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Problems associated with using thermal measurement principles in enzymatic reactions [1]

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Abstract

The primary intention when applying themoanalytical techniques to enzymatic reactions is the derivation of reliable thermochemical and thermokinetic information for selected reactions. The enzymatic oxidation of glucose was selected as a model reaction. Caloric measurements showed that the extrapolated thermal power at $t = 0$ is proportional to the concentration of the substrate in GOD-catalysed glucose oxidation, so that measurement can be restricted to the determination of the initial power. The range of measurable glucose oxidation was initially limited by the oxygen content of the solution (0–1.4 mmol l^{-1}). This concentration range has been expanded considerably by adding mediators. The additional use of mutarotase resulted in an increased reaction rate.

INTRODUCTION

By combining highly specific enzymatic catalysis with the measurement of the reaction enthalpy related to each chemical reaction, it should be possible to produce universal thermal sensors. The first stage in achieving this is to obtain reliable thermochemical and thermokinetic information for selected enzyme-catalysed reactions. As a model, the oxidation of glucose was investigated under the influence of the enzyme glucose oxidase.

The importance of glucose detection in fields such as medicine and the food industry was the influencing factor in the selection of the model reaction. In addition, it was assumed that the chemistry of the oxidation of glucose is so well known that no additional problem would occur. Even the first measurements, however, revealed the complexity of the selected 'model system' so that the above assumption proved to be invalid. Important factors influencing the glucose oxidation include the selection of a suitable buffer system, the oxygen supply in the analysis solution and the

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related question of mediators, as well as the inclusion of the mutarotation equilibrium and the selection of enzymes. Furthermore, catalase can be added to enhance the thermal effect. Reliable caloric measurements were required to assess the mutual relationship between the chemical and thermal aspects of the reaction.

EXPERIMENTAL

The glucose oxidase used (E.C. 1.1.3.4.), from *Aspergillus niger,* had a specific activity of 250 U mg^{-1} (foreign activities, 5 U per mg catalase; 0.05% α-amylase; 0.05% invertase; 0.05% maltase). The specific activity of the catalase used (E.C. 1.11.1.6.), from *Aspergillus niger,* was 1950 U mg^{-1}. The mutarotase, made from *Acinetobacter calcoaceticus* (E.C.5.1.3.3.), had a specific activity of 20 U mg^{-1}. P.a. chemical agents were used throughout in the measurements. Testal buffer (pH 6.86) (M/40, KH_2PO_4/M/40 Na_2HPO_4) was used as a buffer system. The experimental details have been given in ref. 1.

RESULTS AND DISCUSSION

The overall reaction equation of the conversion of β-glucose into D-glucono-δ-lacton or gluconic acid is

$$\beta\text{-glucose} + O_2 \longrightarrow H_2O_2 + \text{D-glucono-}\delta\text{-lacton}$$

$$\text{D-glucono-}\delta\text{-lacton} \xrightarrow{H_2O} \text{D-gluconic acid}$$

The method of quantitative glucose detection is based on the proportionality between the exchanged reaction heat and the converted quantity of glucose. Measurement was performed in an isoperibolic normal-temperature calorimeter. The total exchanged reaction heat, i.e. the reaction enthalpy, is the recorded signal.

The oxidation of glucose under the influence of the enzyme glucose oxidase has been analysed using thermal methods by several research teams [2–7]. However, when looking for reference values for the molar reaction enthalpy of this enzymatic reaction, the only value found is $\Delta_R H = -207$ kJ mol^{-1} measured by Schmidt et al. [7] by flow calorimetry in 1976. To enhance the thermal signal, they added catalase as well as glucose oxidase for the conversion of the hydrogen peroxide. The authors wrongly assumed a reaction enthalpy of -126 kJ mol^{-1} for the hydrogen peroxide decomposition which then led to the value of -80 kJ mol^{-1} which is often cited in the literature [8, 9] as the molar reaction enthalpy of glucose oxidation.

