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A New Method of Investigating the Enzymatic Inversion of Sucrose

BJØRN ANDERSEN and THOR A. BAK

Universitetets Fysisk-Kemiske Institut, Copenhagen

A new way of investigating the enzymatic inversion of sucrose is described. The reaction is followed by measuring the optical rotation of the solution, but instead of taking out samples in which the reaction can be stopped and measuring the optical rotation after the mutarotation of glucose and fructose has stopped, the measurements are performed directly on the reacting solution and a correction for the mutarotation is applied. The expression for the correction term is derived as a second order differential equation which is solved numerically by a step by step integration. The mutarotation constants for glucose and fructose have been determined and using these values and an assumed value for the optical rotation of β-fructofuranose it is shown that the correction term is in accord with experiments.

It is well known that sucrose is hydrolyzed in the presence of acids or the enzyme invertase. During the process the optical rotation (sodium light, 589 mμ) of the solution changes sign and the process is therefore known as the inversion of sucrose.

The classical investigation of the acid catalyzed inversion is due to Wilhelmy [1] who showed it to be a first order reaction or rather deduced the mathematical expression for a first order reaction from the experiments. Since then this reaction has been investigated several times and the mechanism is well known. Contrary to this the mechanism for the enzymatic hydrolysis which takes place at pH 4—7 is not known with certainty, although the reaction has often been investigated.

The results of the many experiments on the enzymatic inversion described in the literature are not all consistent with each other. This is probably due to the fact that the conventional method of following the reaction consists of taking out samples in which the reaction can be stopped and then measuring the optical rotation after the mutarotation has ceased If one does not add base which catalyses the mutarotation it is necessary to wait a long time before the measurements are performed. If on the other hand base is added the measurement must be performed very rapidly since fructose decomposes in alkaline solution.

The first of these possibilities (*i.e.*, stopping the reaction without catalysing the mutarotation) was used by Kjeldahl [2], who investigated the influence of temperature on the enzymatic inversion. He stopped the reaction by adding mercuric chloride which does not affect the mutarotation. In his papers he does not mention how long time he waited before he measured the optical rotation of the samples. The necessary time turns out to be in this case approximately 5 hours [3].

Among the other classical investigations we mention the work of Henri [4]. He used direct measurements of the reaction in the polarimeter tube and obtained in this way a perfectly reproducible curve for the apparent degree of reaction as a function of time, which he took to be an expression for the rate of inversion of sucrose alone. He furthermore found that a first order reaction constant calculated from the experimental data increased rapidly with time and concluded that the reaction is not a first order reaction in contrast to the result found earlier by O'Sullivan and Tompson [5]. The last mentioned authors incidentally were the first to use the above described technique in which mutarotation is catalyzed by the addition of base.

Hudson has discussed the work of O'Sullivan and Tompson critically as well as that of Henri [6,7]. Furthermore he repeated the measurements using the technique of O'Sullivan and Tompson (with Na_2CO_3 instead of KOH) and obtained results which were in agreement with their work.

This technique for measuring the reaction, *i.e.* taking out samples in which the reaction is stopped and the mutarotation is catalyzed, has been used in all subsequent papers such as those by Michaelis and Menten [8], Nelson and Hitchcock [9] and Christiansen and Bergthorsson [12]. It is clear that in this method there are a number of possibilities for making errors for instance in measuring the time when the reaction is stopped or in the dilution of the sample which takes place when the reaction is stopped and during which CO_2 is evolved. Apart from this the method is rather tedious and requires large amounts of enzyme since each sample is about 20 ml. It should be said, however, that the results of Nelson and Hitchcock and those of Christiansen and Bergthorsson are in excellent agreement.

A direct method in which the degree of reaction can be found without stopping the reaction is much to be preferred, but in that case one must take into account that the optical rotation of the solutions is not only a function of the degree of advancement for the reaction

$$S \rightarrow F_\beta + G_\alpha \tag{1}$$

but also depends on how far the reactions

$$F_\beta \rightleftharpoons F_\alpha \tag{2}$$

$$G_\alpha \rightleftharpoons G_\beta \tag{3}$$

have progressed. In these expressions S stands for sucrose G_α means α-glucose and G_β means β-glucose. F_β stands for β-fructofuranose and F_α for the form of fructose which is formed from F_β in solution. The following argument is completely independent of whether F_α is a sixmembered ring or just an optical isomer of F_β.

Below we shall derive an expression for the correction one must apply in order to convert the apparent degree of advancement φ defined as

$$\varphi = (a(t) - a(0))/(a(\infty) - a(0))$$

where $a(t)$ is the optical rotation of the solution at time t, to the true degree of advancement

$$\xi = (S(t) - S(0))/(S(\infty) - S(0))$$

where $S(t)$ denotes the concentration of sucrose at time t. The corrected curve will thereafter be compared with experiments made in the classical way.

