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Experimental data on the energetic flux during glycolytic oscillations in yeast extracts

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Abstract. Calorimetric experiments were performed on yeast extracts in which glycolytic oscillations were occurring. High metabolic fluxes coincided with low amplitudes and with high frequencies in a temperature range from 283 to 303 K, yielding an activation energy of $E_a = 76.8$ kJ mol^{-1} and a mean Q_{10} value of 2.5 ± 0.4. The calorimetrically determined reaction enthalpy $\Delta_f H^{0'}$ of the glycolytic catabolism of glucose revealed two distinct groups of extract preparations, with no intermediate values. The values were -107.5 ± 28.7 kJ mol^{-1} and -30.8 ± 5.7 kJ mol^{-1}, respectively, while -100.0 kJ mol^{-1} was predicted from theoretical calculations. Kreuzberg and Betz[6] predicted rate limiting effects of GAPDH in oscillating extracts. However, when possible effects of GAPDH on frequency, number of oscillations or total heat production were examined, no influence of increasing or reducing the initial GAPDH concentration could be found. The results are discussed with reference to existing models of glycolytic oscillations.
Key words. Calorimetry; heat; glycolysis; oscillatory metabolism; glyceraldehydephosphate dehydrogenase.

Glycolysis plays an important role in the energy supply of most living cells. During this process the concentrations of the metabolites do not always change linearly. Under well defined conditions oscillations are exhibited, which were shown first for yeast cultures by Chance et al.[1]. According to Collatz and Horning biochemical oscillators like the glycolytic system are important for the autodynamic regulation of metabolic pathways[2]. Winfree even thinks that it is reasonable to take into account their possible role as part of the internal clock[3].

Glycolytic oscillations may appear under certain conditions because several key enzymes are allosterically regulated by their substrate and/or their product(s), thus forming a complex network with various feedback mechanisms. It is well known that oscillations only occur in distinct 'windows' of parameter values, whereas glycolysis proceeds monotonously outside these regions. In the present investigations the trehalase-controlled hydrolysis of trehalose to glucose as the main substrate of glycolysis proceeded at such a rate that the requirements for the oscillatory window were met.

The occurrence of oscillating concentrations of metabolites during glycolysis has been investigated with different approaches. Oscillations have been discovered in yeast and yeast extracts[1,4] as well as in muscle extracts[5], algae[6] and blowflies[2]. Optical determination of NAD^+/$NADH^+$ concentrations, and biochemical determinations, yielded data from which mathematical models of glycolytic oscillations were derived[7,8]. However, no direct monitoring of the energetic flux through the glycolytic chain was carried out until 1985[9]. In this paper, we would like to contribute to the latter investigations by offering further data for the glycolytic flux in yeast extracts, obtained from microcalorimetric experiments.

Our special interests may be subdivided into three sections: 1) to compare the experimentally determined reaction enthalpy with values obtained by calculations based on different assumptions, 2) to evaluate the temperature dependence of the calorimetric measurements, and 3) to discuss the role of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a supposedly rate-limiting enzyme additional to phosphofructokinase, as proposed for glycolytic oscillations in algae and yeasts[6].

Theory

According to Hess' law of constant heat sums, the enthalpy $\Delta_f H^{0'}$ of a biochemical reaction such as the fermentation of glucose to carbon dioxide and ethanol can be calculated from the formation enthalpies of its substrates and products:

$$\Delta_f H^{0'} = \text{Sum } \Delta_f H^{0'}(i), \quad (1)$$

$\Delta_f H^{0'}(i)$ being the formation enthalpies of substrates and products at 25 °C and pH 7. However, it is of crucial importance which thermodynamic states of the products are considered. Most of the carbon dioxide will be in the gaseous (g) phase rather than being dissolved in water (aq). Glucose and ethanol are taken as being in the aqueous state. For our calculations we used the following tabulated values for the molar enthalpies of formations $\Delta_f H^{0'}$ (taken from ref. 10 with slight modifications):

glucose (aq): -1262.4 kJ mol^{-1},

ethanol (aq): -287.7 kJ mol^{-1},

carbon dioxide (g): -393.5 kJ mol^{-1}.

When the enthalpy of hydrolysis of trehalose to glucose by the enzyme trehalase is considered to be insignificantly low, calculation of equation (**1**) with these figures yields $\Delta_f H^{0'} = -100.0$ kJ mol^{-1} for the alcoholic fermentation of glucose by yeast. This figure is in contradiction to a value of -126 kJ mol^{-1} reported earlier[9], which was calculated by using $\Delta_f H^{0'} = -413.3$ kJ mol^{-1} for carbon dioxide (aq), assuming it to be completely dissolved. If the glycolytic oscillations are only temporal modifications of an otherwise monotonous catabolism of glucose, and the reaction proceeds under isobaric and isothermal conditions, i.e. without changes in volume and pressure, the total heat produced should be equal to the calculated reaction enthalpy.