The molar reaction enthalpy of hydrogen peroxide decomposition is

TABLE 1

Results of caloric measurements

Enzyme	Mediator	Number of measurements	$\Delta_R H$/kJ mol^{-1}
GOD	Benzoquinone	12	-124 ± 4
GOD	Oxygen	12	-127 ± 4
GOD	$K_3[Fe(CN)_6]$ + air	10	-123 ± 5
GOD	$K_3[Fe(CN)_6]$ + air	10	-125 ± 4
GOD	$K_3[Fe(CN)_6]$ + N_2	4	-125 ± 2
GOD	$K_3[Fe(CN)_6]$ + O_2	4	-127 ± 3
GOD/Catalase	Benzoquinone	7	-223 ± 2

-100 kJ mol^{-1}, which gives a value of -107 kJ mol^{-1} for glucose oxidation, according to Schmidt et al.

Table 1 shows that we determined a value of -125 ± 4 kJ mol^{-1} for the reaction enthalpy of enzymatic glucose oxidation, from more than 50 measurements. The molar reaction enthalpy relates to the entire amount of glucose used. For the molar reaction enthalpy of the reaction with catalase, we obtained a value of -223 ± 2 kJ mol^{-1}, equivalent to a value of -100 kJ mol^{-1} for hydrogen peroxide decomposition, in agreement with the literature [10].

Table 1 also summarizes the reaction conditions. The use of the mediators benzoquinone and hexacyanoferrate was investigated, and gaseous oxygen was also employed. The addition of benzoquinone was particularly advantageous.

According to the mutarotation equilibrium, 62% β-glucose and 38% α-glucose are present in glucose solutions. Therefore, it was first necessary to decide whether only the β component reacts or whether the mutarotation rate is sufficient for the eventual conversion of all the glucose.

Figure 1 represents the calorimetric curves for the measurement with glucose solution and with solid α-glucose. It is evident that the maximum temperature difference, and thus the reaction enthalpy, is independent of the initial state (glucose solution or solid α-glucose). Particularly in the initial phase, the temperature–time curve of α-glucose has a shape that is typical of consecutive reactions.

A further problem of enzymatic glucose oxidation is the requirement of oxygen, i.e. the amount of glucose that can be converted is limited by the quantity of oxidation agents or mediators. Figure 2 demonstrates this with reference to the break points in the calorimetric curves for glucose conversion in an air-saturated solution (a) and in an oxygen-saturated solution (b). The constant increase observed after the break point is independent of the glucose concentration and is obviously controlled by

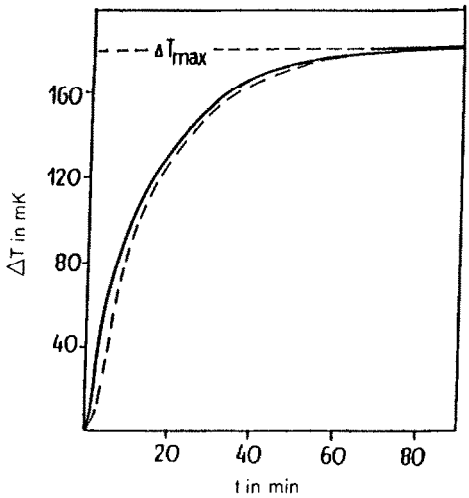

Fig. 1. Temperature–time curves for glucose solution (—) and α-glucose (---); $m = 100$ mg, $v = 62.15$ ml.

the diffusion of oxygen from the gas phase (air or oxygen) to the solution. The ratio of the slopes is approx. 4.8, which is equivalent to the ratio of the O_2 partial pressures or concentrations in the two solutions. Here, the possibility of using this reaction as an oxygen sensor is clear.

For further investigations, sufficient oxidation agents for conversion were made available by using mediators, mainly benzoquinone. Thus, the measurable range of glucose concentration was markedly expanded. Our own measurements were performed in the range of 0.8–18 mmol per l of glucose.

The integral parameter of reaction enthalpy has certain well-known disadvantages when it is employed as a signal variable for a thermal sensor.

