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Thermokinetic investigations into enzyme catalysed glucose oxidation

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Abstract

The kinetic investigation of the enzyme (glucose oxidase) catalysed glucose oxidation was performed by calorimetric monitoring of the reaction. The measurable concentration range was expanded by using benzoquinone as a mediator. A comprehensive kinetic evaluation of the adiabatic calorimetric measurement curves allowed us to determine the Michaelis–Menten constant K_M, the enzyme activity, the reaction rate equations with the corresponding velocity constants, and the functional dependence of the reaction rate on enzyme and glucose concentration.

INTRODUCTION

In a previous study [1], the results of calorimetric measurements of enzyme catalysed glucose oxidation with the mediators oxygen, potassium hexacyanoferrate and benzoquinone were reported.

It was shown that the mediator benzoquinone considerably expanded the measurable concentration range of glucose. This allowed a comprehensive kinetic evaluation of the adiabatic calorimetric curves. Such an evaluation is facilitated by the circumstances that the reaction rate is evidently independent of the benzoquinone mediator concentration, and that because of its high velocity the reoxidation of glucose oxidase is not a substage controlling the overall reaction rate. In the quoted work [1] it was shown that α-glucose is oxidized to β-glucose after mutarotation. Therefore the α/β-mutarotation must be taken into consideration in the kinetic analysis. The determination of glucose by thermal sensors has been described by numerous authors [2] but has received little attention [3]. As a result of the kinetic evaluation, the Michaelis constant K_M, the enzyme activity, the reaction rate equations with their velocity constants, and the

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functional dependence of the reaction rate on enzyme and glucose concentration can be determined. The accurate knowledge of the kinetics is a major precondition for the reliable functioning of a thermal glucose sensor.

MATERIALS AND EQUIPMENT

The details are contained in ref. 1. In a total of 24 calorimetric experiments, the glucose concentration was varied over a range between 0.9 and 18 mmol l^{-1}, and the enzyme concentration (*Aspergillus niger*, Serva) was varied between 50 and 190 mg l^{-1} (enzyme activity about 150 and 260 U mg^{-1}). The amount of benzoquinone was doubled with respect to stoichiometric conversion. A solution consisting of a buffer (phosphate), benzoquinone and glucose was prepared in the calorimetric cell, with the enzyme solution in an ampoule. The reaction was initiated by breaking the ampoule. All measurements and results relate to a calorimeter temperature of 25°C. In this particular case, the shape of the adiabatic temperature–time curve is controlled exclusively by the rate of the chemical reaction and not by heat transfer between the solution and the temperature sensor (within error limits). The half-times of the analysed chemical reactions were 30–220 s, whereas the temperature sensor had a half-time of about 300 ms.

DISCUSSION

The kinetic evaluation was based upon the assumption that the conversion at time t is proportional to ΔT

$$\Delta T = \Delta T_{max} \frac{c_{gl}^{\circ} - c_t}{c_{gl}^{\circ}} \tag{1}$$

where c_{gl}° is the total glucose concentration; c_t is the glucose concentration at time t; $\Delta T_{max} = \Delta T$ at 100% yield.

For the reaction rate, it follows that

$$-\frac{dc}{dt} = \frac{c_{gl}^{\circ}}{\Delta T_{max}} \frac{d\Delta T}{dt} = v \tag{2}$$

First, this equation was used to determine the initial reaction rate v_0 ($t = 0$). The adiabatic temperature–time curves were fitted by means of a cubic spline function and then differentiated.

