Thermochimica Acta, 151 (1989) 69–81
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CALORIMETRIC MEASUREMENTS OF AN INTERMITTENCY PHENOMENON IN OSCILLATING GLYCOLYSIS IN CELL-FREE EXTRACTS FROM YEAST

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SUMMARY

Intermittencies, i.e. quiet non-oscillating periods between oscillating regimes, are described for the first time in glycolytic oscillations and discussed in energy terms. The different regimes in the power-time-curves are of approximately equal energy content and independent of the initial sugar concentration while the total heat produced is a linear function of the sugar concentration.It renders an enthalpy change of -(134±10) kJ per mole glucosyl units in good agreement with data from the literature.

A device for the calorimetric vessel is presented which combines effective stirring of the vessel content with simultaneous measurement of optical absorbance and heat flux.

INTRODUCTION

Among the large number of known oscillating reactions, only a few have been investigated by means of isothermal calorimetry (refs. 1-6). These and other results on oscillating reactions achieved with thermometric experiments (refs. 7, 8) and with adiabatic calorimetry (ref. 9) revealed that a pronounced heat production appears in all systems. Therefore, the measurements of power-time-curves is a promising approach to study the complex dynamics and the overall flux of oscillations in well regulated chemical and biochemical reaction networks.

The variety of biological oscillators, such as the heartbeat, the neural oscillators, the secretory cells (for a review of cellular oscillators see ref. 10) and the oscillations of the glycolytic flux in intact cells or in cell-free cytoplasmic extracts are the most prominent subjects for the investigation of regulation and control phenomena. Many different techniques have been involved. Periodic fluctuations of enzyme activities and metabolite concentrations as well as heat production rates have

004-6031/89/$03.50

been monitored by spectrophotometric and calorimetric means. For the determination of phase relationships between the fluctuations in metabolites and heat fluxes, a calorimetric technique was developed to monitor both signals simultaneously (refs. 11-13). Because of the thermal inertia of the calorimetric system it is necessary to calculate the true time course of the heat production rate from the recorded curve by desmearing techniques. Such a deconvolution of the heat signal has been achieved by Müller and Plesser (ref. 14) applying Fast Fourier Transform, or by Rodriguez et al. (ref. 3) using Laplace transformation.

Oscillating chemical reactions, such as the best-known Belousov-Zhabotinskii reaction, the Briggs-Rauscher reaction, the Bray-Liebhafsky reaction, or the uncatalyzed ABA reactions (ref. 15) are examples of reactions proceeding far from thermodynamic equilibrium. Under such conditions, temporal or spatial structures may appear, indicating autocatalytic and/or strong feedback processes. Numerous investigations revealed that oscillations only occur in distinct "windows", i.e. regions of parameter values outside of which the reaction proceeds in a monotonous manner. In some cases one observes dying away of oscillations and a reappearance after some time, a phenomenon called "intermittency". It might be due to the existence of several windows visited during the course of the reactions or due to a process operating around the borderline of one single "window".

Glycolysis as a sugar metabolizing process can be found in almost all organisms. It consists of a reaction chain of at least 13 enzyme catalysed steps which form a network with various feedback mechanisms (ref. 16). The ubiquitous appearance and the high degree of regulation and control in glycolysis supports the idea that there might be some connections with biological timers.

Here we report new results which have been gained by using a modified TRIFLUX calorimeter allowing for stirring of the cell extract inside the small calorimetric vessel and simultaneous measurement of optical and calorimetric signals. Intermittencies as a new phenomenon in oscillating glycolysis are described for the first time.

METHODS AND MATERIALS

All experiments were run with cytoplasmatic extracts from commercially available bakers yeast. The preparation was slightly modified compared to the recipe described in (ref. 17). The protein content after preparation amounts to about 50 mg/ml, approximately one third of that of intact cells.

