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THE INVERSION OF SUCROSE BY INVERTASE. VIII. AN IMPROVED METHOD FOR PREPARING STRONG INVERTASE SOLUTIONS FROM TOP OR BOTTOM YEAST.

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The Clerget method for estimating sucrose depends upon the measurement of the change in optical rotation which accompanies the hydrolysis of the sugar. Hydrochloric acid is generally used as the hydrolyst although it is known to hydrolyze inulin, raffinose, and some other carbohydrates under the mildest conditions that will accomplish the inversion of sucrose.

In 1881, it was proposed by Kjeldahl[2] to use the enzyme invertase in place of hydrochloric acid in the Clerget method because invertase was regarded as a more nearly specific hydrolyst for sucrose. Although it is now understood that invertase hydrolyzes raffinose, gentianose, and stachyose as well, it seems that these sugars are derivatives of sucrose and that invertase may still be strictly regarded as a specific hydrolyst of sucrose and certain of its immediate derivatives. Kjeldahl used, in one procedure, an aqueous extract of yeast to hydrolyze sucrose, and, in an alternative one, a portion of yeast added to the sucrose solution which was kept at 52° with a little thymol added to prevent alcoholic fermentation. As invertase is rapidly, though incompletely, extracted from yeast by water, its action can be obtained by either of these procedures. Kjeldahl's untimely death prevented the further development of this excellent analytical

[1] Contribution from the Division of Carbohydrate Investigations, Bureau of Chemistry.

[2] Compt. rend. Carlsberg Laboratoire, *Copenhagen*, **1**, 189–95 (1881).

method and it came into notice next in 1886, when O'Sullivan and Tompson,[1] without knowing of Kjeldahl's procedure, recommended one which is substantially the same, the only difference being that thymol is not used and the mixture of yeast and sucrose solution is kept at 55°, at which high temperature alcoholic fermentation is prevented and the action of invertase aided.

Ling and Baker,[2] and more recently Ogilvie,[3] have followed with success O'Sullivan and Tompson's procedure in the estimation of sucrose in a variety of cane and beet products. In 1910, the author[4] sought to improve the method by using in place of yeast, a purified aqueous extract of yeast which had strong inverting power. The idea was that the main objection to the use of invertase, as a hydrolyst in analytical work, came from the uncertainty attaching to the use of a substance of such varying properties as yeast. It seemed that the method would become more dependable if a procedure could be found for preparing a stock solution of invertase which would keep well, have a definitely known, and high, inverting power, and be as free as possible from impurities.[5]

The first method which was tried was the extraction of invertase from living yeast by water, as had been done by Kjeldahl, but the inverting powers of the extracts were too low. The slow autolysis of yeast during several weeks keeping at 10° to 20°, which had been recommended by O'Sullivan and Tompson as a method for preparing aqueous solutions of invertase, was next tried, but while these experiments were in progress, H. E. Berger and the author observed that the liquefaction of pressed yeast, which is one of the changes that is noticeable during its slow autolysis, can be brought about in a few minutes by allowing the vapors of ether or chloroform to act upon living yeast, and a test showed that the fluid which could be filtered from the liquefied yeast had strong inverting power.[6] It became possible, therefore, to substitute in place of O'Sullivan and Tompson's method of preparing invertase by very slow autolysis an excellent and rapid procedure in which the autolysis is accomplished in a few hours by the action of chloroform upon fresh yeast. It was found that

[1] *J. Chem. Soc.*, **49**, 64 (1886); **59**, 46 (1891).

[2] *J. Soc. Chem. Ind.* **17**, 111–4 (1898).

[3] *Ibid.*, **30**, 62–4 (1911); *Int. Sugar J.*, **14**, 89–93 (1912).

[4] *J. Ind. Eng. Chem.*, **2**, 143–6 (1910).

[5] One recalls, in this connection, O'Sullivan and Tompson's statement, made in 1891 (*Loc. cit.*), that "The estimation of cane sugar by means of invertase is, without doubt, a perfectly satisfactory process. The only disadvantage consists in the difficulty of preparing the invertase. Until the recent publication of our paper on invertase, this objection was practically fatal, and it still forms a great drawback to the universal application of the process, as it takes at least three weeks to prepare invertase by our method."

[6] Salkowski (*Z. physiol. Chem.*, **13**, 520 (1889)) has shown that sucrose is rapidly inverted by yeast in the presence of chloroform water.

the aqueous solution which could be filtered from the liquefied yeast could be purified with only a small loss in its inverting power, by clarifying it with an excess of neutral lead acetate, filtering off the precipitate, and removing the excess of lead from the filtrate with potassium oxalate or hydrogen sulfide. This purification of the invertase solution by the use of neutral lead acetate has been confirmed by Euler.[1] The purified solution was then dialyzed thoroughly and used as a stock reagent for inverting sucrose.

