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# A New Method of Investigating the Enzymatic Hydrolysis of Sucrose. II

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The enzymatic inversion of sucrose is followed by measuring the optical rotation of the reacting mixture directly in the polarimeter and by taking out samples, stopping the reaction and then measuring the optical rotation after the mutarotation of glucose and fructose has come to equilibrium. The kinetic equations for the total reaction are set up, and from these a correction formula is derived for the relationship between the apparent degree of the enzymatic reaction and the true degree of the reaction. This correction formula involves only the molar optical rotations of the sugars involved and the rate constants for the reactions, all of which have been measured independently. Finally values of the "true" degree of the reaction, calculated from the "apparent" degree of the reaction, are compared with the directly measured values of the "true" degree of reaction. Good agreement is obtained. We have thus obtained a method for following the enzymatic reaction using only very small amounts of enzyme, and in addition have shown conclusively that only two mutarotations take place.

1. In a previous publication [1] we have discussed the possibility of following the enzymatic hydrolysis of sucrose by measuring the optical rotation of a mixture of sucrose and the mutarotating monohexoses rather than stopping the enzymatic reaction and measuring the optical rotation after the mutarotation has come to equilibrium. It is then necessary to apply a correction formula in order to obtain the degree of advancement of the enzymatic reaction from the directly measured apparent degree of advancement. The correction formula involves numerical integration of the apparent degree of advancement. In the previous publication this was done in a rather complicated fashion, and questions have been raised as to the validity of certain approximations in the kinetic equations used. Furthermore new information concerning the mechanism of the fructose-mutarotation has been obtained [2]. We shall therefore rederive the equations used previously and show how the actual numerical integration may be performed in a much simpler fashion. Finally we shall compare values of the degree of advancement of the enzymatic reaction,

calculated, by use of the correction formula, from experimentally measured values of the apparent degree of advancement, with the experimentally measured values of the true degree of advancement. All experiments have been made on a new type of polarimeter.

2. Nomenclature.

$S_0$ concentration of sucrose at $t = 0$
$S$ » » » » $t = t$
$F_1$ » » $\beta$-fructofuranose
$F_2$ » » $\beta$-fructopyranose
$G_1$ » » $\alpha$-glucose
$G_2$ » » $\beta$-glucose
$\alpha_s$ Molar optical rotation of sucrose *
$\alpha_1$ » » » » $\beta$-fructofuranose
$\alpha_2$ » » » » $\beta$-fructopyranose
$\alpha_m$ » » » » equilibrium mixture of fructose
$\beta_1$ » » » » $\alpha$-glucose
$\beta_2$ » » » » $\beta$-glucose
$\beta_m$ » » » » equilibrium mixture of glucose
$\varphi$ is the apparent degree of advancement } defined below
$\xi$ is the true degree of advancement }
$k_1 = k_F + k_{-F}$
$k_2 = k_G + k_{-G}$

$k_F$ and $k_{-F}$ are the first order rate constants for the reaction $F_1 \rightleftharpoons F_2$, $k_F$ being the rate constant for the forward reaction, $k_{-F}$ that for the reverse reaction. $k_G$ and $k_{-G}$ are likewise the rate constants for the mutarotation of glucose.

We consider the three simultaneous reactions

$$S \rightarrow F_1 + G_1 \tag{2.01}$$

$$F_1 \underset{k_{-F}}{\overset{k_F}{\rightleftharpoons}} F_2 \tag{2.02}$$

$$G_1 \underset{k_{-G}}{\overset{k_G}{\rightleftharpoons}} G_2 \tag{2.03}$$

and have the following differential equations

$$\dot{F}_1 = -\dot{S} - k_F F_1 + k_{-F} F_2 \tag{2.04}$$
$$= -\dot{S} - k_F F_1 + k_{-F}(S_0 - S - F_1)$$

or

$$\dot{F}_1 + k_1 F_1 = -\dot{S} + k_{-F}(S_0 - S) \tag{2.05}$$

similarly

$$\dot{G}_1 + k_2 G_1 = -\dot{S} + k_{-G}(S_0 - S) \tag{2.06}$$

* By the molar optical rotation of a sugar we shall understand the optical rotation exhibited by a solution containing one mole of the sugar per litre, measured in a 10 cm tube, *i.e.* $\alpha_s = [\alpha_s]^{25}_{546} \times M_s/1000$. We assume the optical rotation per mole to be independent of the concentration.