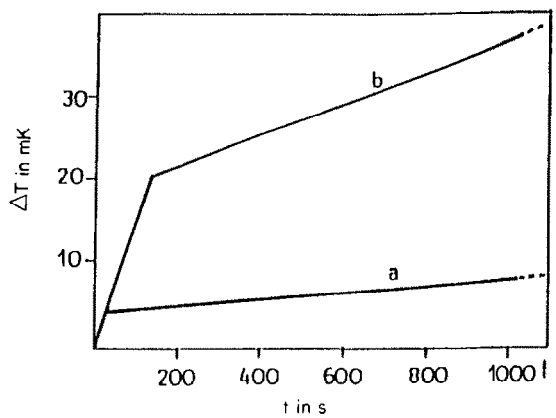

Fig. 2. Calorimetric curves; $c_{Gl} = 7.14$ mmol l^{-1}. Curve (a), air-saturated solution; curve (b) oxygen-saturated solution.

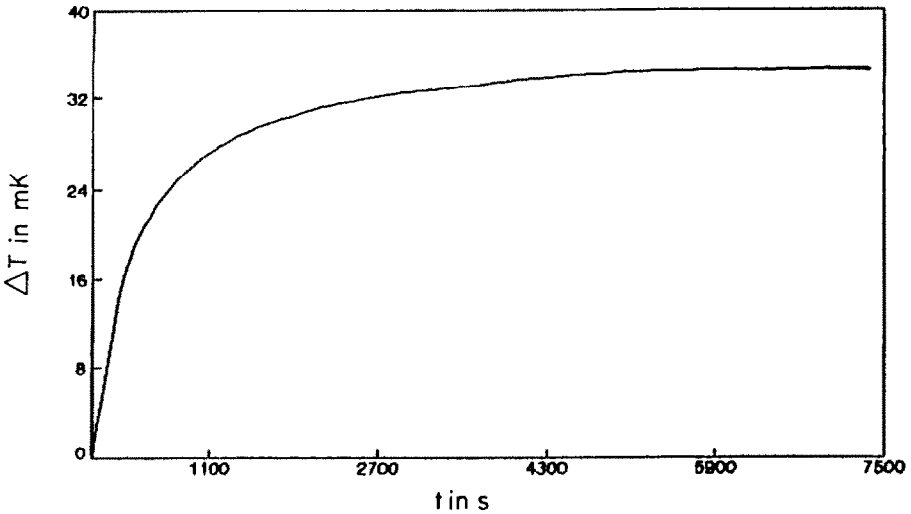

Fig. 3. Adiabatic ΔT–time curve: $m_{GI} = 20$ mg; $v = 62.15$ ml; $m_{GOD} = 3.18$ mg; $m_{Benzo} = 30$ mg.

Therefore, intensive thermokinetic investigations were performed in order to find a signal variable that would be suitable as a thermal sensor of glucose.

The kinetic evaluation assumed justifiably that conversion at time t is proportional to the value of ΔT. Therefore, the initial rate v_0 at time $t = 0$ was first determined. For this, the adiabatic temperature–time curves were smoothed and then differentiated. Figure 3 shows such an adiabatic temperature–time curve obtained with benzoquinone as a mediator.

