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Single cell studies and simulation of cell–cell interactions using oscillating glycolysis in yeast cells

Allan K. Poulsen *, Mai Østergaard Petersen, Lars Folke Olsen

CelCom, Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230 Odense M, Denmark

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Abstract

The observation of oscillations in the concentrations of NADH and other intermediates in glycolysis in dense yeast cell suspensions is generally believed to be the result of synchronization of such oscillations between individual cells. The synchrony is believed to be a property of cell density and the question is: does metabolism in each individual yeast cell continue to oscillate, but out of phase, in the absence of synchronization? Here we have used high-sensitivity fluorescence microscopy to measure NADH in single isolated yeast cells under conditions where we observe oscillations of glycolysis in dense cell suspensions. However, we have not been able to detect intracellular oscillations in NADH in these isolated cells, which cannot synchronize their metabolism with other cells. However, addition of acetaldehyde to a single cell as pulses with a frequency similar to the oscillations in dense cell suspensions will induce oscillations in that cell. Ethanol, another product of glycolysis, which has been proposed as a synchronizing agent of glycolysis in cells, was not able to induce oscillations when added as pulses. The experiments support the notion that the intracellular oscillations are associated with the cell density of the yeast cell suspension and mediated by acetaldehyde and perhaps also other substances.

Keywords: Glycolytic oscillations; Single cell observation; NADH; Acetaldehyde; Microscopy; On-line detection

1. Introduction

Glycolytic oscillations in yeast cell suspensions have, since their discovery in 1957, been studied for nearly 50 years [1]. They were first observed as damped oscillations in the fluorescence signal from NADH in suspensions of starved yeast cells after addition of glucose. The existence of metabolic oscillations are nowadays not only associated with yeast cells, but they have also been reported in many other cell types [2–9]. While glycolytic oscillations in intact yeast cells and in yeast extracts have been studied thoroughly and act as a model for complex behaviour of metabolic systems in general, the mechanism responsible for the oscillations remains unsettled. For the latest reviews on glycolytic oscillations see Refs. [10,11].

In a dense yeast cell suspension with a specific cellular make-up it is possible to record sustained macroscopic oscillations. Consequently, most of the cells should oscillate in concert despite the fact that cells in the suspension differ regarding their size, shape, age, and stage of budding. As cell density is decreased the amplitude of the oscillations decreases gradually, until a critical density is reached where the macroscopic oscillations disappear [12–14]. Synchrony of metabolism only occurs at high yeast cell density. This behaviour is interpreted as mutual interactions among the yeast cells are crucial for the sustained oscillations. Analyses of experimental data and studies of mathematical models of glycolysis indicate that the oscillatory behaviour cannot be ascribed to a single reaction in glycolysis of yeast cells [15–17], but is a property of the entire biochemical pathway. However, acetaldehyde is a very important component to the macroscopic oscillatory behaviour of yeast cells [18,19] and the compound may act as an extracellular messenger, either by itself or together with other glycolytic intermediates [20–22].

Although glycolytic oscillations in yeast cell suspensions are well known, knowledge about the oscillations at the single cell level is very sparse. Chance et al. [23] and Aon et al. [12] have recorded oscillations in individual yeast cells. Using microscopic

* Corresponding author. Tel.: +45 6550 2479; fax: +45 6550 2467.
E-mail address: allanpoulsen@bmb.sdu.dk (A.K. Poulsen).

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techniques they focused on single cells in a suspension of oscillating yeast cells. They discovered that the individual cells continue to oscillate after the macroscopic oscillations have disappeared, but now they oscillate out of phase. They concluded that in suspension the dampening factor of the total population is larger than the dampening factor of the individual cells and that asynchrony makes macroscopic oscillations disappear. The intracellular oscillations and synchrony may arise as a consequence of cell perturbation by e.g. addition of cyanide or other compounds affecting the cell [24] or synchrony may arise due to coupling of individual oscillating yeast cells.

Using a fluorescence microscope we examined and compared cell suspensions and single yeast cells at conditions, which cause glycolytic oscillations in high density yeast cell suspensions. Our approach to studies of single yeast cells differs from previous studies in that we are not focusing on a single cell in a dense suspension. Instead we focus on isolated single cells attached to a coverslip under conditions, where the cell is unable to interact with other cells. The coverslip is mounted in a microscope flow cell and with a constant flow of glucose and cyanide the flow cell acts as a plug-flow reactor. Simulation of cell–cell interactions was done by periodically infusing acetaldehyde or ethanol into the reactor at a frequency similar to the natural frequency of glycolytic oscillations occurring in oscillating yeast cells in high density suspensions at 25 °C. Our experiments show that an extracellular agent is necessary in order to induce oscillations in individual yeast cells and they suggest that acetaldehyde is an important mediator of information regarding glycolytic phase between cells in a cell suspension.