Using the initial conditions $S(0) = S_0$ and $F_1(0) = F_2(0) = G_1(0) = G_2(0) = 0$ we obtain

$$F_1 = -S + \frac{S_0}{k_1}(k_{-F} + k_F e^{-k_1 t}) + k_F e^{-k_1 t}\int_0^t S e^{k_1 t} dt$$

$$F_2 = \frac{S_0 k_F}{k_1}(1 - e^{-k_1 t}) - k_F e^{-k_1 t}\int_0^t S e^{k_1 t} dt \qquad (2.07)$$

$$G_1 = -S + \frac{S_0}{k_2}(k_{-G} + k_G e^{-k_2 t}) + k_G e^{-k_2 t}\int_0^t S e^{k_2 t} dt$$

$$G_2 = \frac{S_0 k_G}{k_2}(1 - e^{-k_2 t}) - k_G e^{-k_2 t}\int_0^t S e^{k_2 t} dt$$

The optical rotation of the reacting mixture is

$$\alpha(t) = \alpha_s S + \alpha_1 F_1 + \alpha_2 F_2 + \beta_1 G_1 + \beta_2 G_2 \qquad (2.08)$$

and using $\alpha(0) = \alpha_s S_0$ and $\alpha(\infty) = \alpha_1 F_1(\infty) + \alpha_2 F_2(\infty) + \beta_1 G_1(\infty) + \beta_2 G_2(\infty)$ we get for $\varphi$, the apparent degree of advancement

$$\varphi = (\alpha(t) - \alpha(0))/(\alpha(\infty) - \alpha(0)) \qquad (2.09)$$

$$\varphi = A\xi + \frac{k_F(\alpha_2 - \alpha_1)}{\frac{k_{-F}\alpha_1 + k_F\alpha_2}{k_1} + \frac{k_{-G}\beta_1 + k_G\beta_2}{k_2} - \alpha_s} e^{-k_1 t}\int_0^t \xi e^{k_1 t} dt$$
$$+ \frac{k_G(\beta_2 - \beta_1)}{\frac{k_{-F}\alpha_1 + k_F\alpha_2}{k_1} + \frac{k_{-G}\beta_1 + k_G\beta_2}{k_2} - \alpha_s} e^{-k_2 t}\int_0^t \xi e^{k_2 t} dt \qquad (2.10)$$

where $\xi = (S_0 - S)/S_0$ is the true degree of advancement of the enzymatic reaction and

$$A = \frac{\alpha_1 + \beta_1 - \alpha_s}{\frac{k_{-F}\alpha_1 + k_F\alpha_2}{k_1} + \frac{k_{-G}\beta_1 + k_G\beta_2}{k_2} - \alpha_s} \qquad (2.11)$$

This expression for $\varphi$ (eqn. 2.10) is exactly the same as the one obtained in the previous article, where we used $F_1 \xrightarrow{k_1} F_M$ instead of $F_1 \underset{k_{-F}}{\overset{k_F}{\rightleftharpoons}} F_2$, where $F_m$ signifies the concentration of "equilibrium mixture" of fructose.

If we consider the reaction $F_1 \underset{k_{-F}}{\overset{k_F}{\rightleftharpoons}} F_2$ we see that at equilibrium $k_F F_1 = k_{-F} F_2$ and the optical rotation of the equilibrium mixture is

$$\alpha_m = \frac{\alpha_1 F_1(eq) + \alpha_2 F_2(eq)}{k_1}$$

When the condition of equilibrium is inserted in this we get the expression for $\alpha_m$

$$\alpha_m = \frac{k_{-F}\alpha_1 + k_F\alpha_2}{k_1} \qquad (2.12)$$

If in the expression for $\varphi$ (eqn. 2.10) we use eqn. (2.12) and the analogous expression for $\beta_m$ we arrive at an expression identical to the one in the previous paper (there we denoted the rotations of the equilibrium mixtures by $\alpha_2$ and $\beta_2$ instead of $\alpha_m$ and $\beta_m$).