According to models currently under discussion there could be a positive correlation between the oscillation periods and the metabolic fluxes as well as the oscillation periods and the amplitudes (pers. commun. Zhi Yuan, Dortmund). Since heat production rates reflect the metabolic flux, microcalorimetry is a suitable method to verify this hypothesis.

The influence of temperature on the metabolic rate can be demonstrated by calculating the Q_{10} value from the heat production rate according to the rule of van't Hoff, or by plotting the natural logarithm of a measure for metabolic flux, e.g. the frequency, versus the reciprocal temperature. The latter is called 'Arrhenius plot' and its slope yields in cases of simple kinetics the activation energy[11].

Three enzymes of the glycolytic chain, hexokinase, phosphofructokinase and pyruvate kinase, catalyze practically irreversible reaction steps and are thus control and regulation points in glycolysis. Recently, the additional role of the GAPDH catalyzed step has been discussed as a rate-limiting step in the glycolytic pathway[6]. Kreutzberg and Betz[6] point out that the higher GAPDH activities would result in a higher substrate flux through the glycolytic chain and therefore lead to a higher oscillation frequency[6]. Such a major role of GAPDH in the glycolytic oscillations is also supported by investigations of Collatz and Horning[2].

Since our experiments show different oscillation frequencies for different extract preparations, the question arises whether this effect could be due to changing GAPDH activities. If it were, a relationship between the GAPDH concentration and the heat flow and the frequency in the extract should be expected, as will be discussed in 'Results' section III below.

Materials and methods

All experiments were carried out with extracts prepared from baker's yeast (Uniferm, Werne, Germany). Prior to extraction, the cells were washed three times, suspended in 0.1 mol l^{-1} potassium buffer and cooled down to 5 °C. Destruction of cells was achieved by 60 s homogenisation with glass beads, using 66 g beads 0.25–0.30 mm in diameter with 20 g of cell suspension (rotation homogeniser and beads were obtained from Braun, Melsungen, Germany). After one hour of centrifugation at about 50,000 *g* the fraction containing the glycolytic enzymes was removed and stored at -18 °C in 1 ml aliquots. These extracts had a pH of 6.4 and an average protein content of 50 mg ml^{-1}, determined by the Bradford method (BIO-RAD laboratories, Munich, Germany). To start the oscillations, 0.45 ml of extract was supplied with final concentrations of 0.48 mmol l^{-1} AMP, 0.37 mmol l^{-1} NAD^+ and varying amounts of trehalose from 30 to 170 mmol l^{-1}. Immediately after mixing, the solution was filled into the calorimetric vessel and transferred to the calorimeter.

Two different twin calorimeters were used for experiments. For the experiments in sections I and II a Triflux-012 instrument (Thermanalyse, Grenoble, France) was used with 1.2 ml vessels which were filled with a volume of 0.615 ml. The sensitivity was 55 ± 2 mV W^{-1}. The second calorimeter, used for section III (Type MS 70, Setaram, Lyon, France) allowed for a 4-fold increase in volume and worked with a sensitivity of 60 mV W^{-1}. Except for the determination of the temperature dependence all experiments were run at 298 K. In order to raise the GAPDH concentration and thus the activity in the extract, purchased GAPDH produced from baker's yeast (low sulfate, 30% citrate buffer salts, SIGMA Chemicals Co., St. Louis, Missouri, USA) was added to well-oscillating extracts. A decrease of activity was achieved by specifically inhibiting GAPDH with iodoacetic acid (Fluka AG, Buchs, Switzerland). All substances were of analytical grade and were purchased directly before use. If not stated otherwise, results given refer to one ml of extract rather than to one ml of solution.

Results and discussion

I Experimentally determined enthalpy of reaction. When calorimetric experiments were performed with different substrate concentrations, a linear relationship between the amount of substrate and the total heat production could be established for all extract preparations up to a substrate concentration of about 330 mmol l^{-1} glucose equivalents, supplied as trehalose (fig. 1). Higher substrate concentrations, however, resulted in a decrease of total heat dissipation. At all substrate concentrations the oscillations ceased at the same heat production rate and thus metabolic flux. The residual heat production after the end of oscillations also remained constant, while the heat produced during the period of oscillations increased linearly with the sugar concentration (fig. 1). The slope calculated from linear regression analysis gave the reaction enthalpy $\Delta_f H^{0'}$. This calcula-