In the first part of the reaction ($t < 150$ s), $d\Delta T/dt$ is almost proportional to t, so that $d\Delta T/dt$ can be reliably determined for $t = 0$. Now, the reaction

TABLE 1

Kinetic data for constant enzyme concentration; reaction volume: 62.15 ml; $M_{gox} = 3.18$ mg

c°_{gl} (mmol l^{-1})	ΔT_{max} (mK)	$(\partial \Delta T/\partial T)_{t=0}$ (mK s^{-1})	$k_2 \times 10^3$ (s^{-1})	$v_0 \times 10^3$ (mmol l^{-1} s^{-1})	$k_1 \times 10^4$ (s^{-1})
0.91	16.39	0.0416	4.09	2.27	8.0
1.86	34.45	0.0831	3.89	4.49	7.4
2.76	44.66	0.106	3.82	6.54	8.5
4.57	90.74	0.206	3.67	10.40	7.5
7.25	128.30	0.284	3.57	16.05	8.4
9.17	167.19	0.359	3.46	19.67	8.4
13.64	253.2	0.488	3.11	26.30	9.4
18.00	315.8	0.552	2.82	31.47	10.4

rate v_0 at $t = 0$ can be calculated, and an evaluation becomes possible of the Michaelis–Menten equation

$$(dc/dt)_{t=0} = v_{max} c^{\circ}_{gl}/(K_M + c^{\circ}_{gl}) = v_0 \tag{3}$$

$$\frac{1}{v_0} = \frac{K_M}{v_{max}} \frac{1}{c^{\circ}_{gl}} + \frac{1}{v_{max}} \tag{4}$$

Table 1 contains the data from a measurement series with constant enzyme concentration (51.4 mg l^{-1}) and activity. The resulting values are graphically represented in Fig. 1 in accordance with eqn. (4) (Lineweaver–Burk diagram).

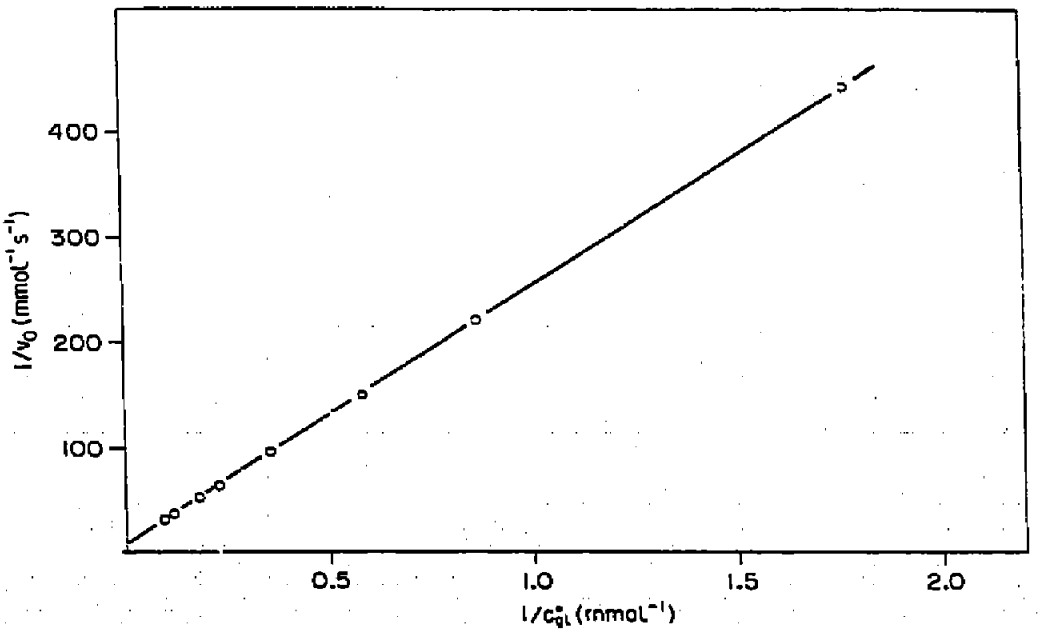

Fig. 1. Lineweaver–Burk diagram.

It is evident that the equation for a straight line is fulfilled. The linear regression produces

$K_M = (41.8 \pm 0.2)$ mmol l^{-1}

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

From v_{max}, an enzyme activity of 125 U mg^{-1} is calculated. The same value was obtained by photometric measurement. The K_M value is in the same range as known ones. Values between 12 and 33 are known for glucose oxidase (god) from *Aspergillus niger* [4–6].