Figure 4 shows the reaction power plotted against time. In the first part

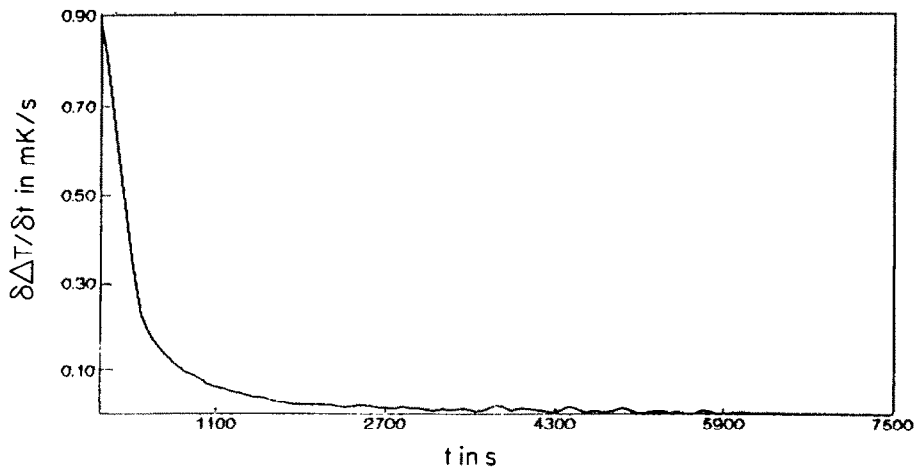

Fig. 4. Thermal power–time curve: $m_{GI} = 20$ mg; $v = 62.15$ ml; $m_{GOD} = 3.18$ mg; $m_{Benzo} = 30$ mg.

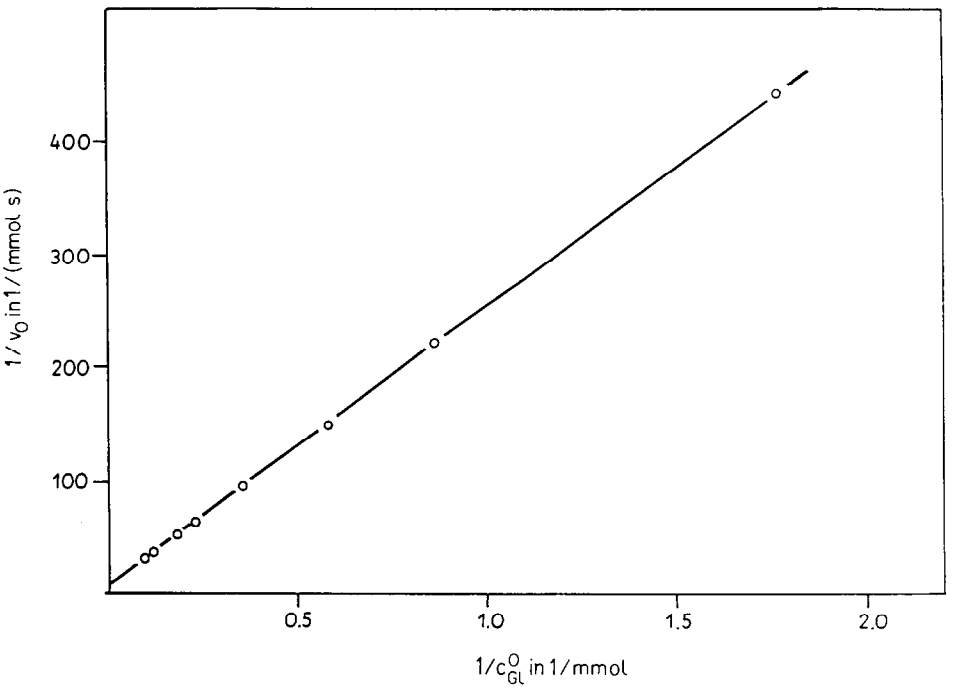

Fig. 5. Lineweaver–Burk diagram.

of the reaction, up to approx. 150 s, the reaction power is almost proportional to time, so that v_0 can be determined with certainty.

Figure 5 shows the evaluation of the Michaelis–Menten equation in the Lineweaver–Burk diagram: a straight line is obtained. From the linear regression, we obtain

$K_m = 41.8 \pm 0.2$ mmol l^{-1}

$v_{max} = 106.4 \pm 0.2$ μmol l^{-1} s^{-1}

The enzyme activity can be calculated on the basis of the maximum reaction rate. In our own measurements, it was 125 U mg^{-1}, in agreement with the results of photometric analysis.

For further kinetic analysis, it was important to understand that not only β-glucose but also α-glucose is converted after mutarotation. For this reaction, we have developed a kinetic model [11] in which the velocity constants k_1 for $\alpha \rightarrow \beta$ conversion and k_2 for the actual glucose oxidation can be calculated.