EXPERIMENTAL

All polarimetric measurements were performed at 25.0°C in a Zeiss "Kreis-polarimeter 0.01°" using a 40 cm polarimeter tube in a thermostated jacket. The kinetic experiments were made in solutions which contained 10 g of sucrose * in 100 ml of solution. The solution was 0.005 M with respect to sodium acetate and 0.005 M with respect to acetic acid. In two experiments samples were taken out and the reaction was stopped by means of 2 ml 0.2 M Na_2CO_3; in two experiments the reaction was followed directly in the polarimeter tube. Fig. 1 shows a curve for the average of the last two experiments and the average points obtained by the first method.

The rate constants for mutarotation of glucose and fructose were also determined by dissolving the two sugars in the same buffer as above and measuring the optical rotation as a function of time. The slope of the line which is obtained by plotting the natural logarithm of the rotation minus final rotation *versus* the time is the sum of the rate constants for the forward and backward first order reactions. For these sums which are the so called mutarotation constants we obtained

(Fructose) $k_F = 0.2750$ min^{-1}

(Glucose) $k_G = 0.02473$ min^{-1}

as an average of two determinations which deviated less than one in thousand.

THE CORRECTION FORMULA

In order to derive the relation between the two degrees of advancement discussed above, we use that instead of considering the reactions

$$F_\beta \rightleftharpoons F_\alpha \qquad \text{and} \qquad G_\alpha \rightleftharpoons G_\beta$$

we may consider the reactions

$$F_1 \rightarrow F_2 \qquad \text{and} \qquad G_1 \rightarrow G_2$$

in which F_1 denotes F_β and F_2 the equilibrium mixture of F_β and F_α. Similarly G_1 denotes α-glucose and G_2 the equilibrium mixture of α and β-glucose. The last equation simply expresses that instead of considering the approach to equilibrium as the net result of two opposing first order reactions we may (formally) consider it as an irreversible transformation of a pure compound to an equilibrium mixture. The first order rate constants for the latter processes are the sums of the rate constants for the opposing reactions $\alpha \rightleftharpoons \beta$. The rate constants for the reactions $F_1 \rightarrow F_2$ and $G_1 \rightarrow G_2$ are therefore the first order mutarotation constants k_F and k_G which we have determined experimentally.

* The sucrose was a high grade product kindly given to us by "De Danske Sukkerfabrikker".

We have therefore the equations

$$S \rightarrow F_1 + G_1$$
$$F_1 \rightarrow F_2$$
$$G_1 \rightarrow G_2$$

and using the same symbols for concentration we have the differential equations

$$\dot{F}_1 + k_F F_1 = -\dot{S}$$
$$\dot{G}_1 + k_G G_1 = -\dot{S}$$

with the initial values $S(0) = S_0$, $G_1(0) = G_2(0) = F_1(0) = F_2(0) = 0$. These equations are first order linear equations with the formal solution

$$F_1 = -S + S_0 e^{-k_F t} + k_F e^{-k_F t} \int_0^t S e^{k_F t} dt$$

$$F_2 = S_0(1 - e^{-k_G t}) - k_G e^{-k_G t} \int_0^t S e^{k_G t} dt$$

and analogous expressions for G_1 and G_2.

The total optical rotation of the reaction mixture is

$$\alpha(t) = \alpha_S S + \alpha_1 F_1 + \alpha_2 F_2 + \beta_1 G_1 + \beta_2 G_2$$

where the α's and β's are the optical rotations of the different species in arbitrary units. Using that $\alpha(0) = S_0 \alpha_S$ and $\alpha(\infty) = (\alpha_2 + \beta_2) S_0$ we can form the expression for the apparent degree of advancement defined as

$$\varphi = (\alpha(t) - \alpha(0))/(\alpha(\infty) - \alpha(0))$$

We obtain

$$\varphi = (1-a_1-a_2)\xi + a_1 k_1 e^{-k_1 t} \int_0^t \xi e^{k_1 t} dt + a_2 k_2 e^{-k_2 t} \int_0^t \xi e^{k_2 t} dt$$

where $\xi = 1 - S/S_0$ is the true degree of advancement and

$$a_1 = (\alpha_1 - \alpha_2)/(\alpha_S - \alpha_2 - \beta_2)$$
$$a_2 = (\beta_1 - \beta_2)/(\alpha_S - \alpha_2 - \beta_2)$$

The value of a_2 ($a_2 = 0.544$) can be calculated from data in the literature about the specific rotation of the sugars, but a_1 cannot be calculated *a priori* since the specific rotation of the form of fructose which is liberated from sucrose is not known.