As ether and chloroform had each caused yeast to autolyze very rapidly and give up its invertase, it became of interest to test the action of other neutral volatile liquids for the purpose of learning which substance would cause yeast to liberate the most invertase. Ether, chloroform, ethyl acetate, acetone, carbon tetrachloride, carbon disulfide, kerosene oil, and toluene were tried, and it was found that the rapidity of the liquefaction of the yeast varied considerably and that toluene caused the liberation of far more invertase than did chloroform and that toluene was not surpassed by any other of the liquids in this respect.[2] Accordingly, I have substituted toluene for chloroform in the method for preparing invertase, with an important increase in the inverting strength of the resulting purified extracts. These extracts we now use as stock reagents for the hydrolysis of sucrose.

The procedure which is used for the preparation of invertase solutions may be understood from the following record. Ten kilos of pressed top fermentation yeast, from the Corby Company, Langdon, D. C., were kneaded well with 10 liters of tap water and 500 cc. of toluene[3] at room temperature. Liquefaction of the yeast was noticeable in a few hours

[1] *Z. physiol. Chem.*, **73**, 338 (1911); also "General Chemistry of the Enzymes," English, 1912 edition, p. 26.

[2] After our experiments had been completed, it was learned that such volatile liquids have been used in "processes for obtaining the contents of yeast cells," according to the British patent to Ransford, No. 8722, April 27, 1901, and the United States patents to Hess, No. 785,733 and No. 785,734, March 28, 1905. The processes which are described in these patents are evidently intended for extracting soluble albumin from yeast and attention was not directed to obtaining a maximum yield of enzymes such as invertase. In these patents, there is no distinction made between the low extraction of invertase which results from the action of chloroform and the high extraction which results from the action of toluene. We have found that the use of ethyl acetate, which is mentioned in Hess's patent as particularly suitable for obtaining the contents of yeast cells, gives an extract which is very weak in invertase and quite unsuitable for our purpose.

[3] Nelson and Born (THIS JOURNAL, **36**, 395 (1914)) have recently prepared invertase from yeast by allowing the compressed yeast to autolyze slowly at room temperature during two to six weeks, toluene being added to prevent bacterial growth. This procedure differs from the one here described in that we thoroughly saturate the yeast with toluene, after mixing it with an equal weight of water to aid the saturation, and thereby obtain a far more rapid autolysis than they produced.

and on the third day a test of the activity of the filtered extract from the autolysis showed that 5 cc. of it would invert half the sucrose in 50 cc. of a 9% sucrose solution, acidified with 2 drops of glacial acetic acid and kept at 30°, in 5.9 minutes. Other experiments have repeatedly shown that the extract exhibits considerable activity on the day immediately following that upon which the autolysis was started, but we were unable to test this extract so early. The yeast was allowed to autolyze two days longer, when it was found that the extract would invert half the sucrose, under the conditions recorded above, in 5.6 minutes. On the third day the value was found to be 6.4 minutes, showing some loss in activity. Neutral lead acetate was then added until no further precipitate formed and the activity was thereby reduced to 6.8 minutes. Upon treating the filtrate with hydrogen sulfide to remove the excess of lead, the activity rose to 6.3 minutes, which shows that there is no considerable permanent loss in activity due to the use of lead acetate as a clarification agent and that the temporary loss of activity from the first precipitation is caused by a retarding action of the excess of dissolved lead acetate on the invertase. It was found, in confirmation of this view, that the addition of lead acetate to a purified invertase solution, in which no precipitate formed, caused a marked loss in activity.

The preparation of invertase from bottom fermentation or brewer's yeast by the method which has been described shows some points of difference, which may be understood from the following record. Twelve kilos of pressed brewer's yeast from the Heurich Brewing Company, Washington, D. C., were kneaded well with 12 liters of tap water and 750 cc. of toluene at room temperature. On the succeeding day the filtered extract showed an activity of 7.2 minutes and on successive following days the number of minutes required for half inversion were 5.7, 4.3, 3.8 and 3.4. At this point, the lead acetate was added and the activity became 4.0, at which value it remained after the excess of lead had been precipitated by hydrogen sulfide. It will be observed that brewer's yeast furnishes an invertase solution which is almost twice as strong as that yielded by top yeast.

The next step in the preparation of the solutions of invertase is a thorough dialysis. It is advisable to carry this out immediately because the extracts are markedly acid before dialysis and often lose activity rapidly in this condition. Neutralization causes a formation of color which does not disappear in a subsequent dialysis. The most suitable membrane for the dialysis which has been found is a collodion sac which may be formed on the inside of a glass cylinder about 5 cm. in diameter and 35 cm. long, resembling a large test tube. The collodion solution, which is of Pharmacopeia strength, is poured into the dry test tube, filling it, and wetting

the walls evenly. The solution is then poured back into the stock bottle, and the film which coats the test tube is allowed to dry with even thickness while the tube is gently turned during five or ten minutes. When the film has dried until it is no longer sticky, the test tube is filled with water and the film soaked for a few minutes. The water is then poured out, the film is loosened at the top from the tube, and water is run in between film and tube at the loosened place. The pressure of the water loosens the entire film and it may readily be removed from the test tube. In order to dialyze the invertase solutions, they are poured into the collodion sacs and these are immersed in running tap water, a layer of toluene to retard bacterial action being on the surface of the solutions. Dialysis is frequently complete overnight, the time required depending much upon the quality of the membrane.