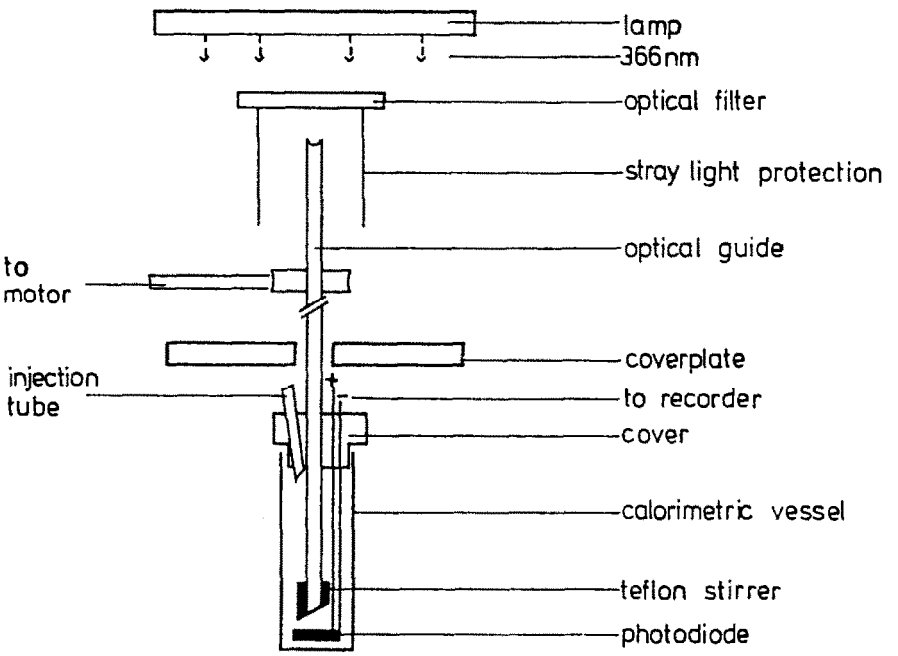

Fig. 1. Device for stirring and simultaneous measurements of heat production and optical density.

For an experiment equal volumes (15 µl) of potassium phosphate [1M], NAD [20mM] and AMP [15mM], but varying amounts of the disaccharide trehalose as sugar source (calculated in glucosyl equivalents for a better comparability with other results) were added to 450 µl of extract. All substances had been dissolved in 0.1 M pH 6.4 potassium phosphate buffer.

For each experiment a stable baseline was determined with empty calorimetric vessels. Immediately before injection the extract was thawed and the final mixture prepared. The experiments were then started by injection of the reaction mixture with an actual volume of 300 or 500 µl, respectively, independent of the

amount of trehalose. Injection of such a small volume does not affect the baseline as could be shown by control injections with distilled water.

The modified calorimeter is a differential (twin) isothermal isoperibolic batch calorimeter, type "TRIFLUX" from Thermanalyse/Grenoble. The sensitivity amounts to 65 μV/mW, the active volume of the vessels to 1.2 ml, reduced to approximately 1 ml by the modification.

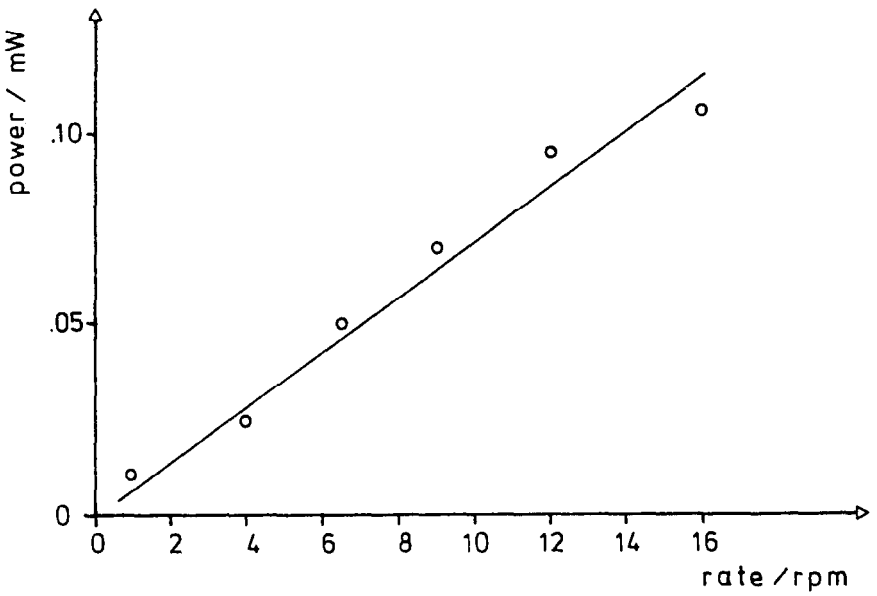

Fig. 2. Heat production rate as function of stirring rate.