For the further kinetic analysis of the temperature–time curves, it was important to know that not only β-glucose but (after mutarotation) also α-glucose is converted. Therefore, the following reaction scheme was assumed.

$$\alpha\text{-glucose} \xrightarrow{k_1} \beta\text{-glucose} \xrightarrow{k_2} \text{gluconic acid}$$

If a reaction of first order is assumed for both steps, the concentration on c_s of the gluconic acid at time t will be

$$c_s = c_\alpha^\circ\left(1 + \frac{k_2 e^{-k_1 t} - k_1 e^{-k_2 t}}{k_1 - k_2}\right) + c_\beta^\circ(1 - e^{-k_2 t}) \tag{5}$$

where c_α is the concentration of α-glucose, and c_β is the concentration of β-glucose.

The reaction enthalpy of the mutarotation can certainly be neglected in comparison with the oxidation of the β-glucose. Thus, no thermal effect that could possibly have been connected to mutarotation was measured when pure α-glucose was dissolved in a buffer solution; it holds that

$$\frac{c_s}{c_{gl}^\circ} = \frac{\Delta T}{\Delta T_{max}} \tag{6}$$

Using eqn. (5), it follows that

$$\Delta T = \frac{c_\alpha^\circ}{c_{gl}^\circ}\Delta T_{max}\left(1 + \frac{(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})}{(k_1 - k_2)}\right) + \frac{c_\beta^\circ}{c_{gl}^\circ}\Delta T_{max}(1 - e^{-k_2 t}) \tag{7}$$

here c_α° and c_β° are the concentrations for the mutarotation equilibrium, where $K_{Mu} = c_\beta^\circ/c_\alpha^\circ = 1.63$, or $c_\alpha^\circ/c_{gl}^\circ = 0.38$ and $c_\beta^\circ/c_{gl}^\circ = 0.62$. In eqn. (7), the first term refers to the conversion of α-glucose to gluconic acid and the second term to that of β-glucose. For the starting phase, the first term can be neglected: ΔT is controlled exclusively by the conversion of β-glucose.

For $t \rightarrow 0$

$$\Delta T = 0.62\Delta T_{max}(1 - e^{-k_2 t}) \tag{8}$$

or

$$\frac{\partial \Delta T}{\partial t} = 0.62\Delta T_{max} k_2 e^{-k_2 t} \tag{9}$$

and for $t = 0$

$$\left(\frac{\partial \Delta T}{\partial t}\right) = 0.62\Delta T_{max} k_2 \tag{10}$$

The combination of eqns. (2) and (10) produces

$$v_0 = 0.62 c_0 k_2 \tag{11}$$

i.e. the velocity constant k_2 can be calculated from v_0 (see Table 1). As expected, k_2 decreases with increasing glucose concentration in accordance with the Michaelis–Menten equation

$$0.62 k_2 = \frac{v_{max}}{K_M + c_{gl}^{\circ}} \tag{12}$$

To determine the velocity constant k_1 for a conversion of more than 80% eqn. (7) can be simplified to

$$\Delta T = 0.38\Delta T_{max}\left(1 + \frac{k_2 e^{-k_1 t}}{k_1 - k_2}\right) + 0.62\Delta T_{max}$$

$$= \Delta T_{max}\left(1 + \frac{0.38 k_2 e^{-k_1 t}}{k_1 - k_2}\right)$$

or, in linearized form

$$\ln\left[2.632\frac{\Delta T_{max} - \Delta T}{\Delta T_{max}}\right] = \ln\left(\frac{k_2}{k_2 - k_1}\right) - k_1 t \tag{13}$$

This equation allowed a comparatively reliable determination of k_1.

The values are given in Table 1. The k_1 value is in the range that is known for base-catalysed α/β-transformation (phosphate buffer) [7]. The obvious increase of k_1 with growing glucose concentration is due to shortcomings in the model: according to eqn. (12), particularly at a high glucose concentration, k_2 is not constant but increases with increasing conversion. In our model this is reflected by the above increase of k_1.