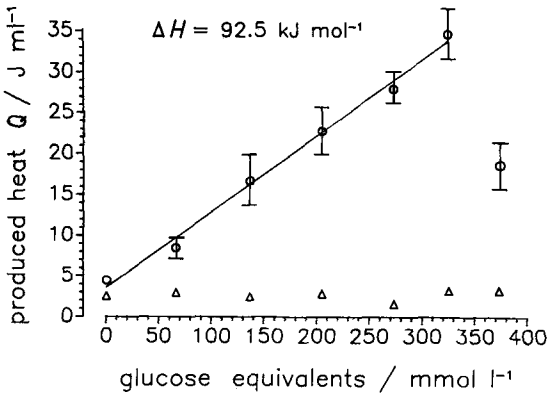

Figure 1. Total heat production as a function of substrate concentration and determination of the reaction enthalpy $\Delta_f H^{0'}$ in different experiments of the same extract preparation. Added trehalose is given as glucose equivalents. Circles represent the total heat production (mean value and S.D., n = 3), triangles represent the remaining heat production after the oscillations have ceased.

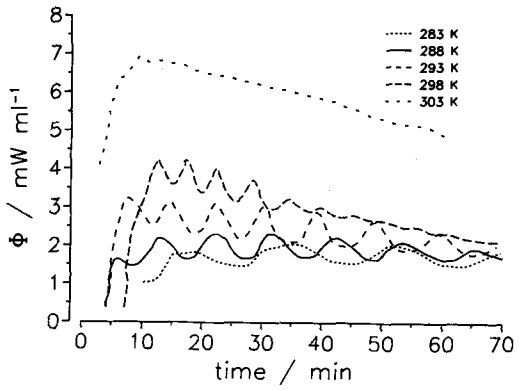

Figure 2. Effect of different temperatures on the heat flux in five experiments with a single extract. Different temperatures are represented by different dashings of the graphs.

tion was performed for 25 extract preparations at 6 substrate concentrations each, in the same manner as demonstrated for a single extract preparation in figure 1. Considering the calculated significantly different values, two groups of extracts could be distinguished (Student's t-test, $\alpha = 0.01$). In group A (n = 17) the reaction enthalpy per glucose equivalent amounted to -107.5 ± 28.7 kJ mol^{-1} in good agreement with the theoretical value, while in group B (n = 8) only -30.8 ± 5.7 kJ mol^{-1} of heat were produced. No extracts with reaction enthalpies between these two values were ever found, as was also published earlier by Plesser and Lamprecht[11]. Metabolic fluxes in both groups were calculated in glucose equivalents from the experimental reaction enthalpy and the heat production rate at the moment when oscillations ceased. In group A a value of 0.38 ± 0.18 mmol l^{-1} min^{-1} was found, while group B gave 1.66 ± 0.55 mmol l^{-1} min^{-1}. Since all added sugar has been metabolized at the very end of the experiments (enzymatic test for starch and glucose), flux calculations were performed with the same enthalpy value; $\Delta_f H^{0'} = 107.5$ kJ mol^{-1}, for all extracts. The results were 0.43 ± 0.06 mmol l^{-1} min^{-1} in group A and 0.47 ± 0.03 mmol l^{-1} min^{-1} in group B. These values were in good agreement with previous results[12], but did not explain the missing heat dissipation in group B.

There must be a striking difference in the physiological state of the two groups of extracts, which could only be detected by detailed biochemical analysis. Plesser and Lamprecht[11] introduced a model for similar differences with experiments using trehalose as energy supply. They described the existence of two forms of the trehalose-degrading enzyme trehalase which could account for the differences in heat evolution in various extract preparations[13].

II Temperature dependence. Calorimetric experiments were performed at different temperatures, resulting in a change of oscillation frequency and heat flux amplitude. While the total heat production remained constant according to the added amount of substrate, the heat flux as well as the frequency rose with increasing temperature. Taking the heat flux as a measure for the metabolic flux, the experiments clearly demonstrated that low metabolic fluxes and low oscillation frequency coincided with high oscillation amplitudes in the heat flux, while high metabolic fluxes were connected with short periods and low amplitudes. Figure 2 shows the effect of different temperatures for one extract. Plotting the natural logarithm of the oscillation frequencies versus the reciprocal experimental temperature ('Arrhenius plot') rendered an activation energy $E_a = 76.8$ kJ mol^{-1}. The slope of the Arrhenius plot yielded a regression coefficient of r = 0.987, thus indicating simple kinetics for the overall process in this temperature range. The calculation of Q_{10} according to the law of van't Hoff between 298 and 303 K gave $Q_{10} = 2.77$ for the frequency and $Q_{10} = 2.15$ for the heat flux. However, since these two values were not significantly different from each other they have been averaged to 2.5 ± 0.4. The experiments could not be continued above 303 K because of precipitation of proteins in the solution.

