polarimeter tube but it was then necessary to observe the rotation in a much shorter tube (50 mm.), because the addition of the caustic alkali caused the solution to become cloudy.

TABLE VII.—APPARENT RATE OF INVERSION OF CANE SUGAR AT 30° BY STRONG INVERTASE.

Time (t). Min.	Rotation (without alkali).	$\frac{1}{t}\log_{10}\frac{r_0-r_\infty}{r-r_\infty}$.	Time (t).	$\frac{1}{t}\log_{10}\frac{r_0-r_\infty}{r-r_\infty}$.
0.....	33.50	.....	..	.....
3.....	11.88	0.099	..	.....
4.....	7.32	0.099	..	.....
5.....	4.77	0.093	..	.....
6.....	2.70	0.088	..	.....
7.....	1.67	0.081	..	.....
8.....	0.61	0.076	..	.....
9.....	—0.35	0.072	..	.....
10.....	—1.35	0.069	..	.....
13.....	—3.57	0.063	0	Start.
16.....	—4.90	0.057	3	0.0323
19.....	—6.03	0.054	6	0.0334
23.....	—7.15	0.050	10	0.0336
29.....	—7.92	0.044	16	0.0287
30.....	—8.22	0.045	17	0.0307
∞.....	—10.22	0.000	..	.....
		Average.....		0.0317

TABLE VIII.—RATE OF MUTAROTATION OF GLUCOSE IN THE SOLUTION OF TABLE VII.

Time (t).	Rotation (r).	$\frac{1}{t}\log_{10}\frac{r_0-r_\infty}{r-r_\infty}$.
0	93.75	.....
1 min	90.90	0.0284
3	84.96	0.0315
5	80.40	0.0306
7	76.32	0.0304
9	72.85	0.0301
15	68.01	0.0284
∞	48.75	.....
	Average	0.0299

The real rate of inversion in the experiment was very great: a portion of the solution in which the invertase had been destroyed by heating within three seconds to 80° after acting for only half a minute on the sugar showed that 72 per cent. of the sugar had been inverted in this time. This would indicate that the rate of inversion was so great that the solution may be considered to be practically free of cane sugar after the first minute. As the mutarotation of the fructose is also practically completed within the first ten minutes according to the values of its rate found in the previous section, the change of rotation of the solution after this time is here considered as due only to the mutarotation of the glucose and accordingly in column four of Table VII are given the values of the velocity constant of the portion of the change of rotation which occurred after the first ten minutes, this portion of the change of rotation being considered an independent unimolecular reaction. The values so obtained are sufficiently constant to prove that this portion of the apparent inversion is a unimolecular reaction. Concerning the value of the velocity coefficient here found it is to be noted that the rate of mutarotation of glucose in this solution was greater than the rate for pure water because the yeast liquor contained free acetic acid due to the large quantity of acid yeast liquor used, but the rate was accurately measured by dissolving anhydrous glucose in a mixture of water and yeast liquor in the same proportions as were used in the inversion and the data are given in Table VIII. The free acidity of the solution as shown by a comparison of the observed rate of mutarotation with the formula of the previous section was about 0.05 normal.

The agreement between the velocity-coefficient of the apparent inversion in its later stages (0.032) and that of the mutarotation of glucose in the solution (0.030) shows clearly that the two reactions are identical, and that at 30° the difference between the real and the apparent curves of inversion is due almost entirely to the glucose. Column three of Table VII gives the values of the unimolecular velocity coefficient cal-

culated under the assumption that the rotation is the true measure of the extent of inversion, and the regular and large variation of this coefficient is quite apparent.

By the aid of equations (5) and (6) of section 7, together with the values of k_2 and k_3 found in the previous section, the amounts of fresh glucose and fresh fructose that are present at any instant in the solution which is undergoing inversion may be calculated. Now the difference in rotation at any instant between the apparent curve of inversion and the real curve, here called the drop of rotation, is determined by the amounts of fresh glucose and fresh fructose in the solution at that instant and by the difference between the specific rotations of the fresh and stable forms of these sugars. As has just been shown, at 30° the drop of rotation is due almost entirely to the glucose and as the quantity of fresh glucose present at any instant can be calculated and as the drop in rotation can be measured, the specific rotation of the fresh glucose can be found. In the following tables the experiments of Tables III and VII of this article together with that of Table IV of the previous article,[1] are used as the basis of such calculations. In the first column of each is given the time (t) since the beginning of the inversion, in the second the drop (D) or difference in rotation between the apparent and real inversion curves at the time t, in the third the number of mols. (W) of fresh glucose per mol. of cane sugar at the start that are present in the solution at this time as calculated from equation 5, Section 7, using the values of k_1 and k_2 that are given at the head of each table, and in column four are given the similar values for fresh fructose. In the fifth column the values of the specific rotation of the fresh glucose are calculated from a formula which can be derived by simple reasoning, specific rotation = $52.5 + \frac{66.5}{r} \cdot \frac{342}{180} \cdot \frac{D}{W}$, 52.5 being the specific rotation of stable glucose, 66.5 the specific rotation of cane sugar, r the rotation of the solution of the experiment before any inversion took place, 342 and 180 the molecular weights of cane sugar and glucose respectively, D the drop in rotation of column 2, W the molal per cent. of fresh glucose from column 3. In this calculation it is assumed, as has been mentioned, that the fresh fructose is so small in amount at any instant compared with the quantity of fresh glucose then present, that the drop may be considered to be due entirely to the fresh glucose. A comparison of columns 3 and 4 shows that this assumption is fully justified in these experiments at 30°.