On the basis of eqn. (7), the whole temperature–time curve can be calculated from the determined k_1 and k_2 values. Figures 2 and 3 show a

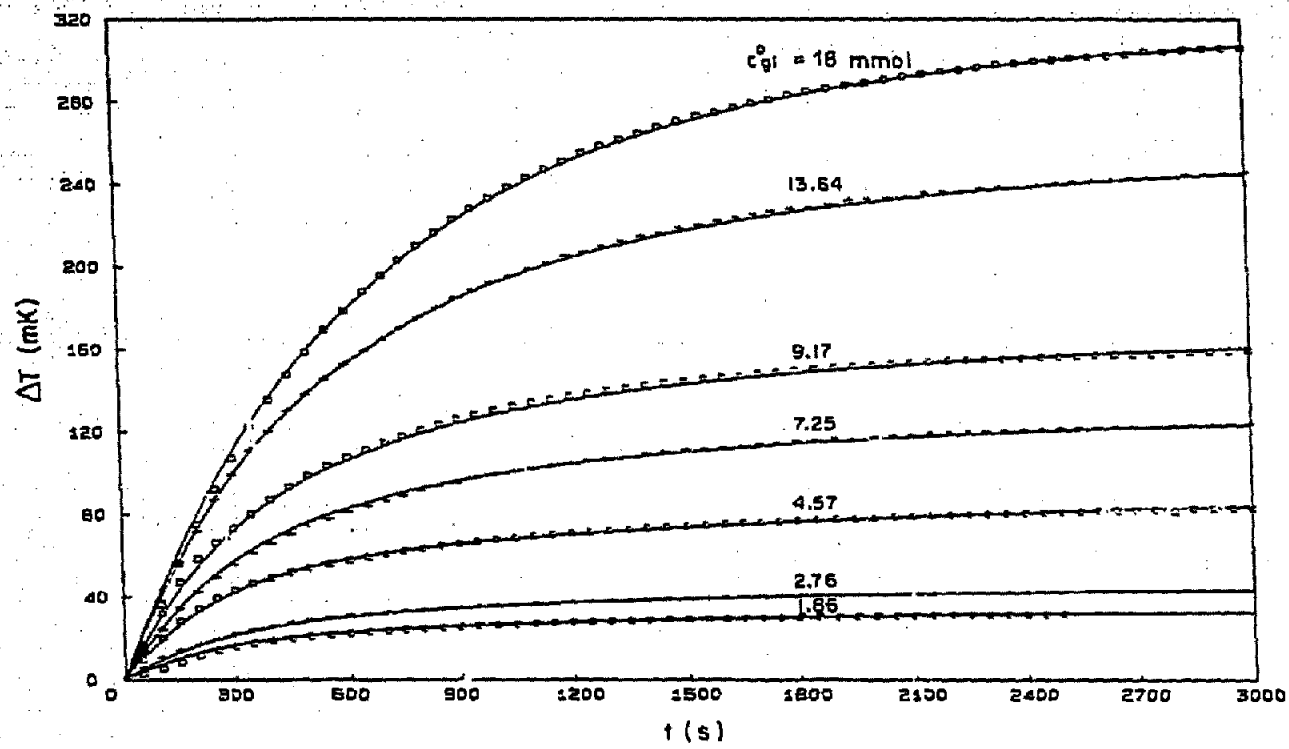

Fig. 2. Adiabatic ΔT–time curves; $c_{gcal} = 51.4\ mg\ l^{-1}$ (□, + exp. values, — calculated).