The optical signal is measured at a wavelength of 366 nm by means of a Sylvania F8W/BLB lamp as light source, an UG1-UV-filter (SCHOTT GLAS), a quartz rod as light guide and a BPx91B-silicium-photodiode (Siemens) for detecting the signal with a sufficient sensitivity at the chosen wavelength. Both signals are monitored by a two channel recorder (B 41, Kipp&Zonen) and by an Atari 1024-micro-computer with an analog to digital converter and a floppy disk for data storage. The calorimeter is run at 298 K.

Since the monitoring of different parameters and the later injection of biochemical compounds require a continuous and effective stirring, we designed a stirring mechanism different from that shown in (refs. 12, 13): The light guide, a quartz rod 130 mm in length and 2 mm in diameter with a teflon propeller at the lower end, can be rotated at different rates by means of an electro motor mounted next to the calorimeter. This setup is shown in Fig. 1.

Stirring, of course, results in an additional heat production depending on the stirring rate and slightly - due to the varying viscosity - on the type of the specimen. Fig. 2 shows the additional heat flux in extracts as a function of the stirring rate.

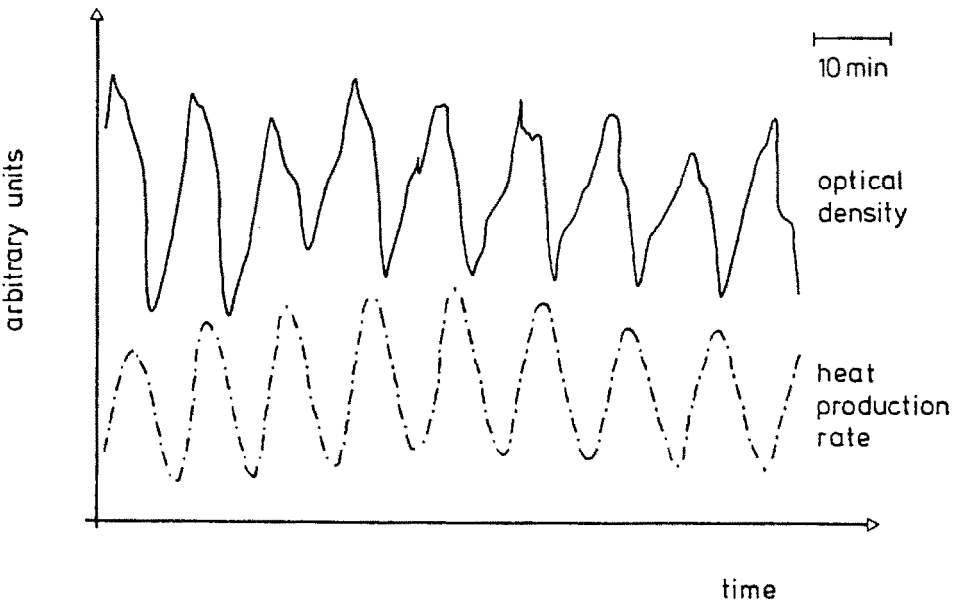

Fig. 3. Example for simultaneous recording of heat flux and optical absorbance in an extract from yeast.

A significant difference between cytoplasmic extracts and water could not be observed, so that an error due to different extract preparations should be negligible.

In yeast extracts a moderate stirring rate of 6 rpm adds a heat flux of about 30 μW, i.e. approximately 3 to 5 % to the biochemical signal. Thus, the baseline has to be corrected for

the additional constant heat flux caused by stirring. A number of calibration experiments showed that the stirring rate does not affect period and amplitude of the oscillations.

