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Biophysical Chemistry 72 (1998) 49–62

Biophysical
Chemistry

Sustained oscillations in glycolysis: an experimental and theoretical study of chaotic and complex periodic behavior and of quenching of simple oscillations

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Revision received 16 January 1998; accepted 13 February 1998

Abstract

We report sustained oscillations in glycolysis conducted in an open system (a continuous-flow, stirred tank reactor; CSTR) with inflow of yeast extract as well as glucose. Depending on the operating conditions, we observe simple or complex periodic oscillations or chaos. We report the response of the system to instantaneous additions of small amounts of several substrates as functions of the amount added and the phase of the addition. We simulate oscillations and perturbations by a kinetic model based on the mechanism of glycolysis in a CSTR. We find that the response to particular perturbations forms an efficient tool for elucidating the mechanism of biochemical oscillations. © 1998 Elsevier Science B.V. All rights reserved

Keywords: Glycolysis; NADH oscillations; Chaos; Response to perturbations; Model of glycolysis

1. Introduction

Glycolytic oscillations have been observed in extracts from various types of cells since 1964 [1,2]. In these studies, the reaction was conducted in a closed system with addition of trehalose to bring about a continuous production of glucose [3] or in a semi-open system with inflow of some substrate (usually glucose) [4]. Under such conditions, oscillations may continue for a long time but are fundamentally transient, which excludes observation of

a number of interesting phenomena. A system in which enzymes were retained with a semipermeable membrane has been used for muscle extract [5].

In this paper we demonstrate that it is possible to run glycolysis in a genuine CSTR long enough to establish and maintain a well-defined oscillatory dynamical state of the system, to determine the dependence of the oscillations on external conditions, and to characterize the system through perturbation experiments.

One purpose of the paper is to describe in detail the experimental procedures for making the extract and running the experiments (in Section 2). However, our main interests are to characterize glycolysis as a dynamical system and to contribute to determining the

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kinetics of the pathway as a whole. We investigate the dependence of the pattern of oscillation on external parameters (in Section 3) and the response of the oscillating system to perturbations (in Section 4).

We find in Section 3 that glycolytic oscillations in a CSTR can be simple periodic, complex periodic, or chaotic, depending on the external conditions. These results confirm a previous report [6]. It is significant that a CSTR experiment roughly may simulate the operating conditions for glycolysis in a living cell.

The dependence of patterns of oscillation on external parameters, can be used to improve models of glycolysis. However, our primary experimental tool for modeling glycolysis will be its response to perturbations. In fact, it is possible to deduce information about the kinetics of the entire system from the experimentally measured dynamical behavior. This has been demonstrated for inorganic reactions like the Belousov–Zhabotinsky reaction [7,8].

The method is based on perturbations of the system in a state of simple periodic oscillations. With small harmonic oscillations (near a supercritical Hopf bifurcation) one can always ‘quench’ the oscillations by instantaneous addition of a definite amount of a species in a definite phase of the oscillation. The idea of quenching of chemical oscillations and the theory underlying the method is described briefly in Section 4.1 and in more detail in [8].

We have not yet found suitable experimental conditions for a proper quenching analysis of glycolytic oscillations, but we show in Section 4.2 that it is still possible to obtain a response similar to quenching with additions of some substrates of glycolysis in states of oscillations of rather large amplitude. The results of such experiments may help modeling glycolysis even though a systematic, quantitative analysis cannot yet be carried through.

An important goal of our work with glycolysis is ultimately to model the kinetics of the pathway, so our analysis must necessarily build on the known mechanism. The kinetics of some of its steps for some enzymes have been intensively studied. But the complex kinetics of the entire pathway is not known for any specific type of living cell in a well defined state.

As a first step, we therefore consider a model of glycolysis based on the well-known mechanism, using the kinetic constants for yeast enzymes, estimat-

ing other constants, and using the remaining ones as adjustable parameters that are varied to fit the experimental data best possible. We show in Section 5 that the model obtained this way can reproduce several features of the experiments including complex oscillations.

2. Experiments

2.1. Preparation of extract

Running glycolysis in a flow reactor requires a sufficient supply of extract of uniform quality. In our experiments, cell-free extract is prepared from (Danish) commercial baker’s yeast (*Saccharomyces cerevisiae*) according to Refs. [9–11]. A batch is obtained from 250 g yeast by the following procedure (all operations are carried out at a temperature of 4°C unless otherwise stated). The yeast is washed in 400 ml 0.1 M potassium phosphate buffer (pH 4.5) and centrifuged for 10 min at $4000 \times g$. This step is repeated once.