These experiments show that the fresh glucose has a specific rotation of 100–125°. There are three forms of glucose known, α-anhydrous glucose and its monohydrate, and β-anhydrous glucose. Of these the

[1] THIS JOURNAL, 30, 1164 (1908).

TABLE IX.—CALCULATED SPECIFIC ROTATION OF FRESH GLUCOSE.

Experiment A. $k_1 = 0.00542$, $k_2 = 0.0167$. $k_3 = 0.186$, $r = 24.50$ (From previous article).

Time. Min. (t).	Drop in rotation D.	Fresh glucose. (w).[1]	Fresh fructose. (x).	Calc. sp. rotation of fresh glucose.
30	2.58°	0.176	0.021	128
60	3.05	0.175	0.018	142
90	1.75	0.137	0.010	118
110	1.15	0.112	0.008	106
130	0.94	0.089	0.006	109

Experiment B. $k_1 = 0.0153$, $k_2 = 0.0167$, $k_3 = 0.186$, $r = 12.20$ (From Table III).

5	1.18	0.148	.....	135
15	2.18	0.304	.....	127
25	2.48	0.352	.....	125
35	2.42	0.340	.....	126
50	1.97	0.280	.....	125
65	1.39	0.210	.....	121
90	0.70	0.116	.....	113

Experiment C. $k_1 = 1.11$, $k_2 = 0.030$, $k_3 =$ approx. 0.40 (From Table VII).

13	6.65	0.395	.....	116
16	5.32	0.319	.....	115
19	4.19	0.258	.....	114
23	3.07	0.194	.....	112
29	2.30	0.126	.....	121
30	2.00	0.117	.....	117

first two have the same specific rotation which has been found by a great many observers to lie between the limits 100–115°,[2] and the third has the specific rotation +20°. There is no question on comparing these values with that found above that the fresh glucose which is first formed in the inversion of cane sugar by invertase is α-glucose. Similar experiments on the drop of rotation at lower temperatures where the mutarotation of fructose takes place more slowly will enable one to determine the specific rotation of the fresh fructose. These experiments will be performed as soon as cool weather sets in.

10. *Summary.*—The contents of this article may be summarized as follows:

(1) On account of the neglect of the mutarotation of the invert sugar in studying the inversion of cane sugar by invertase a series of erroneous conclusions on the laws of the action of this enzyme has become widely accepted. These conclusions are directly opposed to the results of a classical investigation by O'Sullivan and Tompson on the action of invertase. The measurements of the present article show clearly that

[1] In calculating w it is to be remembered that since the velocity-coefficients are here expressed in terms of decimal logarithms, it is necessary to substitute 10 for e in equations 5 and 6.

[2] Lippmann. *Die Chemie der Zuckerarten* I, pp. 283–4.

the conclusions of O'Sullivan and Tompson are correct. Their work has been extended as described below.

(2) Data on the velocity of mutarotation of glucose and fructose at 30° in water and in solutions of hydrochloric acid and of invertase are given. Invertase does not affect the rates of mutarotation while acids accelerate them strongly. This fundamental difference between the action of acids and invertase on the products of the inversion of cane sugar causes the action of invertase, as measured polarimetrically, to be apparently irregular as compared with the action of acids. This is due to the influence of the mutarotation of the invert sugar on the polarimetric reading and when this difficulty is corrected the inversion of cane sugar by invertase proves to be a catalytic reaction of the first order. The disagreeing results of O'Sullivan and Tompson and their critics are thus explained.

(3) The acceleration of the mutarotation of glucose at 30° by hydrochloric acid is such that the rate is a linear function of the concentration of the hydrogen ions, but in the case of the similar acceleration for fructose a minimum rate occurs in weakly acid solutions, which is similar to but much more pronounced than the minimum that has been previously found for glucose at the lower temperature 25°.

(4) The action of invertase is very strongly accelerated by the addition of minute traces of acid to the neutral solution but further small additions of acid are without further effect. Data on this unexplained phenomenon are given.

(5) The inversion of cane sugar by invertase is accurately proportional to the concentration of the invertase in both dilute and concentrated sugar solutions.

(6) A theory of the separate steps in the inversion by invertase is developed and from it in combination with data on the differences between the real and the apparent polarimetric curves of inversion it is shown that the glucose which is liberated from the cane sugar by invertase has the specific rotation 100–125° and must therefore be the form which is usually designated α-glucose (specific rotation 106°). A similar determination of the form in which fructose is liberated from cane sugar is planned. The same method can be applied to a determination of the forms in which the various hexoses are liberated from the glucosides and the *di-* and *tri*-saccharides by the action of enzymes, and in this way light may be thrown on the constitution, and possibly the synthesis, of these sugar derivatives.