From the integral equation for $\xi$ (eqn. 2.10) we obtain a differential equation connecting $\varphi$ and $\xi$ in the same way as previously, *i.e.*, integrating once and twice with respect to time and eliminating the remaining integrals between the three equations. It is, of course, still the same differential equation as the one in the previous paper, namely

$$\ddot{\xi} + \left[k_1 + k_2 + \frac{k_F(\alpha_2-\alpha_1) + k_G(\beta_2-\beta_1)}{\alpha_1 + \beta_1 - \alpha_s}\right]\dot{\xi} + \frac{k_1k_2}{A}\xi = \frac{\ddot{\varphi}}{A} + \frac{k_1+k_2}{A}\dot{\varphi} + \frac{k_1k_2}{A}\varphi \tag{2.13}$$

but we shall now solve it in a different manner.

The solution of the homogeneous equation is

$$\xi = C_1e^{\lambda_1 t} + C_2e^{\lambda_2 t} \tag{2.14}$$

where $\lambda_1$ and $\lambda_2$ are the roots of the characteristic equation.

A solution of the inhomogeneous equation then becomes

$$\xi = \frac{e^{\lambda_2 t}}{A}\int_0^t \frac{e^{\lambda_1 t}}{W}(\ddot{\varphi} + (k_1+k_2)\dot{\varphi} + k_1k_2\varphi)dt - \frac{e^{\lambda_1 t}}{A}\int_0^t \frac{e^{\lambda_2 t}}{W}(\ddot{\varphi} + (k_1+k_2)\dot{\varphi} + k_1k_2\varphi)dt \tag{2.15}$$

with the Wronski determinant

$$W = (\lambda_2-\lambda_1)e^{(\lambda_1+\lambda_2)t}$$

Integrating by parts once and twice and using that $\varphi(0) = 0$ we obtain

$$\xi = \frac{\dot{\varphi}(0)}{A}\left\{e^{\lambda_1 t} - e^{\lambda_2 t}\right\} + \frac{\varphi(t)}{A} + \frac{\lambda_2^2 + (k_1+k_2)\lambda_2 + k_1k_2}{A(\lambda_2-\lambda_1)}e^{\lambda_2 t}\int_0^t \varphi e^{-\lambda_2 t}dt - \frac{\lambda_1^2 + (k_1+k_2)\lambda_1 + k_1k_2}{A(\lambda_2-\lambda_1)}e^{\lambda_1 t}\int_0^t \varphi e^{-\lambda_1 t} \tag{2.16}$$

The initial conditions are $\xi(0) = 0$ and $\dot{\xi}(0) = A^{-1}\dot{\varphi}(0)$ as can be seen from the integral equation (2.10). Combining (2.14) and (2.16) using these conditions leads to the following expression for $\xi$:

$$\xi = \frac{\varphi}{A} + \frac{\lambda_2^2 + (k_1+k_2)\lambda_2 + k_1k_2}{A(\lambda_2-\lambda_1)}e^{\lambda_2 t}\int_0^t \varphi e^{-\lambda_2 t}dt - \frac{\lambda_1^2 + (k_1+k_2)\lambda_1 + k_1k_2}{A(\lambda_2-\lambda_1)}e^{\lambda_1 t}\int_0^t \varphi e^{-\lambda_1 t}dt \tag{2.17}$$

Remembering that $\lim_{t\to\infty} e^{\lambda t}\int_0^t \varphi e^{-\lambda t}dt = -\frac{1}{\lambda}$ it is easily verified that $\xi(t) \to 1$ as $t \to \infty$.

Eqn. (2.17) expresses the actual degree of advancement of the enzymatic reaction in terms of the apparent degree of advancement, the molar optical rotation of the sugars, and the rate constants for the reactions involved, which can all be measured independently.

3. The rate constants and molar optical rotations for the glucoses are well known,[3] as is the optical rotation for $\beta$-fructopyranose, and the rate constants and optical rotation for $\beta$-fructofuranose have been determined recently by one of us [2].

In a mutarotation experiment, starting with one mole of pure $F_2$, the following equations apply

$$F_1 \underset{k_{-F}}{\overset{k_F}{\rightleftharpoons}} F_2 \qquad F_1 + F_2 = 1$$

$$F_1 = \frac{k_{-F}}{k_1}(1-e^{-k_1 t}) \; ; \; F_2 = e^{-k_1 t} + \frac{k_F}{k_1}(1-e^{-k_1 t}) \tag{3.01}$$

$$\alpha(t)-\alpha_m = e^{-k_1 t}\frac{k_{-F}}{k_1}(\alpha_2-\alpha_1) \tag{3.02}$$

or

$$\ln(\alpha(t)-\alpha_m) = -k_1 t + \ln\,(k_{-F}(\alpha_2-\alpha_1)/k_1) \tag{3.03}$$

Here $\alpha_m$ is the molar optical rotation after equilibrium is reached, defined by eqn. (2.12).