The dialyzed solutions are colorless, without taste or odor, develop none upon boiling, and yield on the average about two-tenths of 1% of solid material when evaporated to dryness on a water bath. Numerous tests have shown that there is no loss of invertase during the dialysis, but the absorption of water by the solutions causes some dilution. The dialyzed solutions may be preserved with toluene at room temperature and in distinction from the dialyzed solutions of other enzymes, they preserve their activity well. During the first month's keeping the solutions show no loss in activity and at the end of a year the activity is about half lost. Their inverting strength is enough to cause the hydrolysis of sucrose solutions over night, under usual analytical conditions, and is many fold larger than that of the invertase solution which was previously recommended, made by hastening the autolysis of yeast with chloroform. The dialyzed solutions we have concentrated and even dried without loss in activity by boiling at not over 30° in a vacuum. This work will be described later, when it is hoped that a description of the solid material, which must include the invertase, can be made.

It may be noted that, though the dialyzed invertase solutions have an unusually strong power of hydrolyzing sucrose, test has shown them to be without any action on α-methyl-glucoside, thus confirming the generally accepted view that this substance is not hydrolyzed by invertase. They also show no action upon solutions of maltose or lactose. An interesting observation concerning raffinose was noted. H. S. Paine and T. S. Harding noticed that an invertase solution, which the latter prepared from *top* fermentation yeast, obtained from the yeast factory of the Corby Company, Langdon, D. C., changed the specific rotation of a solution of anhydrous raffinose from $+123°$ to $+63.9°$. On the other hand, a similar invertase solution which was prepared from *bottom* fermentation yeast, obtained from the Heurich Brewing Company, Washington, D. C., changed

the rotation from +123° to +14.9°.[1] This interesting difference we attribute to the presence of an enzyme in the invertase solution from bottom yeast which carried the hydrolysis of raffinose a step beyond that which invertase causes. Probably invertase and melibiase are in the solution which was prepared from bottom yeast, while melibiase is lacking in that made from top yeast. This view corresponds to the well known fact that top yeast ferments raffinose to alcohol and melibiose, and bottom yeast ferments it completely to alcohol. T. S. Harding and the writer have worked out an analytical method for estimating raffinose in solutions containing other sugars, by utilizing the difference in rotation which is observed when the solution is acted upon, first by invertase from top yeast, and then by invertase and melibiase from bottom yeast.

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THE INVERSION OF SUCROSE BY INVERTASE. IX. IS THE REACTION REVERSIBLE?[2]

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The synthetic production of sucrose by various plants, ultimately from carbon dioxide and water, is a firmly founded fact, though it is not positively known what substances are the intermediate products in the synthesis. Many plants also synthesize an enzyme of unknown composition called invertase, which can accomplish the hydrolysis of sucrose to glucose and fructose. Thus the synthesis of sucrose may proceed in plants along with its decomposition by invertase. If the last stage in the synthesis should prove to be the uniting of glucose and fructose to yield sucrose, this change would represent the reverse reaction to the hydrolysis of sucrose by invertase. It has been suggested at various times that the enzyme invertase can accomplish such a synthesis of sucrose from glucose and fructose, and that therefore the inversion of sucrose by invertase is a balanced or reversible reaction. The present investigation was undertaken for the purpose of obtaining experimental evidence upon this question, partly because the subject is of some general interest and partly

[1] The values 63.9° and 14.9° correspond almost exactly to those (64.1° and 14.9°) which have been calculated by C. A. Browne ("Handbook of Sugar Analysis," p. 737) for the two steps that are to be expected in the hydrolysis of raffinose, which may be represented as the reactions

$$\underset{\text{(raffinose)}}{C_{18}H_{32}O_{16}} + 2H_2O = \underset{\text{(melibiose)}}{C_{12}H_{22}O_{11}} + \underset{\text{(fructose)}}{C_6H_{12}O_6} + H_2O = \underset{\text{(glucose)}}{C_6H_{12}O_6} + \underset{\text{(galactose)}}{C_6H_{12}O_6} + \underset{\text{(fructose)}}{C_6H_{12}O_6}$$

[2] Read at the Minneapolis meeting of the American Chemical Society in December, 1910.

[3] Contribution from the Division of Carbohydrate Investigations, Bureau of Chemistry.