The yeast is resuspended in 500 ml 0.1 M potassium phosphate buffer (pH 6.5) and bubbled with air for 3.5 h at room temperature (using a pump of capacity 400 ml/h). The suspension is centrifuged for 10 min at $4000 \times g$, and the sediment resuspended in 200 ml 0.1 M potassium phosphate (pH 8.0).

The yeast cells are ruptured using a bead-beater with 0.5 mm glass beads as follows. The bead-beater is turned on for 30 s, then off for 30 s, and this cycle is repeated ten times. The resulting suspension is then centrifuged for 30 min at $13\,000 \times g$. Subsequently, the supernatant is centrifuged for 1 h in an ultracentrifuge at $100\,000 \times g$. A clear phase in the middle of the centrifugation tube is taken out with a syringe. (the cell-free yeast extract). The extract is freeze-dried (duration about 15 h), and then stored in a freezer at -18°C until it is used. It can be stored in this way for several months. The protein concentration is about 300 mg/g freeze-dried extract, as determined by the biuret method.

Before use, the ability of a sample of extract to produce oscillations is tested with trehalose: 100 μl extract containing 20 mg protein is mixed with 300 μl 0.1 M potassium phosphate buffer (pH 6.5) and 25 μl 1.5 M trehalose is added, and the concentration of

nicotinamide adenine dinucleotide (NADH)₂ is monitored as described below.

2.2. Experimental setup for the flow experiments

Sustained oscillations of glycolysis were obtained in a continuous-flow stirred tank reactor, CSTR, with inflow from two stock solutions, A and B, and outflow of surplus reaction mixture. Solution A contains 100 mM glucose dissolved in doubly ion-exchanged water. Solution B contains the extract dissolved in 0.1 M potassium phosphate buffer (pH 6.5) with 10 mM magnesium sulfate. The concentration of protein in solution B is typically about 10 mg/ml, and at such low protein levels, we found no oscillations unless NADH and adenosine triphosphate (ATP) were added to the solution [10] whereas with a concentration about 20 mg/ml in the stock solution, addition of NADH and ATP is unnecessary.

For setting up the experiment, the following procedure is used. The freeze-dried yeast extract is dissolved to a desired protein concentration, and NADH and ATP are added to the desired concentrations. Typical concentrations of NADH and ATP are 0.5 mM and 3.3 mM, respectively. In experiments with a protein concentration about 20 mg/ml in the stock solution, addition of NADH and ATP is unnecessary. The two stock solutions are placed in ice baths to keep them cold during an experiment. All the chemicals are of analytical grade. Cold, doubly de-ionized water is used.

The reactor is a $1 \times 1 \text{ cm}^2$ quartz cuvette placed in a thermostatic brass jacket, by which the temperature of the reaction mixture is kept constant at the chosen temperature (typically 30.0°C). The jacket is covered with a Plexiglas lid. The two stock solutions are pumped into the reactor through separate Teflon tubes and are mixed in the cuvette. The volume of the reaction mixture is kept constant at 1.7 ml by removing surplus liquid with a vacuum pump through a glass tube. Above the reaction mixture there is a gas phase containing atmospheric air. The reaction mixture is stirred at 440 rev./min or 880 rev./min to give a homogeneous solution in the reactor.

The thermostated reactor is placed in an HP8452A diode array spectrophotometer from Hewlett-Packard. The oscillations are observed continuously by monitoring the absorption of light at 340 nm using the

absorption at 400 nm as a reference. The absorbance difference measured is assumed to be related to the concentration of NADH. The light beam passes through the reaction mixture near the bottom of the reactor. The data are recorded by a computer connected to the diode array spectrophotometer.

To maintain the system in a well-defined dynamical state, stable and constant flows of reactants of the reaction from a reliable pump system are essential. We have used two different systems for pumping the reactants into the reaction mixture, either a peristaltic cartridge pump or computer controlled stepper motor driven piston burets (1.0 ml). No essential difference in the observed oscillatory behavior of glycolysis results from using either of the two different pump systems.

2.3. Running the CSTR experiments

Before each experiment a fresh stock solution B is made as described above. The solution is filtered through a Millipore filter. The two cold solutions are allowed to warm up in the tubes leading to the reactor.

In all of the experiments reported here, the rates of the flow from the two stock solutions were chosen equal. Therefore, the concentrations of all reactants of glycolysis in the reactor are half their concentrations in the stock solutions. To get sensible initial conditions, the reactor is filled with 0.85 ml of solution B and 0.85 ml double ion-exchanged water before the flows are started. In this way, we have investigated the oscillatory behavior of the system as a function of the total flow rate.