A plot of $\ln(\alpha_t-\alpha_m)$ *versus* time therefore gives a straight line. From this experiment we therefore obtained $\alpha_m$, $k_1$ and $\alpha_2$ which was the initial optical rotation. In order to obtain the value of $\alpha_1$ one could in principle have done a mutarotation experiment starting with pure $F_1$, but this is not obtainable as a solid as is $F_2$. Instead we obtained the remaining constants from an inversion experiment with a large concentration of enzyme.

In an inversion experiment, starting with pure sucrose, we have

$$S \xrightarrow{k_s} G_1 + F_1 \; ; \; F_1 \underset{k_{-F}}{\overset{k_F}{\rightleftharpoons}} F_2 \; ; \; G_1 \underset{k_{-G}}{\overset{k_G}{\rightleftharpoons}} G_2 \tag{3.04}$$

If we assume the enzymatic splitting of sucrose to be a first order reaction we have

$$S = S_0 e^{-k_s t} \tag{3.05}$$

and

$$\dot{F}_1 = k_s S_0 e^{-k_s t} + k_{-F}F_2 - k_F F_1 \tag{3.06}$$

then the solution, with the initial condition $F_1(0) = 0$, is

$$F_1 = \frac{S_0 k_{-F}}{k_1} - \frac{S_0(k_s-k_{-F})}{k_s-k_1}e^{-k_s t} + \frac{S_0 k_F k_s}{k_1(k_s-k_1)}e^{-k_1 t} \tag{3.07}$$

$$F_2 = \frac{S_0 k_F}{k_1} + \frac{S_0 k_F}{k_s-k_1}e^{-k_s t} - \frac{S_0 k_F k_s}{k_1(k_s-k_2)}e^{-k_1 t}$$

The analogous equations apply for the two forms of glucose.

This leads to the following expression for $\alpha(t)-\alpha(\infty)$, the optical rotation of the reacting solution minus that of the final equilibrium mixture,

$$\alpha(t)-\alpha(\infty) = S_0 e^{-k_s t}\left\{\alpha_s + \frac{k_{-F}\alpha_1 + k_F\alpha_2 - k_s\alpha_1}{k_s-k_1} + \frac{k_{-G}\beta_1 + k_G\beta_2 - k_s\beta_1}{k_s-k_2}\right\} + \frac{S_0 k_F k_s}{k_1(k_s-k_1)}(\alpha_1-\alpha_2)e^{-k_1 t} + \frac{S_0 k_G k_s}{k_2(k_s-k_2)}(\beta_1-\beta_2)e^{-k_2 t} \quad (3.08)$$

After a short time the first term in eqn. (3.08) can be neglected, and after a somewhat longer time the second term can be neglected, since $k_1 \gg k_2$. For large times we therefore have

$$\alpha(t) - \alpha(\infty) \approx \frac{S_0 k_G k_s}{k_2(k_s-k_2)}(\beta_1-\beta_2)e^{-k_2 t} = \beta(t) \quad (3.09)$$

which allows us to calculate $k_2$ and the pre-exponential factor from the measured values of $\alpha(t)-\alpha(\infty)$ for large times. Using these values $\beta(t)$ was calculated for smaller times and subtracted from $\alpha(t)-\alpha(\infty)$. For reasonably large times the remaining term now satisfies the equation

$$\alpha(t)-\alpha(\infty)-\beta(t) \approx \frac{S_0 k_F k_s}{k_1(k_s-k_1)}(\alpha_1-\alpha_2)e^{-k_1 t} \quad (3.10)$$

which allows the determination of $k_1$ (which is known also from the previous experiment) and the pre-exponential factor. Using eqn. (2.12) the latter is written as

$$S_0 \frac{(\alpha_1-\alpha_m)}{1-(k_1/k_s)}$$

and we see that we can now determine $\alpha_1-\alpha_m$. The necessary value of $k_s$, which actually gives rise only to a comparatively small correction term, was obtained from the initial rate of the sucrose inversion. Knowing $\alpha_1$, $\alpha_2$, $\alpha_m$ and $k_1$, we were able to obtain $k_{-F}$ and $k_F$ from the definition of $k_1$ and eqn. (2.12).