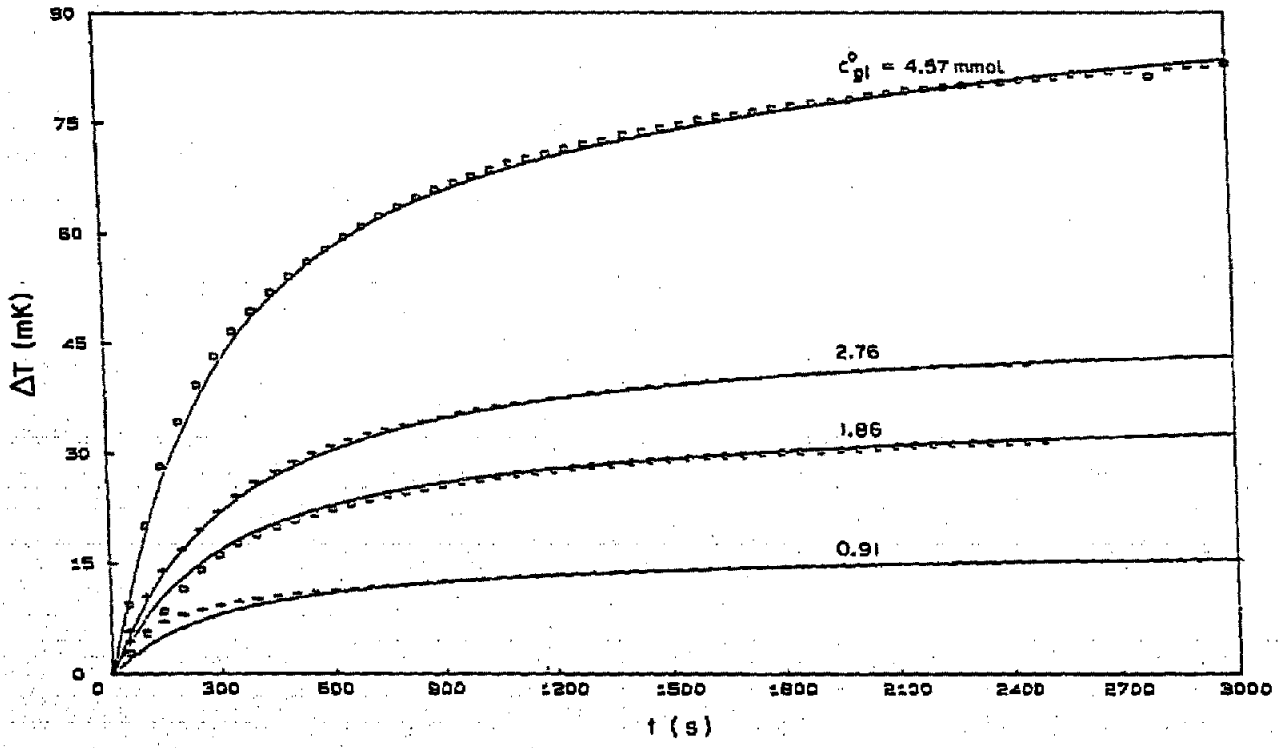

Fig. 3. Adiabatic ΔT–time curves; $c_{gcal} = 51.4\ mg\ l^{-1}$ (□, + exp. values, — calculated).