RESULTS

As a first result we present in Fig. 3 the simultaneous recording of the optical and the calorimetric signal in the first part of an experiment on glycolytical oscillations. The thermal inertia of the calorimeter was not corrected in this example. The optical signal, upper trace in Fig. 3, shows significant irregularities generated by vibrations of the rotating glas rod as well

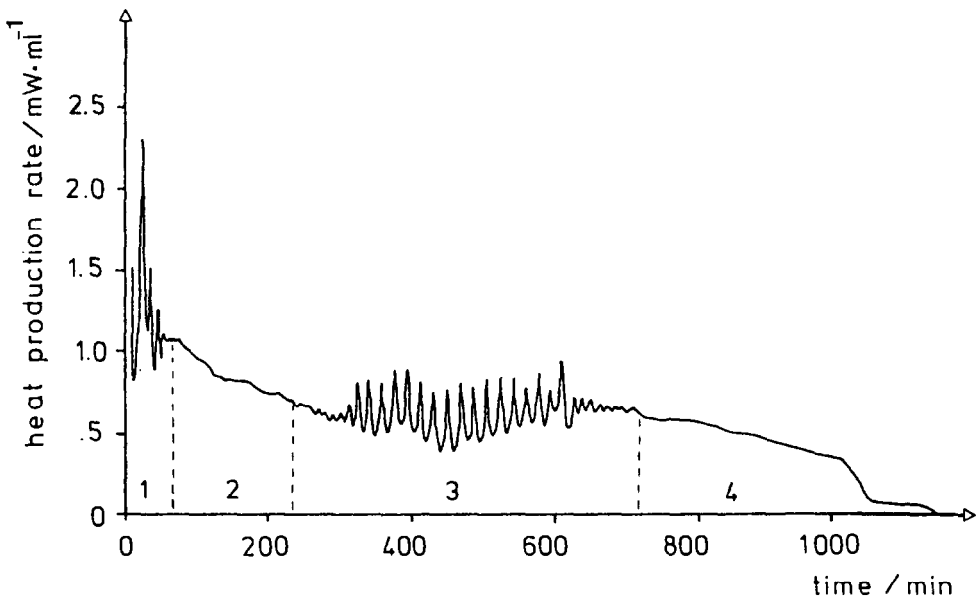

Fig. 4. Different regimes of heat production in an extract from yeast exhibiting an intermittency. 1) first oscillatory regime, 2) intermittency, 3) second oscillatory regime, 4) nearly constant plateau and decrease of the signal to the baseline. (Initial trehalose concentration: 154 μmol glucosyl equivalents)

as by problems with the isolation of the cabling of the photodiode. Therefore phase relationships and possible phase shifts will be discussed in detail elsewhere. Here we concentrate on the phenomenon of intermittencies.

The "normal" power-time-curve of glycolytic extracts from yeast consist of an oscillating period, a non-oscillating plateau regime and a final drop to the baseline (refs. 2, 6). Now we observed in addition intermittencies in several extract preparations. That means, the oscillations cease after a few periods and reappear after a certain interval of time. The power-time-curve of such an experiment is given in Fig.4, where four regimes can be distinguished: 1) a first oscillatory regime, 2) an intermittency, 3) a second oscillatory regime, 4) a non-oscillating decay regime with a nearly constant heat production rate which finally decreases to the baseline. Curves with the same structure have been measured by optical absorbance.