The constants we have obtained are given in Table 1.

When we insert the values from Table 1, the characteristic equation, from eqn. (2.13),

$$\lambda^2 + \left[k_1 + k_2 + \frac{k_F(\alpha_2-\alpha_1) + k_G(\beta_2-\beta_1)}{\alpha_1+\beta_1-\alpha_s}\right]\lambda + \frac{k_1 k_2(\alpha_m+\beta_m-\alpha_s)}{\alpha_1+\beta_1-\alpha_s} = 0 \quad (3.11)$$

becomes

$$\lambda^2 + 1.93155\lambda + 0.06980491 = 0 \quad (3.12)$$

The roots are

$$\lambda_1 = -1.89471, \qquad \lambda_2 = -0.0368420$$

and so eqn. (2.17) becomes

$$\xi = 13.1373\varphi - 0.0151865e^{-0.0368420t}\int_0^t \varphi e^{0.0368420t}\,dt - 22.215617e^{-1.89471t}\int_0^t \varphi e^{1.89271t}\,dt \quad (3.13)$$

*Table 1.* All constants apply at25.0°C. The wavelength used is 546 mμ.

| | | |
|---|---|---|
| $\alpha_S$ | 26.73 | deg/mole |
| $\alpha_1$ | 0.31 | » |
| $\alpha_2$ | −27.70 | » |
| $\alpha_m$ | −18.86 | » |
| $\beta_1$ | 23.80 | » |
| $\beta_2$[b] | 4.05 | » |
| $\beta_m$ | 11.23 | » |
| $k_F$ | 0.14687 | $min^{-1}$ |
| $k_{-F}$ | 0.06773 | » |
| $k_1$ | 0.2146 | » |
| $k_G$[a] | 0.015756 | » |
| $k_{-G}$[a] | 0.009004 | » |
| $k_2$ | 0.02476 | » |

[a]) calculated from data given in Ref.[3]
[b]) calculated from $\beta_2 = (k_2\beta_m - k_{-G}\beta_1)/k_G$

The numbers in this equation are only a function of the temperature and the wavelength used (through the optical rotations). They are neither a function of the concentration of sucrose nor of the concentration of enzyme. If the experimental conditions for the measurement of $\varphi$ were such that there was any amount of glucose or fructose or both present, the equation would still apply, provided they had come to mutarotation equilibrium when the experiment started.

4. Polarimetric measurements were performed at 25.0°C in a 10 cm polarimeter tube with a thermostated jacket. The polarimeter was a Zeiss "Lichtelektrisches Polarimeter" which allowed readings of the optical rotation with an accuracy of 0.005°. The wavelength used was 546 mμ (the green Hg-line). The kinetic experiments were made in solutions which contained 10 g of sucrose in 100 ml of solution. The solutions were 0.005 M with respect to sodium acetate and 0.005 M with respect to acetic acid. The pH of the solutions was 4.75 which is the optimum pH for the enzyme.

The enzyme solution contained 4 mg β-fructofuranosidase [4] per ml. In both experiments the reaction vessel contained 500 ml of the sucrose solution and 500 μl of the enzyme solution. In the first type of experiment samples of 20 ml were taken out and the reaction was stopped by introducing the sample into a flask containing the necessary amount of $Na_2CO_3$. These flasks were prepared by evaporating 1 ml of 0.5 M $Na_2CO_3$ leaving the $Na_2CO_3$ as a film inside the flask. The $Na_2CO_3$ immediately stops the enzyme catalyzed reaction and catalyzes the mutarotation of the glucose and the fructose. The polarimetric readings were taken 15 min after the reaction was stopped. In the other type of experiment one sample was taken out and the reaction was directly followed in the polarimeter at intervals of 30 sec.

Since a volume of 10 ml is necessary for a polarimetric reading in the thermostated tube it will be seen that under the above conditions an experiment of the first type will use 2.0 mg of enzyme while the second type can be performed with only 0.04 mg of enzyme.