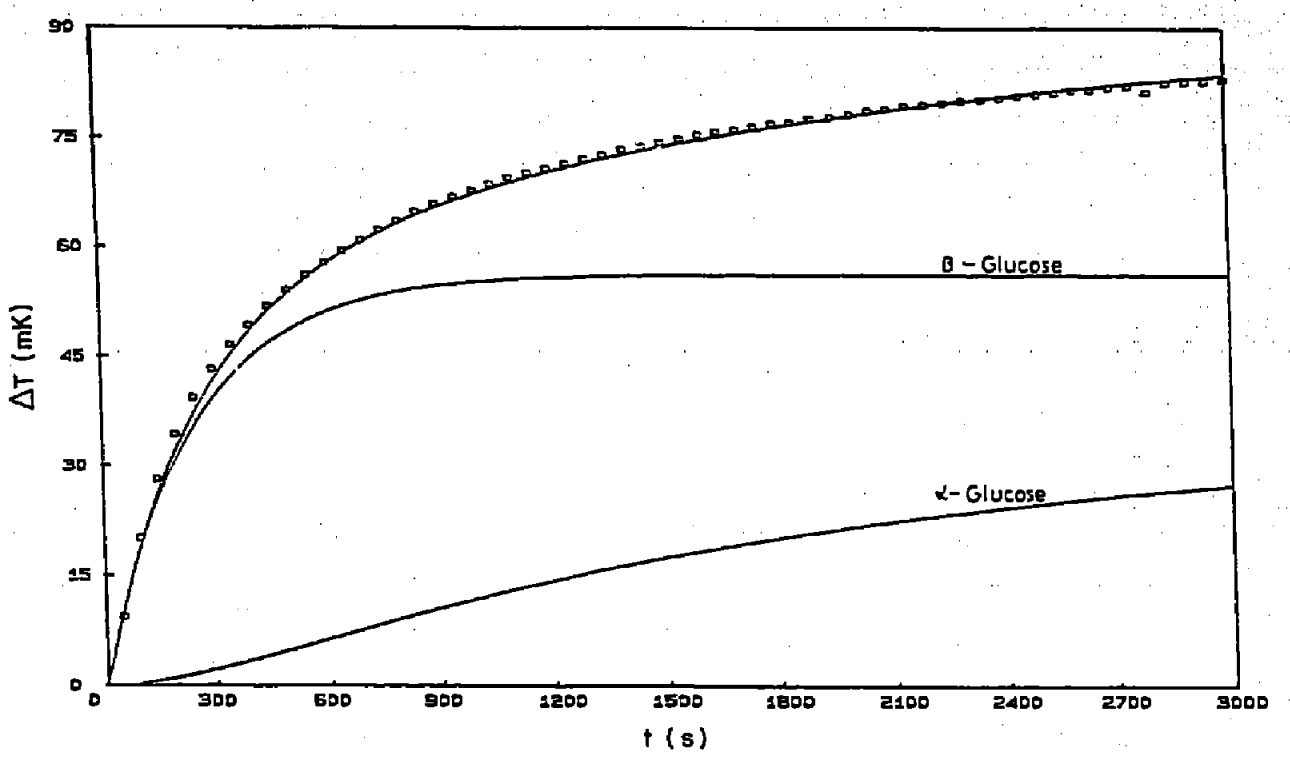

Fig. 4. Adiabatic ΔT–line curves; separate representation of the two fractions α-glucose and β-glucose; $c_{god} = 51.4\ mg\ l^{-1}$; $c_{gl}^{\circ} = 4.57\ mmol\ l^{-1}$ (□ exp. values, — calculated).

very good agreement of the experimental and calculated curves. Figure 4 gives an example of a separate representation of the two fractions, α-glucose and β-glucose. The oxidation of β-glucose is completed within about 16 min, whereas the mutarotation/oxidation of α-glucose requires reaction times up to 100 min.

After it has been established that the initial reaction rate is proportional not to the overall glucose concentration but only to the β-glucose fraction, only the concentration of β-glucose can be used in the Michaelis–Menten eqn. (3). This leads to a reduction of the Michaelis constant by the factor 0.62 ($K_M = 26\ mmol\ l^{-1}$); however, there are no consequences for the model calculation and further considerations.

In a second test series, the concentration of god was varied for two constant glucose concentrations ($c \approx 7.14$ and $13.4\ mmol\ l^{-1}$). Table 2 gives the kinetic data, and three selected temperature–time curves are represented in Fig. 5. As expected, the velocity constant k_2 is proportional to the god concentration (Fig. 6). The increase averaged by the least-squares method is

$$c_{gl}^{\circ} = 7.14\ mmol \qquad \frac{k_2}{c_{god}} = 0.131 \times 10^{-3}\ l\ s^{-1}\ mg^{-1}$$

$$c_{gl}^{\circ} = 13.14\ mmol \qquad \frac{k_2}{c_{god}} = 0.111 \times 10^{-3}\ l\ s^{-1}\ mg^{-1}$$