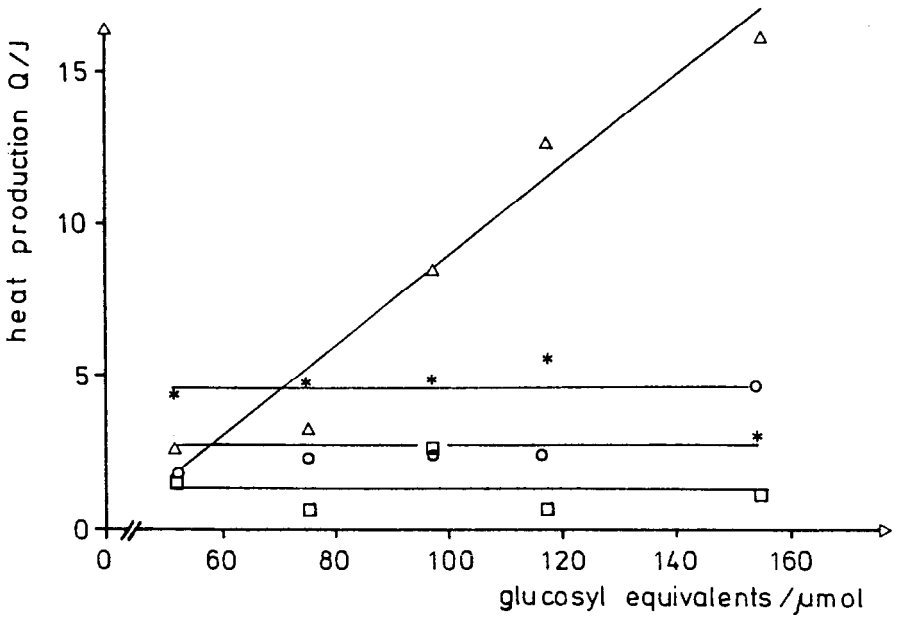

Fig. 5. Total heat produced in the different regimes of an intermitting yeast extract. *: First oscillatory regime; o: regime of intermittency; Δ: second oscillatory regime; □: decay regime.

Intermittencies have been only observed at higher trehalose concentrations. The threshold depends on the preparation, and lies above 50 µmol glucosyl equivalents for the preparation used for the experiments of Fig. 5.

A series of experiments with the same extract preparation and varying amounts of trehalose renders an enthalpy change of -(134 ± 10) kJ/mol glucose equivalents. This figure is in good agreement with former extract preparations exhibiting no intermittencies (ref. 2) as well as with data from the literature for a complete catabolic degradation of sugar to ethanol and carbon dioxide (ref. 18).

The total heat produced during the different regimes is plotted as a function of initial sugar concentration in Fig. 5. We observe trehalose concentration independent levels for the regimes one, two and four. The only sugar concentration dependent part of the power-time-curve is the regime after the intermittency whose heat content as well as its number of oscillations increase linearly with the amount of sugar added.

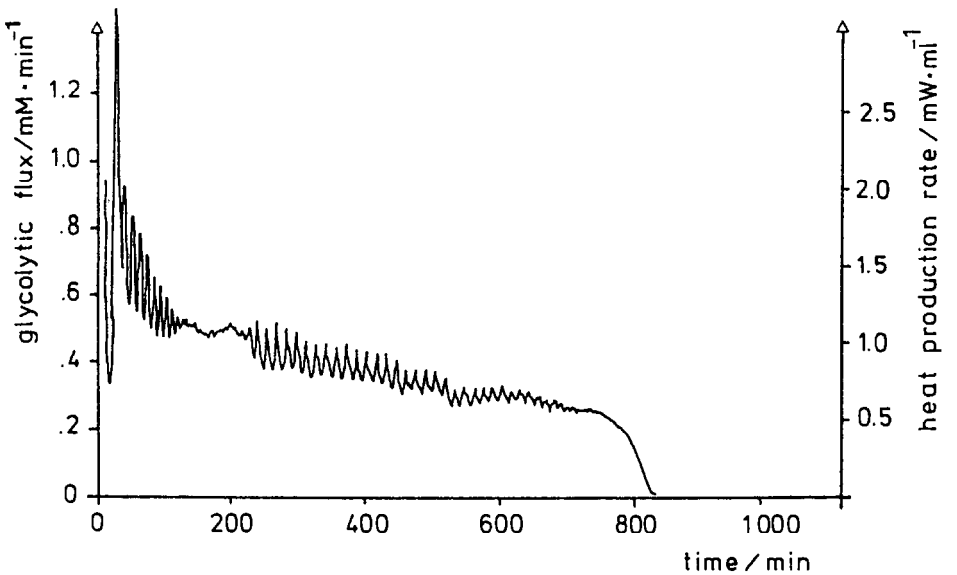

Fig. 6. Glycolytic flux, i.e. turnover of glucosyl equivalents per time, during an experiment with an intermitting extract. For comparison the heat production rate is given at the right hand side. (Initial trehalose concentration: 117 μmol glucosyl equivalents.)

Since there is a proportionality between heat flux and glycolytic flux, we can calculate the glycolytic flux from the measured heat production rate during an experiment using the

known enthalpy change. Thus, power-time-curves as shown in Fig. 4 transform into Fig. 6 for the glycolytic flux, dividing the heat production rate by the enthalpy change.

In a number of experiments from different preparations we observed a remarkable coincidence with respect to the glycolytic fluxes at the end of the various regimes. Each regime ceases at a distinct glycolytic flux as demonstrated in Fig. 7.