Figure 6 gives an example of the calculations made on the basis of this model. The conversion of α-glucose and β-glucose are represented separately. The oxidation of β-glucose is complete after approx. 16 min,

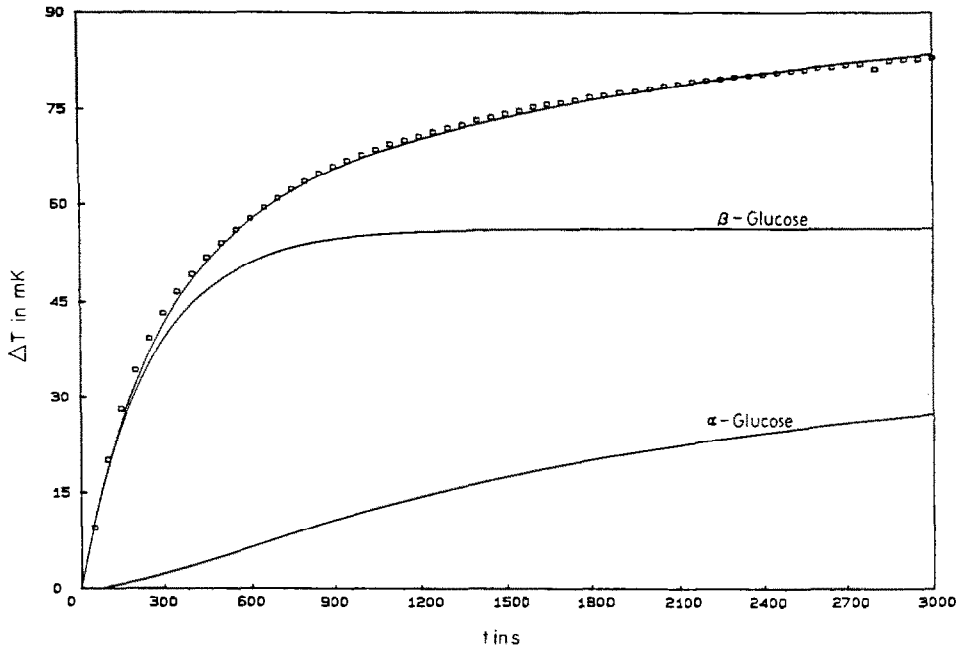

Fig. 6. Adiabatic ΔT–time curves for the two fractions α-glucose and β-glucose: $c_{GOD} = 51.4\ mg\ l^{-1}$; $c^{\circ}_{Gl} = 4.57\ mmol\ l^{-1}$; □, exp. values; —, calculated.