2. Materials and methods

Yeast cells, *Saccharomyces cerevisiae* diploid strain X2180, were grown, harvested, and the suspension was tested for its ability to oscillate prior to the experiments with an experimental setup described earlier by Poulsen et al. [24]. When glucose in the growth medium was depleted, the cells were washed twice with 100 mM potassium phosphate buffer (Merck, Germany), pH 6.8 (centrifugation, 5 min at 5000 rpm, GSA Sorvall, USA), resuspended in the same buffer to a cell density of 10% (w/w) and starved for 3 h on a rotary shaker at 30 °C. After starvation, the cells were kept at +5 °C until use.

2.1. Fluorescence microscopy

NADH can be considered as a naturally occurring fluorescent probe, which enables us to examine metabolism inside living cells. For the measurements of fluorescence from individual yeast cells an inverted Leica microscope DMIRE2 equipped with a Hamamatsu C7190 EB-CCD camera was used. The camera allows for high-sensitive recording of images acquired at low exposure time. The microscope is equipped with a filter cube (Leica, +A4) consisting of two filters. Excitation of NADH was done using a bandpass filter (360/40) and emission was recorded through a bandpass filter (470/40). The light source was a 75 W Hg-lamp connected to the microscope. Images and movies were recorded by the software "Image-Pro plus 5.1". Images were captured at regular intervals of 3 s. A high-speed shutter (*Smart*Shutter, Sutter Instrument Company, USA), which was only open a few milliseconds each time a picture was taken, ensured that the cells were exposed to a minimum of light from the Hg-lamp.

2.2. Immobilization of yeast cells

Yeast cells for single cell experiments were immobilized on polylysine-coated coverslips. 100 μl of a 0.1 mg/ml polylysine solution was transferred to a coverslip, which had been washed, first in acetone and then in distilled water. When the solution was dry, eventually by use of a vacuum oven at 50 °C, the coverslips were ready to use or they were stored dry at room temperature. The polylysine coated coverslips can be stored for several months. 100 μl of a yeast cell suspension was transferred to a polylysine-coated coverslip and stored on ice. After 20–30 min the coverslip was flushed with 100 mM potassium phosphate buffer, pH 6.8, in order to remove excess yeast cells leaving a monolayer of immobilised cells on the coverslip.

2.3. Flow chamber

To study individual yeast cells in a microscope under well-defined conditions we used a flow chamber in aluminum and acrylic. The chamber was constructed in our workshop and is shown in Fig. 1. Using the flow chamber it is possible to expose the cells to a well-defined environment. A steady flow of e.g. a solution of glucose in buffer ensures that the cells are exposed to a constant glucose concentration while compounds excreted by the cells are continuously removed. The flow through the flow chamber is controlled by pumps connected to a computer. While the cells are exposed to well-known and controllable extracellular conditions we record the fluorescence signal from intracellular NADH.

We introduced glucose, potassium cyanide, ethanol, and acetaldehyde using infusion pumps (Harvard Apparatus,

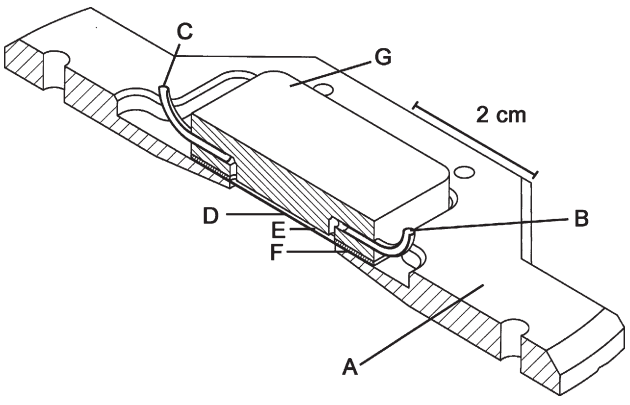

Fig. 1. Schematic drawing of the flow cell used for the inverted fluorescence microscope. The figure is a cut away, which leaves the flow path open. Aluminum base (A); inlet (B); outlet (C); glass coverslip 24 × 24 mm, on which yeast cells are immobilised (D); flow path (E); silicone membrane (F); and top of acrylic plastic (G). The flow cell has a total volume of 25 μl.