2.4. Perturbation experiments

Once sustained, stable oscillations have developed, a perturbation experiment is carried out by adding a specific amount of a substrate at a specific phase of the oscillations. A small amount of a 0.1 M solution of the substrate to be added is made fresh every day and kept at 4°C. The chosen amount is quickly injected into the reaction mixture with a syringe at the selected phase of the oscillations.

The perturbation experiments are carried out using one feed stream containing 100 mM glucose and another one containing extract with 10 mg/ml protein

and NADH, ATP, and Mg^{2+} added to give concentrations 0.5 mM, 3.3 mM, and 10 mM, respectively. The stirring rate is 880 rev./min and the temperature 30.0°C. The total specific flow rate is 2.4×10^{-2} /min (residence time 42 min). The oscillations have a period of about 13 min. The oscillations and the results of the perturbations are reproducible and do not depend on the batch of extract.

3. Patterns of oscillations

3.1. Simple periodic oscillations

Glycolytic oscillations in a CSTR can be maintained as long as the supply of reactants lasts. Fig. 1 shows an example of a long run of oscillations with constant flows of reactants. The oscillations begin 1 h after the initiation of the experiment and last until the supply of extract is used up (approximately 33 h). The simple relaxation oscillations with a period of 14 min were obtained with a flow rate of 2.2×10^{-2} /min and other conditions as described in Fig. 1.

The pattern of oscillations depends on the flow rates of the two feed streams, the concentration of protein and other reactants, and on the temperature. We have investigated the dependence of the pattern on some of these parameters. In all experiments, the flow rates of the two flows (extract and glucose) are equal.

The dependence of the oscillations on the flow rate

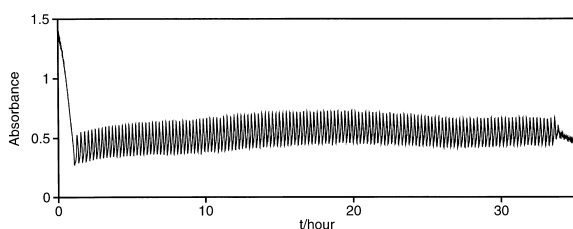


Fig. 1. Simple periodic oscillations. Experimental time series showing the absorbance of light at 340 nm relative to that at 400 nm in glycolysis conducted in a CSTR at 30°C. The total specific flow rate is kept constant at 2.2×10^{-2} /min throughout the experiment, with equal inflow rates of 100 mM glucose and of yeast extract having a protein concentration of 9.7 mg/ml, [ATP] = 2.9 mM, [NADH] = 0.5 mM and $[\text{Mg}^{2+}] = 10$ mM. The oscillations start after a short induction period and continue for about 33 h, until the supply of extract is used up. The period of oscillation is about 14 min.

is exemplified by the sequence of time series shown in Fig. 2. For low specific flow rates, $j = 1.2 \times 10^{-2}$ /min, almost harmonic oscillations with small amplitude can be observed, Fig. 2a. With a higher signal to noise ratio, these would have been ideal for the perturbation experiments.

At higher flow rates, the pattern changes to relaxation oscillations. At the same time, the period of oscillation becomes much larger, from less than three minutes in Fig. 2a to about 15 min in Fig. 2b where the flow rate is 1.5×10^{-2} /min. With increasing flow rates, the period increases still further, and the fraction of a period spent in states of high [NADH] increases. These changes are illustrated in Fig. 2c,d, where the flow rates are 2.4×10^{-2} /min and 2.7×10^{-2} /min, respectively. (The level of NADH is high in the phase of oscillation where the enzyme reaction catalyzed by phosphofructokinase (PFK), is active [12].)

At a flow rate of 3.0×10^{-2} /min, the oscillations have been replaced with a stationary state, as shown in Fig. 2e. The experiments suggest that the period of the oscillations may go to infinity at a finite flow rate.

3.2. Complex regular oscillations

Besides the simple periodic oscillations shown in Figs. 1 and 2, glycolysis in a CSTR can also exhibit more complex behavior. Fig. 3 shows two examples. They are run at flow rates between those of small-amplitude oscillations like Fig. 2a and relaxation oscillations like Fig. 2b, namely 1.9×10^{-2} /min in Fig. 3a and 2.1×10^{-2} /min in Fig. 3b. Other experimental conditions are comparable to those of Fig. 2 but not identical. They are described in the legend to Fig. 3.

The oscillations in Fig. 3b seem to be periodic. (Small irregularities due to external disturbances are almost inevitable.) It shows two small and one large peak per period (and is similar to the pattern shown in Fig. 1b of Ref. [6], which has one small and one large oscillation per period).