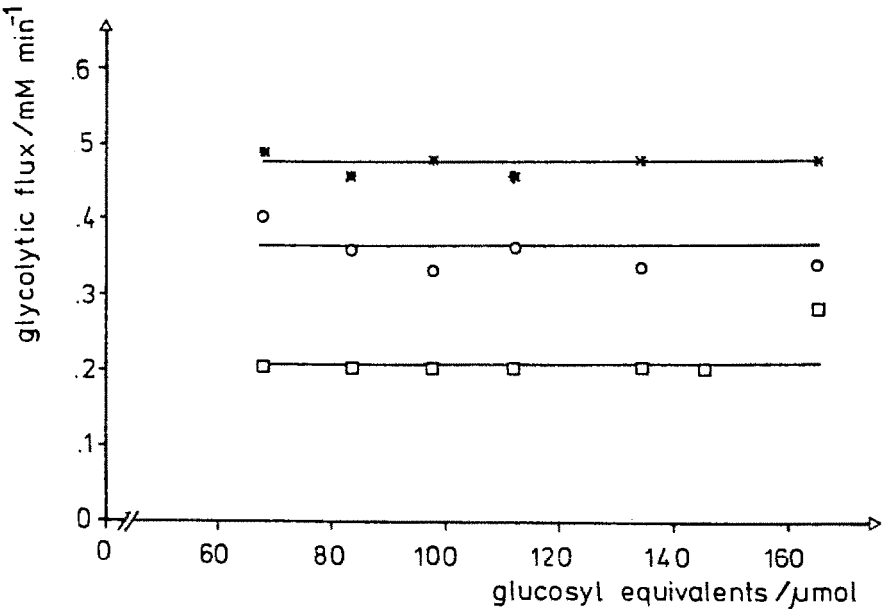

Fig. 7. Diagram of the glycolytic flux at the transition points between the different regimes of an intermitting extract. *: end of first oscillatory regime; o: end of intermittency; □: end of second oscillatory regime.

The results obtained by our experiments can be summarized as follows:

* In some preparations of extracts from yeast we observe two regimes of oscillations which are separated by an intermittency.
* The linear increase of the total heat produced is due to the second oscillatory regime. The other three regimes exhibit a constant heat output (Fig. 5).
* The oscillation frequencies are the same in both oscillatory regimes.

* The number of oscillations varies in the second oscillatory regimes among the different preparations of extracts, but it always increases linearly with the applied amount of sugar.
* The different regimes are entered or left at the same level of substrate flux independently of the initial sugar concentration (Fig. 7).
* The total amount of heat produced during the experiment is a linear function of the initial sugar concentration (Fig. 5) as observed in non-intermitting extracts.

DISCUSSION

Intermittencies have been observed in many oscillating reactions and even been monitored calorimetrically for Belousov-Zhabotinskii and for ABA reactions (ref. 1). But to our knowledge intermittencies have never been seen in oscillating glycolytic extracts. This may partly explain our difficulties to establish a recipe for producing such intermittencies. A yet unknown parameter must be responsible for the appearance or the lack of intermittencies. In seemingly identical experiments with specimens of the same extract no intermittencies are observable some weeks after preparation.

The results summarized in Fig. 5 and Fig. 7 can be interpreted in the light of the activation/deactivation mechanism of trehalase, the influx regulating enzyme (refs. 2, 19), and of a bifurcation diagram with two oscillatory "windows" as theoretically investigated in (ref. 20). The bifurcation diagram (Fig. 8) describes the various dynamic states of the glycolytic system in a parameter plane given by the sugar input rate (ordinate) and the sink rate coefficient (abscissa). The sink rate coefficient is the proportionality factor between the rate of product output, the sink rate, and the product concentration. The input rate is an experimentally and theoretically well defined parameter in contrast to the sink rate coefficient. A correlation of this coefficient with an experimentally accessible parameter of the glycolytic network is difficult. However, a linear relationship between the sink rate and the product concentration simplifies the theoretical considerations and model simulations enormously (refs. 20,21).

The schematic bifurcation diagram given in Fig. 8 separates the parameter plane in two oscillatory (hatched areas), and one

non-oscillatory domain (plain area). The shape of the power-time-curve is most sensitive to the track followed by the glycolytic system in the bifurcation diagram in the catabolism of a given pool of trehalose.