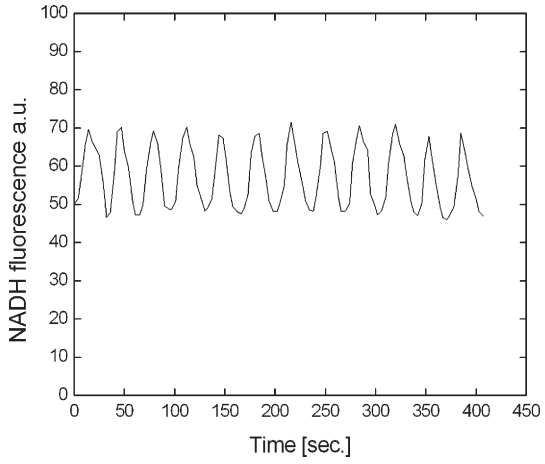

Fig. 2. Illustration of the waveform of oscillations of acetaldehyde perturbations used. A flow of 100 mM potassium phosphate buffer, pH 6.8, at 500 μl/min was constantly pumped through the flow cell. A 10 mM NADH solution was periodically added at 17 μl/min. Each period had a duration on 36 s; 18 s flow of NADH solution followed by 18 s without addition of NADH.

Massachusetts, USA, model 22) equipped with Hamilton syringes (Hamilton, Reno, Nevada, USA). The pumps were connected to a personal computer and a home-made software allowed us to control the rate of infusion of compounds. We tested the effect of infusion and mixing in the flow cell by periodic infusion of an NADH solution into a constant flow of phosphate buffer. Fig. 2 shows the fluorescence trace of NADH when it is added in repeated cycles and infused for a period of 18 s and shut off for a period of 18 s.

2.4. Perturbation of cells

The cells in the flow chamber are exposed to a constant flow of a 100 mM phosphate buffer, pH 6.8 containing 30 mM glucose and 5 mM KCN, unless otherwise stated. The flow rate through the flow cell was set to 500 μl/min giving a retention time of 3.0 s. Acetaldehyde was infused in cycles with a period of 36 s corresponding to the natural frequency of NADH oscillations in dense yeast cell suspension. Acetaldehyde concentration in flow chamber never exceeded 5.5 μM. Acetaldehyde was dissolved in buffer and the flow rate was set at 17 μl/min when it was on.

2.5. Data analysis

Images of the cells were analyzed subsequent to the experiments. We performed the analysis using two different softwares specially developed for this assignment. PRIMOX, a programme developed by EURISCO Research and Development, Denmark, automatically identified and selected a number of yeast cells in the picture. The fluorescence intensity in pixels, representing the respective yeast cells in each of the pictures in the film, was then recorded and analyzed. Another programme, CellAnalysis, has been developed by Dr. Ivar Balslev, Maersk Mc-Kinney Møller Institute for Production Technology, University of Southern Denmark, Odense. This program also has the option of automatic identification of yeast cells in the pictures, but in addition it allows the user to select and compare light intensity of different areas in that picture, e.g. a cell and its surroundings.

3. Results and discussion

A single yeast cell is less than 10 μm in diameter and its small size makes single cell observations demanding regarding technical and methodical issues. Furthermore, cellular metabolism works on a timescale of seconds, which necessitates fast recording of any intracellular event. The amount of NADH in one single cell, and hence the fluorescence intensity of that cell, is low. To ensure that we could observe glycolytic oscillations in single yeast cells using our microscope setup we started out studying high density yeast cell suspensions. A train of glycolytic oscillations was initiated by addition of glucose and cyanide while the stirred suspension was monitored by spectrofluorometry. A sample of the oscillating yeast cell suspension was injected into the flow chamber and the fluorescence due to NADH was measured using fluorescence microscopy. We tried to single out and focus on individual cells in the suspension; however, light scattering from other cells will inevitably affect the measurement and it is almost impossible to distinguish NADH fluorescence in a particular cell from that in cells in the close surroundings. The experiment is similar to the one conducted by Aon et al. [12] and typical recordings of the fluorescence from the fluorescence spectrometer and the fluorescence microscope are shown in Fig. 3. Note that the two traces are recorded simultaneously. The cells in the fluorescence microscope flow chamber were maintained under similar conditions to those in the fluorescence spectrometer, except that the flow chamber was not stirred. The cells were not