The pattern in Fig. 3a may be characterized as growing small oscillations ending in a large one, repeated more or less periodically. Such complex oscillations have previously been observed in glycolysis as short transients in a system where only glucose is flown (at a very low rate) into a reactor containing cell-free extracts (a pseudo-open system)

[13]. In chemical systems run in a CSTR, such patterns are not uncommon [14] and may perhaps be associated with a saddle focus, which under suitable

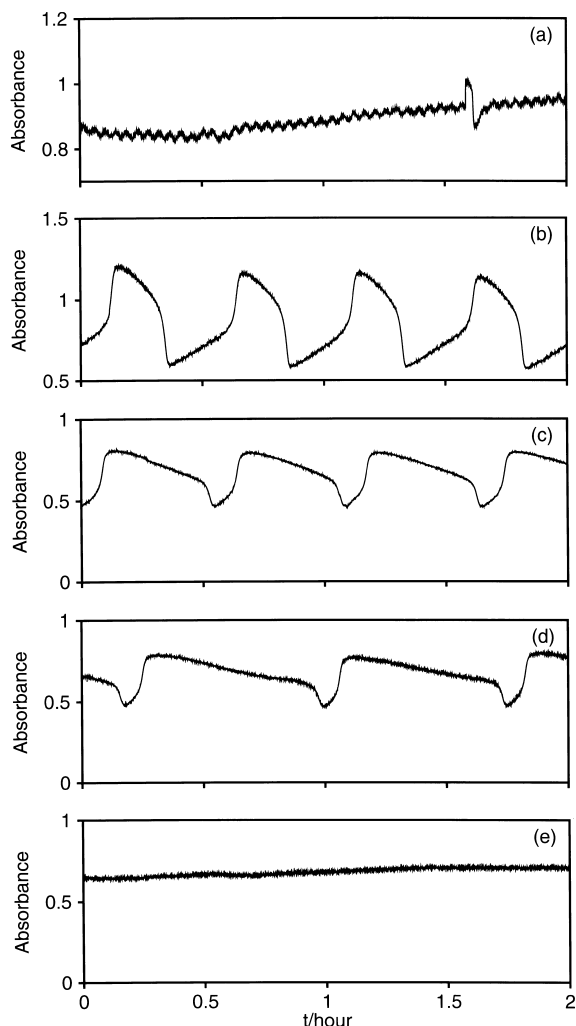


Fig. 2. Dependence of pattern on flow rate. Experimental time series showing the absorbance of light at 340 nm relative to that at 400 nm in glycolysis conducted in a CSTR at 30°C. All five time series were obtained with the same stock solutions and with equal inflow rates of 100 mM glucose and of yeast extract having a protein concentration of 10.0 mg/ml, [ATP] = 3.3 mM, [NADH] = 0.5 mM and $[Mg^{2+}] = 10$ mM. The five experiments differ in the total specific flow rates as follows: (a) 1.2×10^{-2} /min, (b) 1.5×10^{-2} /min, (c) 2.4×10^{-2} /min, (d) 2.7×10^{-2} /min and (e) 3.0×10^{-2} /min. (a) Small-amplitude oscillations are shown. They are almost harmonic but rather noisy (and there is a disturbance after about 1.6 h). For increasing flow rates, the period of oscillation becomes longer (b–d), and at the highest flow rate (e), the state is stationary.

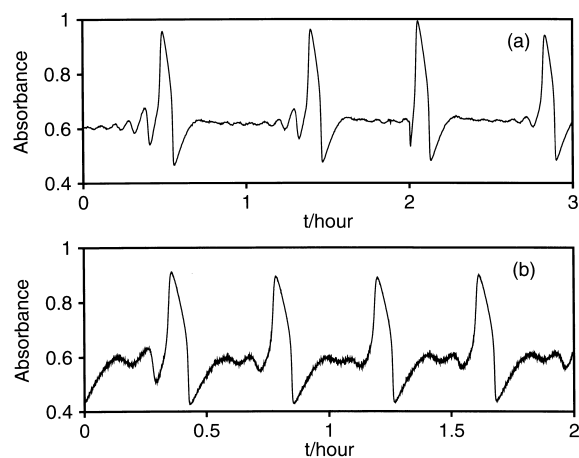


Fig. 3. Complex oscillations. Experimental time series showing the absorbance of light at 340 nm relative to that at 400 nm in glycolysis conducted in a CSTR at 30°C. The two time series were obtained with the same stock solutions and with equal inflow rates of 100 mM glucose and of yeast extract having a protein concentration of 9.5 mg/ml, [ATP] = 2.5 mM, [NADH] = 0.6 mM and $[Mg^{2+}] = 10$ mM. The two experiments differ in the total specific flow rate which is 1.9×10^{-2} /min in (a) and 2.1×10^{-2} /min in (b).

conditions can give rise to a sort of chaos described by Šilnikov [15].