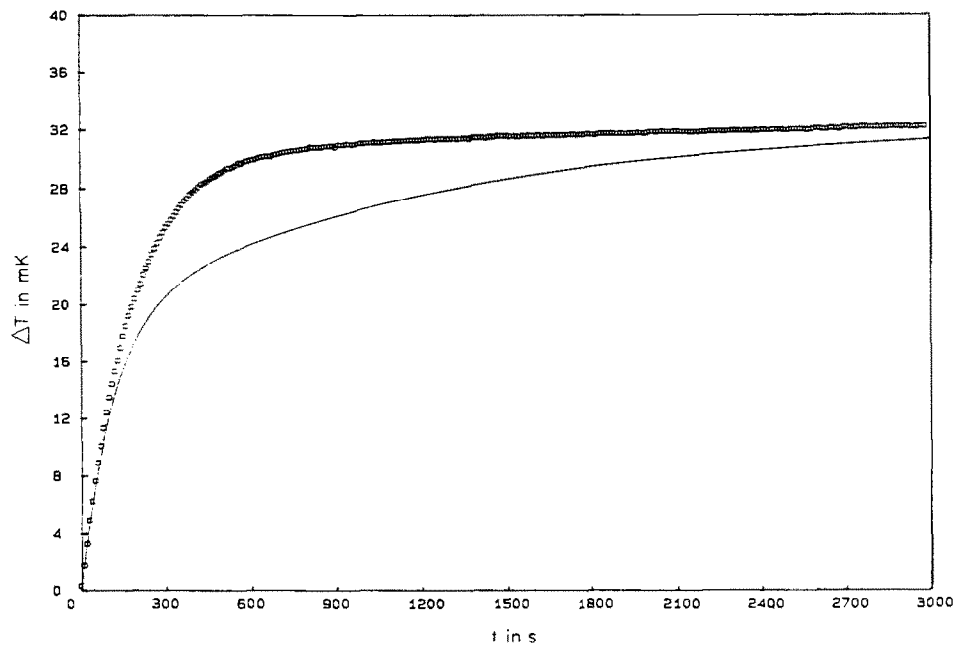

Fig. 7. Adabatic ΔT–time curves: $c_{GOD} = 55.4\ mg\ l^{-1}$; $c_{Gl} = 1.85\ mmol\ l^{-1}$; —, without mutarotase; □, $c_{Mu} = 83\ mg\ l^{-1}$.

whereas the mutarotation/oxidation of α-glucose requires up to 100 min. Therefore, the initial reaction rate, which is decisive for a thermal glucose sensor has to be related not to the total amount of glucose but to the concentration of β-glucose only. A consequence of this is that the Michaelis constant is reduced by a factor of 0.62, i.e. ($K_m = 26$ mmol l^{-1}).

Because the problem of mutarotation has not received the attention it deserves in the literature on enzymatic glucose oxidation, we have investigated glucose oxidation after addition of mutarotase.

Figure 7 shows two temperature–time curves, one for the measurement without mutarotase (solid line) and one with addition of mutarotase (5 mg per 60 ml cell content). It is clear that the addition of mutarotase results in a considerable increase in the reaction rate. This is particularily interesting with respect to the increase in the detection limit or the measurement accuracy. The reaction is essentially complete after 500 s if mutarotase is added. The parameters of the two enzymes, K_m and the activity, can be derived from the kinetic evaluation.

CONCLUSIONS

The most important result of this work has been the reduction of the thermal detection to the registration of the initial reaction power. Figure 8 represents the proportionality between the initial thermal power, as a suitable parameter for a thermal sensor, and the glucose concentration.

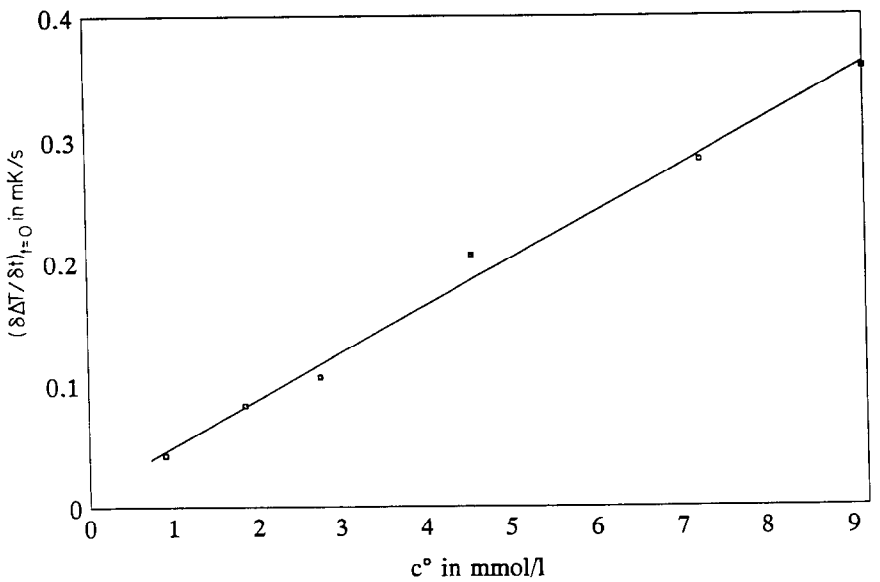

Fig. 8. Initial thermal power–concentration curve: $v = 62.15$ ml; $m_{GOD} = 3.18$ mg.

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