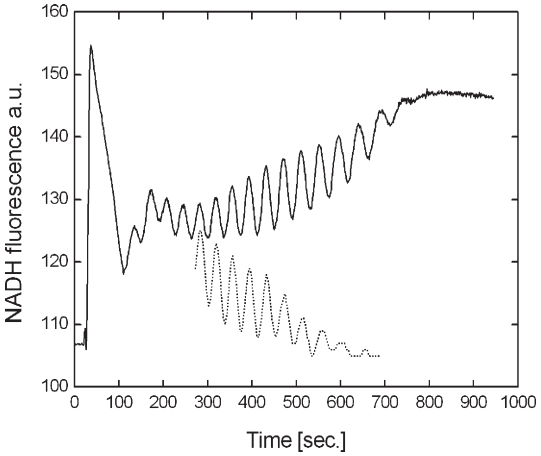

Fig. 3. Comparison of glycolytic oscillations obtained from a stirred and dense yeast cell suspension studied using a spectrofluorimeter as described in Ref. [24], and from focusing on a single cell in suspension using a microscope. The solid line shows the trace of fluorescence due to intracellular NADH measured by the spectrofluorimeter. Yeast cells were suspended in 8 ml of 100 mM potassium phosphate buffer, pH 6.8, to a concentration of 35.4 mg/ml dry weight. At time $t = 11$ s 100 μl of buffer containing glucose and KCN was added to the suspension, giving final concentrations of 20 mM and 5 mM, respectively. After 120 s a sample (200 μl) of the suspension was removed, transferred to the flow cell, we focused on a single cell in the suspension, and the fluorescence due to intracellular NADH was measured using the fluorescence microscope (dotted line). The experiment was performed at 25 °C.

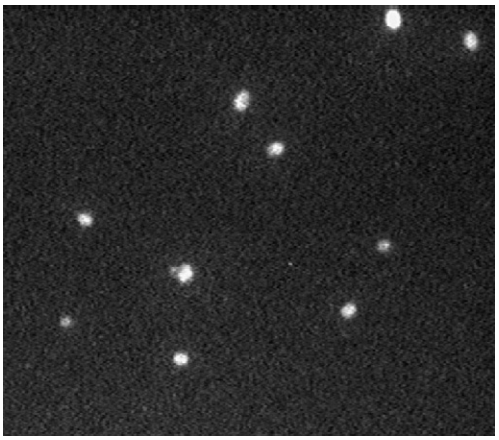

Fig. 4. A number of immobilised yeast cells on polylysine coated coverslip in the flow chamber. The emitted light is caused by NADH fluorescence. It is seen that the cells are well separated on the coverslip. The fluorescent cells are recorded by an EB-CCD camera from Hamamatsu at 630× magnification.

immobilized and there was no flow of solutes or cells through the chamber.

The frequency of glycolytic oscillations is very sensitive to temperature [25]. Therefore, any heat source, such as the light beam through the flow cell, might cause the temperature in the sample to rise. However, we found that the temperature in the flow chamber filled with water never increased more than 2 °C under extreme conditions. The latter means constant exposure of light from the Hg-lamp during a period of 30 min without any flow of liquid through the flow cell. This allowed us to compare glycolytic oscillations recorded using the ordinary spectrofluorimeter observing several millilitres of high density yeast cell suspensions and oscillations recorded using the flow cell and studied through the microscope.

Our experiments therefore show that each cell in a dense yeast suspension oscillates in phase with the other cells as suggested by Aon et al. [12] and Ghosh et al. [26]. So far, published experimental data concerning glycolytic oscillations in individual yeast cells have dealt with observations of individual cells in oscillating high density suspensions of yeast cells [12,27]. However, this does not answer the question if a single cell in a low-density yeast suspension oscillates, but out of phase with the other cells in the suspension. To solve this question it is necessary to examine single cells, which are not affected by the presence of other yeast cells. Immobilisation of cells on polylysine coated coverslips allows us to examine single cells. The cells were immobilised in a monolayer and well distributed on the coverslip as shown in Fig. 4. Data are collected from the entire cell profile and cannot discriminate between different cell compartments such as cytoplasm and mitochondria. However, *S. cerevisiae* is a Crabtree-positive yeast cell in which cyanide-insensitive respiration is unlikely and mitochondrial NADH does not contribute to the oscillations [28,29].