TABLE 2

Kinetic data for constant glucose concentration; reaction volume: 62.13 ml

c_{gl}° (mmol l^{-1})	c_{god} (mg l^{-1})	$(\partial\Delta T/\partial t)_{t=0}$ (mK s^{-1})	$k_2 \times 10^3$ (s^{-1})	$k_1 \times 10^4$ (s^{-1})
7.17	51.18	0.476	6.34	9.8
7.17	51.18	0.500	6.67	8.9
7.17	51.34	0.499	6.66	8.9
7.15	83.15	0.891	11.88	9.1
7.15	112.9	0.979	13.05	7.4
7.14	144.8	1.42	18.9	7.9
7.11	185.5	1.85	24.6	8.0
13.45	51.18	0.89	5.87	10.6
13.43	83.19	1.32	8.73	10.4
13.42	112.9	1.92	12.7	10.1
13.40	144.8	2.44	16.1	9.8
13.36	193.3	2.80	21.4	9.5

With these values, an enzyme activity of 249 or 228 U mg^{-1} respectively was calculated on the basis of eqn. (12).

The difference is in agreement with the sequence of the experiments: the measurements for $c_{gl}^{\circ} = 7.14$ mmol l^{-1} were performed with a fresh god

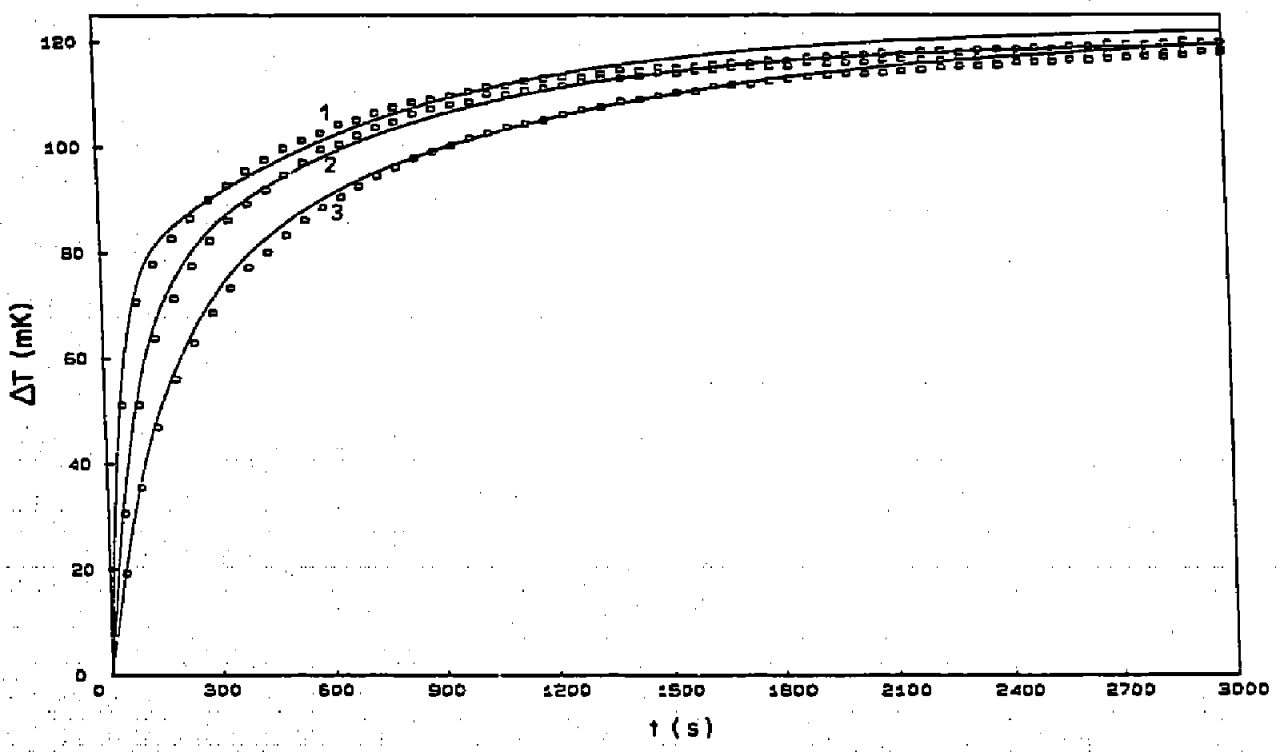

Fig. 5. Adiabatic ΔT–time curves (□ exp. values, — calculated); $c_{gl}^{\circ} = 7.14$ mmol l^{-1}. 1: $c_{god} = 185.5$ mg l^{-1}; 2: $c_{god} = 112.9$ mg l^{-1}; 3: $c_{god} = 51.18$ mg l^{-1}.