III Effect of varying GAPDH concentrations. Assuming an initial GAPDH activity of 382 U ml^{-1} for baker's yeast, as found for *Saccharomyces carlsbergensis*[14], GAPDH was progressively added up to 900 U ml^{-1} prior to oscillation experiments. No effect of the surplus GAPDH could be detected on the total heat production or the frequency and the number of oscillations, at any investigated amount of added activity.

To investigate the influence of decreasing GAPDH activity, we specifically inhibited GAPDH with iodoacetic acid at concentrations up to 0.5 mmol l^{-1}. Such inhibition experiments have been performed before, but only with concentrations of more than 3 mmol l^{-1}, which caused a complete inhibition of GAPDH[1]. Since possible rate-limiting effects might occur at some critical

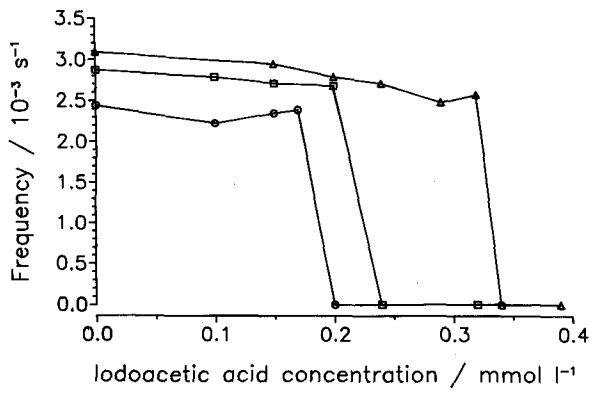

Figure 3. Effect of the iodoacetic acid concentration on the oscillation frequency for three different extract preparations (△, □, ○).

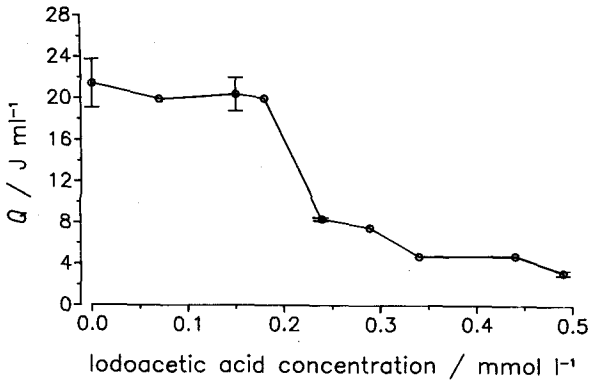

Figure 4. Influence of the iodoacetic acid concentration on the total heat production. The values represent the mean of 2 to 4 extract preparations. S.D. is marked only if n = 4.

concentrations below 3 mmol l^{-1}, we inhibited GAPDH progressively. When very small amounts of iodoacetic acid were added, heat flux and oscillation frequency remained unaffected (fig. 3). At final concentrations of 0.2 to 0.35 mmol l^{-1} iodoacetic acid, however, the oscillations ceased. The variability of this distinct inhibition concentration for different extract preparations was probably due to variations in initial GAPDH concentrations among the extracts. In all cases a remaining heat signal of 4 J ml^{-1} could be detected after the collapse of the oscillations caused by the addition of iodoacetic acid, as demonstrated in figure 4. Since iodoacetic acid specifically inhibits GAPDH, this remaining heat represented the heat produced by the reactions preceeding the GAPDH in the glycolytic chain.

Summarizing, neither decreasing nor increasing GAPDH concentrations or activities could reveal significant changes in heat production, frequency or number of oscillations. Complete poisoning of the GAPDH, resulting in a disruption of the glycolytic chain, indicates that GAPDH is essential for the oscillations, but that no further regulating effect is achieved with varying concentrations or activities of GAPDH. According to our understanding, this excludes any possible rate-limiting effect of GAPDH in glycolytic oscillations.

In contrast, Horning and Collatz described the GAPDH to be the flux limiting step in the glycolytic chain[15]. According to their theory, addition of GAPDH must result in a higher flux through the chain and therefore cause a higher oscillation frequency of NAD^+ /$NADH^+$ concentrations. In this case, a reduction of the GAPDH concentration by poisoning should decrease the metabolic flux through the chain and reduce the oscillation frequency. None of these theoretical effects could be observed in our experiments. Since neither addition of GAPDH nor reducing GAPDH activity affected the oscillation frequency, and since the oscillations were only terminated by a complete poisoning of GAPDH with iodoacetic acid, we cannot describe GAPDH as flux-limiting in glycolytic oscillations.

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