The experimental values of $\varphi$ did not extrapolate to $\varphi \to 0$ for $t \to 0$. By using the method of least squares on a second degree polynomial through the first ten experimental points it was found that the curve would go smoothly through the origin if we assumed that the reaction had started 0.39 min before the stopwatch was started. The 500 μl of enzyme solution is introduced into the 500 ml of sugar solution by means of a pipette and immediately the

*Table 2.* $\varphi$ is the apparent degree of advancement. $\xi_{calc}$ is the true degree of advancement calculated from the $\varphi$'s and eqn. (3.13). $\xi_{exp}$ is the measured true degree of advancement.

| $t$ | $\varphi$ | $\xi_{calc}$ | $\xi_{exp}$ |
|---|---|---|---|
| 0 | 0.0000 | 0.0000 | 0.0000 |
| 10 | 0.0938 | 0.2007 | 0.1900 |
| 25 | 0.2838 | 0.4421 | 0.4389 |
| 30 | 0.3463 | 0.5122 | 0.5116 |
| 40 | 0.4631 | 0.6343 | 0.6358 |
| 50 | 0.5662 | 0.7318 | 0.7353 |
| 60 | 0.6534 | 0.8061 | 0.8110 |
| 70 | 0.7248 | 0.8608 | 0.8659 |
| 80 | 0.7818 | 0.8989 | 0.9055 |
| 100 | 0.8613 | 0.9441 | 0.9490 |
| 120 | 0.9097 | 0.9655 | 0.9688 |

reaction vessel is shaken vigorously for 1 min, the stopwatch being started 10 sec. after the shaking was commenced. Experimentally, therefore, the time at which the reaction starts is not well defined, and we believe that this definition of a zero point leads to a more accurate description.

The experimental points for $\varphi$ were taken in groups of 7 at 0.5 min intervals around every 5 min up to 100 min, thereafter only at 5 min intervals until virtual completion of the reaction (180 min). The groups of seven points were smoothed by a third degree polynomial [5] as were also the latter points. From the smoothed values the values at $t - 0.39$ min were interpolated using a five point Lagrange interpolation formula.

The experimental points for $\xi_{exp}$ were taken at 2 and 3 min intervals up to 30 min, then at 10 min intervals up to 100 min, and finally a value at 120 min was recorded. The experimental points extrapolated to $\xi = 0$ at $t \to 0$, but in view of what was said above we consider this as a lucky coincidence. Since no differentiation or integration was to be done on $\xi(t)$, no smoothing was performed here.

From the values for $\varphi$, corresponding values for $\xi$ were calculated using eqn. (3.13). The integrals were calculated numerically, the integral involving $\lambda_1$ being converted to a series of differences, and the integral involving $\xi_2$ being calculated as the numerical solution of a differential equation. These procedures have been described previously [6]. The values for $\xi$ calculated in this way are given in Table 2, together with the experimental values for $\varphi$, and also the experimental values for $\xi$.

5. It is seen from Table 2 that apart from $\xi$ at $t = 10$ min the difference between the calculated and the experimental values of $\xi$ is about 0.005. Although the uncertainty in $\xi$ in a single experiment is less than that, the difference between the corresponding $\xi$-values in two presumably identical experiments will usually be of that order. Since we are testing the method by comparing two experiments we feel that the agreement is quite satisfactory.

The method which we have developed here has the obvious advantage of being less enzyme-consuming than the ordinary way of following the reaction, but in addition to that it confirms the value attributed to the optical rotation

of the furanose form of fructose. Since the detailed mechanism of the inversion does not enter into the calculations at all, the method of course gives no information about this mechanism, not even to the extent of deciding for instance whether oligosaccharides are formed. The only aim of this work has been to get $\xi(t)$ from the measured $\varphi$-values in as simple a manner as possible.

It could be argued that since after all we only want the kinetic data in order to find one (or several) mechanisms which are compatible with the experiments, we might as well from the various possible theoretical $\xi(t)$ calculate the theoretical $\varphi(t)$ which then could be compared directly with the measured values of the apparent degree of reaction. This is, however, not a simple matter, since it is known that usually for steady state kinetics it is not $\xi$ which is a simple function of $t$, but rather $t$ which can be written as a simple function of $\xi$. This appears also to be the case for the inversion and we believe that the present method in spite of its apparent complexity, is the simplest to use when conditions are such that only small amounts of enzyme may be used.

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