The high flow of buffer through the flow cell (retention time 3.0 s) and the fact that only few cells are attached to the coverslip, ensure that each cell is exposed to constant surroundings and are not affected by other cells. Glucose and cyanide at the concentrations used here would have caused metabolic oscillations in a dense yeast cell suspension. However, in spite of numerous attempts, we did not observe any oscillations in NADH fluorescence in isolated single cells (data not shown).

As mentioned previously, it has been speculated that acetaldehyde [19,20,22] or ethanol [12] acts as a cellular messenger causing yeast cells in suspension to synchronize their metabolism. Richard et al. [22] reported that the concentration of extracellular acetaldehyde oscillated between 40 μM and 100 μM with the same frequency as oscillations in the concentration of NADH. This frequency corresponds to a period of 36 s at 25 °C. We simulated this by adding acetaldehyde to immobilised yeast cells at a frequency similar to glycolytic oscillations. Within a period of 36 s we infused an acetaldehyde solution to the system for 18 s. When the concentration of acetaldehyde in the flow cell is modelled as a CSTR (*c*ontinuous-flow *s*tirred *t*ank *r*eactor) and we assume that mixing is ideal, the profile of the increasing and decreasing acetaldehyde concentration has an almost sinusoidal shape. This is supported by the experiment shown in Fig. 2 in which we measure the periodic infusion of NADH into the constant flow of buffer through the flow cell.

Acetaldehyde diffuses readily through the cell membrane and a high-density oscillating yeast cell suspension responds promptly to perturbations with amounts in the micromolar range [18,22,30]. We periodically perturbed single yeast cells with acetaldehyde in the low micromolar range at concentrations a magnitude of order lower than reported for suspensions [18,19,22]. As seen in Fig. 5 single yeast cells respond immediately to an oscillating acetaldehyde concentration. During the perturbations cells were exposed to a maximum concentration of acetaldehyde of 5.5 μM.

The cellular oscillations in dense yeast cell suspension occurring when glucose and cyanide are added are not forced by an external oscillating source of acetaldehyde generated by a certain experimental setup. They represent an integral part of the biochemical system, regulation of glycolysis, and the environment. This is emphasized if we discontinued the external

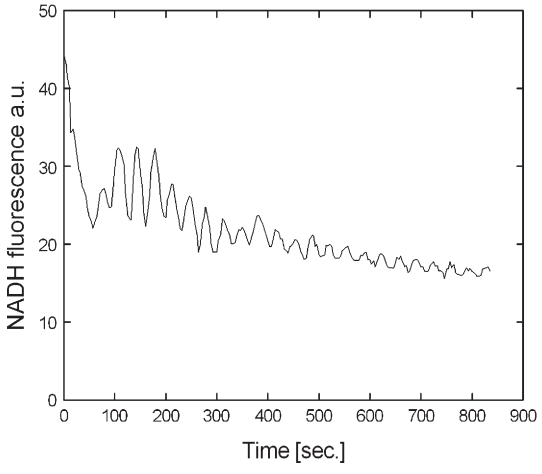

Fig. 5. Fluorescence microscopic measurements of intracellular NADH in an immobilised yeast cell subjected to a constant flow of phosphate buffer containing glucose and cyanide as described in Materials and methods. Acetaldehyde is infused in cycles with a period of 36 s corresponding to the natural frequency of NADH oscillations in dense yeast cell suspension. Acetaldehyde concentration never exceeded 5.5 μM.

forcing of single cells. When acetaldehyde addition was stopped the glycolytic oscillations did not stop immediately. Instead the oscillations gradually died out as shown in Fig. 6. This shows that the recorded oscillations are not just caused by an external forcing of the activity of alcohol dehydrogenase converting acetaldehyde to ethanol under consumption of NADH. The oscillations are an integral part of an excitable system. Alcohol dehydrogenase is not a major point of regulation in glycolysis and a simple displacement of the chemical equilibrium around this enzyme alone does not cause these dampened oscillations in NADH. Instead the displacement caused by a very low acetaldehyde concentration has a ripple effect on glycolysis as a complex metabolic system via NADH.

Additionally, we tried to force the single cells with ethanol, which is another product of glycolysis and direct product of alcohol dehydrogenase. Ethanol diffuses easily through the cell membrane and is a potential mediator of glycolytic oscillations. We attempted to force the cells with up to 500 μM ethanol at max. concentration. As can be seen in Fig. 7 oscillations could not be induced by such forcing. The trace of NADH is similar to the traces obtained when a flow of cyanide and glucose is added to non-perturbed single cells. From this experiment we conclude that it is doubtful that ethanol is responsible for the synchronization of glycolytic oscillations between cells, since an oscillating ethanol concentration has no effect on the immediate behaviour of glycolysis.