The above integral equation giving φ in terms of ξ is not useful since φ is the quantity which is easy to measure. Differentiating the equation once and twice with respect to time and eliminating the remaining integrals between the three equations we can, however, obtain a differential equation expressing ξ in terms of φ. It is

$$(1-a_1-a_2)\ddot{\xi} + (k_1+k_2-a_1k_2-a_2k_1)\dot{\xi} + k_1k_2\xi = \ddot{\varphi} + (k_1 + k_2)\dot{\varphi} + k_1k_2\varphi$$

Table 1. The table shows the agreement between the experimental values of ξ (t) and the calculated values. The figures in brackets have been used to determine the initial slope of $\xi_{expt}(t)$.

t (min)	5	10	15	20	40	70
ξ_{expt}	0.065	0.127	0.188	0.242	0.453	0.682
ξ_{theor}	(0.065)	(0.127)	(0.188)	0.238	0.461	0.670

When φ is known as a function of t this equation can be integrated numerically to give ξ. Since φ is found experimentally it will be necessary to use smoothed out values of it to get reasonable values for its derivatives. In the actual experiment φ was measured every minute in the larger part of the experiment and we used smoothing by fives to get a table of φ with five minute intervals. Even then the second derivative was rather erratic and we therefore introduced the new variable $z = \varphi - (1-a_1-a_2)\xi$ obtaining the equation

$$\ddot{z} + \frac{k_1+k_2-a_1k_2-a_2k_1}{1-a_1-a_2}\dot{z} + \frac{k_1k_2}{1-a_1-a_2}z = \frac{k_1a_1+k_2a_2}{1-a_1-a_2}\dot{\varphi} + \frac{k_1k_2(a_1+a_2)}{1-a_1-a_2}\varphi$$

in which the second derivative is absent on the right hand side. Using the smoothed out values for φ the derivative was calculated using a five point differentiation formula.

Had a_1 been known the procedure would now have been to use a power series expansion for φ in the neighbourhood of $t = 0$ obtained by least square curve fitting to obtain a power series expansion for ξ near $t = 0$. From this a sufficient number of ξ values could be obtained to start a step by step integration of the equation for z. Such an integration could for instance follow the method of Milne [10,11] which we used in the calculations described below.

As it is, a_1 is not known and we have therefore used the initial slope of the curve $\xi = \xi(t)$ to determine a_1. Using this value $a_1 = 0.228$ the corrected curve

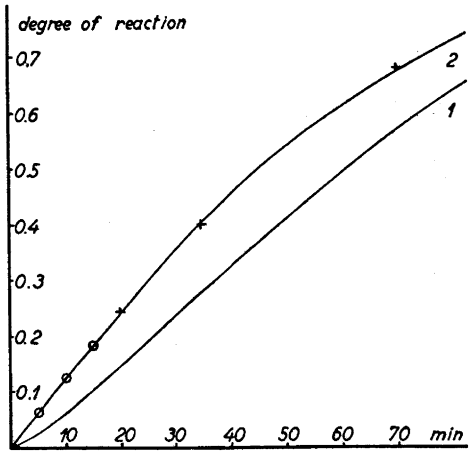

Fig. 1. Curve No. 1 shows the experimental values of $\varphi(t)$, curve No. 2 shows $\xi_{theor}(t)$ calculated using curve No. 1. O are the values of ξ_{expt} which have been used in determining the initial slope of $\xi_{expt}(t)$, + are the other values of ξ_{expt}.

on Fig. 1 has been calculated. Since this curve has been fitted to the experimental data near the origin it is not surprising that it gives a good fit there, but even for $\xi \simeq 0.7$ the disagreement between the calculated values as given in Table 1 and the experimental points is only about 2 % which is only slightly more than the experimental uncertainty. By using a_2 as a parameter which is fitted to give the best agreement on the average the disagreement could be made even smaller and furthermore this would lead to a more precise determination of a_2 and thereby of the optical rotation of fructose as it is formed from sucrose. Such calculations would require calculating facilities which are not at our disposal at the present time but we hope to return to the problem later. Here our primary aim has been to show that a correction of this type is possible but as a byproduct we have obtained a possibly not very accurate value for the optical rotation of β-fructofuranose which from the value of $a_1 = 0.228$ is found to be $-70.8°$ in contrast to the rotation $-95.75°$ found for the equilibrium mixture.

In using this method it is of the utmost importance that φ is known with great accuracy so that its derivatives can be determined with reasonable accuracy. When this is achieved on the other hand, our method presents several advantages. Firstly it is more accurate than the classical method since all errors due to stopping the reaction and diluting the samples are avoided, and more experimental points can be obtained. Secondly the experimental part of it is much quicker so that the method will be faster, provided calculating facilities are available. Finally the method is far less enzyme-consuming than the previous methods. Since earlier experiments always were carried out using enzyme mixtures which were easily obtained the problem of enzyme consumption was not serious. When one wants to use highly purified invertase as we plan to do later the problem becomes important, however, and this has indeed been one of the reasons for developing this method.

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