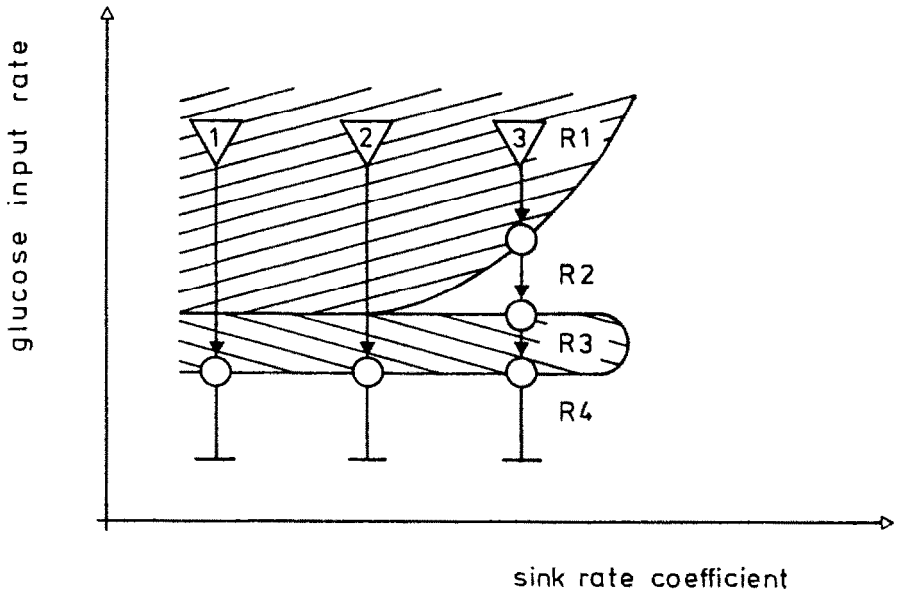

Fig. 8. Schematic diagram of three possible tracks and their bifurcation points (O) during the catabolic degradation of a sugar pool by glycolysis. 1: "Usual" oscillating glycolysis without intermittency as given in Fig. 1A of (ref. 6). 2: Oscillating glycolysis without intermittency as shown as Fig. 1B of (ref. 2). 3: Oscillating glycolysis with intermittency as shown in Figs. 4 and 6 in this paper.

The glucose input rate at the beginning of an experiment is determined by the activity of the two forms of the trehalose splitting enzyme trehalase. During the course of time the active form is transformed by a phosphatase into the much less active form called "cryptic". This process is of course accompanied by a drop of the glucose input rate down to the maximum rate of the stable "cryptic"" form of the enzyme. An extract exhibits an intermittency during the decrease of enzyme activity if the product removal in the specimen corresponds to a value of the sink rate coefficient as schematically given for track three in Fig. 8. Oscillations with large amplitudes appear in region R1

followed by an intermittency when the input rate crosses the region R2; Oscillations show up again in Region R3. This small region covers just the maximum rate of the "cryptic" trehalase. The lower boundary is approached and the system enters the non-oscillatory region R4 if the input rate decreases further due to the exhaustion of the trehalose pool. This behaviour explains the results given in Fig. 5. The energy content of the first oscillatory regime and of the intermittency is controlled by the deactivation of that amount of trehalase which is in the active form of the enzyme at the beginning of the experiment, a process independent of the trehalose concentration.

In contrast, the second oscillatory regime is totally controlled by the trehalose concentration. The bifurcation diagram determines a critical rate at which the system leaves the oscillatory region R3 and enters the non-oscillatory region R4. The critical rate is reached - due to the trehalase rate law - at a critical trehalose concentration. It depends only on the regulatory properties of the glycolytic system. Therefore the only trehalose dependent regime is the second oscillatory one in which the trehalose pool is burned down from any size to the critical concentration.

The boundary between R3 and R4 in Fig. 8 has been drawn as a horizontal line because for all extracts investigated so far, intermitting as well as non-intermitting ones, a critical rate of 0.21 mM/ml has been found, a value close to that measured by a direct glucose injection technique (ref. 22).

Further details of the dynamics of the glycolytic system during the bifurcation can be evaluated when precise data of the simulataneously recorded rates of heat production and optical absorbances are available.

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