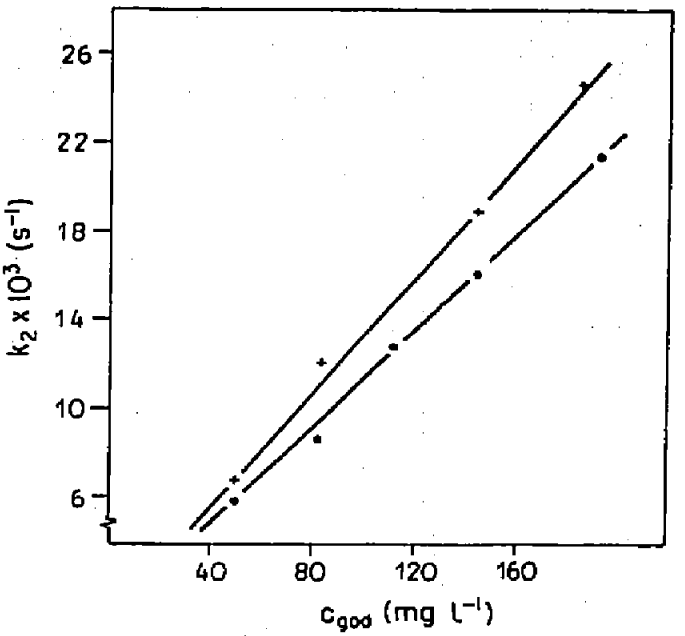

Fig. 6. Dependence of k_2 on the god concentration.

solution with an activity of 250 U mg^{-1}, whereas in the investigations for $c^{\circ}_{gl} = 13.4$ mmol l^{-1} the god solution had somewhat aged. From the scatter of the k_2 values it is evident that the enzyme activity can be determined from one calorimetric measurement with a relative error of about ±5%.

Under identical conditions of glucose concentration and enzyme activity, the temperature–time curves of the glucose oxidation with the mediators benzoquinone and oxygen respectively are almost superimposable in the initial phase, ending at the saturation concentration of oxygen (see [1]); thus, the presented model and results can in principle be applied to a conversion with oxygen. As the reaction rate is independent of the mediator concentration, it does not seem justified to assume the existence of a Michaelis constant with respect to O_2 [8].

The results and calculations show that, by means of suitable and realistic enzyme activities, the conversion rate of β-glucose can be increased in such a way that the β-glucose is practically completely converted (>99.9%) after about 5 min. However, this has next to no effect on the reaction rate of the α-glucose. The reaction rate asymptotically approaches zero, i.e. a conversion of 99% is achieved after about 60 min and a conversion of 99.9% is achieved only after about 100 min. Under the prevailing conditions, a thermometric glucose determination (determination of the reaction heat under substance-constant conditions) is therefore impossible. It also becomes understandable why the $\Delta_R H$ value measured by heat flux calorimetry [9] is smaller by some 7% than the value measured by us.

However, this does not have any consequences with respect to glucose determination by thermal sensors in moving-substance media, since eqn.

(7) shows the heat flow rate to be proportional to the glucose concentration at every moment. On the other hand, strict linearity is guaranteed only for a glucose concentration $c_{gl}^{0} < 0.1K_M$. It appears to make sense (and is already known [10]) to increase the mutarotation rate $\alpha \rightarrow \beta$ by adding mutarotase. This should lead to a marked increase of $(\partial \Delta T/\partial t)$ and thus to a better detection limit. Investigations are currently being initiated.

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