We have demonstrated that oscillations in extracellular acetaldehyde at very low concentration are sufficient to generate metabolic oscillations in an isolated single yeast cell and that a perturbation of the glycolytic system is a necessity for the oscillations to occur. Glycolysis in single cells does not oscillate unless the cell is surrounded by many other cells in a dense suspension. The fact that glycolytic oscillations may be induced in individual yeast cells by forcing the extracellular concentration of acetaldehyde to oscillate and that ethanol does not have an effect on the intracellular NADH level suggests that acetaldehyde can be considered as a sort of messenger. Acetaldehyde carries information from one cell to another and thereby

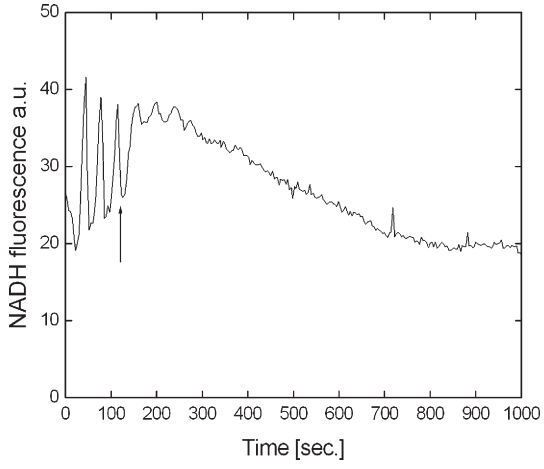

Fig. 6. The periodic infusion of acetaldehyde is stopped after 120 s (indicated by the arrow). The intracellular oscillations dampen out. Experimental conditions as in Fig. 7.

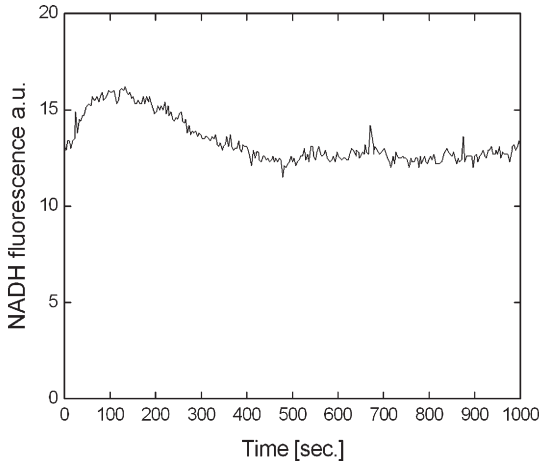

Fig. 7. NADH trace of a yeast cell exposed to glucose, cyanide and periodic infusion of ethanol. The ethanol was dissolved in phosphate buffer at concentrations up to 15.1 mM and added in cycles corresponding the natural frequency of NADH oscillations. The flow was 17 μl/min giving a max. ethanol concentration on 500 μM.

synchronizes glycolytic oscillations in cell suspension. This may also explain in part why oscillations are not observed in cell suspensions of low density. Here the concentration and probably the amplitude of extracellular acetaldehyde becomes too low to synchronize oscillations between individual cells. Another finding that points to acetaldehyde as the synchronizing agent is that periodic infusion of ethanol is not able to induce glycolytic oscillations.

While our results cannot explain why oscillations are not seen in a single cell subjected to a flow of glucose and cyanide they clearly demonstrate that the cells are excitable. They respond immediately to a low concentration of acetaldehyde and when the pulsating inflow of acetaldehyde is stopped the oscillations do not stop immediately.

The oscillations in NADH observed in dense yeast cell suspensions are accompanied by oscillations in acetaldehyde and it is believed that these acetaldehyde oscillations serve to synchronize oscillations in the individual cells in the suspensions [19,22]. This is supported by our results. However, at the same time it is important to notice, that even though oscillations in acetaldehyde concentration are able to introduce oscillations in the glycolytic pathway in living yeast cells, the oscillations seen in suspension represent a property of the system. Hence, it is probably the interplay between many constituents of the biochemical system, which helps maintaining the oscillations in glycolysis and determines their frequency not the oscillating acetaldehyde concentration alone [15].

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