3.3. Chaotic oscillations

In fact, we have also observed completely irregular oscillations which undoubtedly are chaotic (whether of Šilnikov type or not) [6]. Fig. 4 shows an experiment which was started at a flow rate where simple periodic oscillations developed. After approximately 7 h (during which the rate was changed once), the flow rate was decreased from 1.3×10^{-2} /min to 1.2×10^{-2} /min. As a result, chaotic oscillations appeared with irregular patterns somewhat similar to those of Fig. 3. The chaotic oscillations continued for approximately 20 h until the supply of extract was exhausted.

True chemical chaos can only exist in an open system where oscillations can be sustained by inflow of reactants. In fact, the possibility of observing chaos was an incentive to the present work. In Ref. [6], we observed unforced chaos in glycolysis, evidenced by a positive Lyapunov exponent as well as other features discussed there. The possibility of chaos has been suggested on the basis of abstract enzyme models at

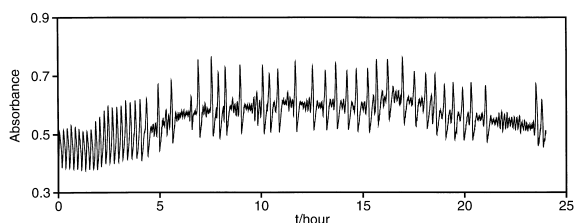


Fig. 4. Chaotic oscillations. Experimental time series showing the absorbance of light at 340 nm relative to that at 400 nm in glycolysis conducted in a CSTR at 30°C. Inflow of 100 mM glucose and of yeast extract having a protein concentration of 9.9 mg/ml, [ATP] = 3.1 mM, [NADH] = 0.5 mM and $[Mg^{2+}] = 10$ mM. The two inflows have the same rate, and the total specific flow rate was initially 2.1×10^{-2} /min. After about 2 h, flow rate is changed to 1.3×10^{-2} /min, and after another interval of 2 h, the flow rate is fixed at 1.2×10^{-2} /min. Whereas the oscillations at the high flow rates are simple periodic, they are highly irregular, presumably chaotic, at the low level selected after 4 h.

least for 15 years [16–18]. Chaotic oscillations have been induced in a semi-open system by time dependent forcing with a periodically varying flow of glucose [19,20].

A difficulty of demonstrating chaos is that irregularities may be caused by external perturbations as well as by the intrinsic dynamics. A number of features support the assumption that Fig. 4 represents chaos in glycolysis. The fact that the experiments at other parameter values show completely regular, periodic oscillations which can be simple or complex depending on parameters (as in Figs. 2 and 3, respectively) shows that the experiments are very well controlled. The complex patterns that make up the irregular oscillations of Fig. 4 are not themselves due to external disturbances because they also appear as part of regular periodic oscillations. The experiment of Fig. 4 also demonstrates that the irregular oscillations can be switched on simply by a small change of flow rate.

3.4. Summary of flow experiments

The experiments described so far have all been run at 30°C. The concentrations of ATP and NADH in the extract differ somewhat, whereas the protein concentration is almost the same in all the experiments. If we disregard the variation of [ATP] and [NADH] (which may be important), we may summarize the main features of the dependence of patterns on the flow rate as follows.

For very low flow rates there is a stable stationary state. At higher flow rates this state bifurcates (by a supercritical Hopf bifurcation) to small-amplitude oscillations. At still higher flow rates, there first appears an interval of complex oscillations at certain levels of [ATP] and [NADH]. Subsequently these change into simple periodic relaxation oscillations of increasing period. At sufficiently high flow rates, these are replaced by a stable stationary state.

In addition to the experiments reported so far, we have also investigated the influence of temperature on the pattern of oscillations. For three different temperatures (24°C, 29°C and 35°C), we found the same overall picture of oscillation patterns as a function of flow rate, but with a shift towards higher flow rates for increasing temperature. (We do not show these results.) A similar tendency has also been observed in the Belousov–Zhabotinsky reaction [21].

4. Perturbation of glycolytic oscillations

4.1. Quenching of oscillations and phaseless sets

The purpose of the perturbation experiments initiated in this paper is to obtain experimental data that can be used to model glycolysis. To get useful quantitative data, it is preferable to work with small-amplitude oscillations near a supercritical Hopf bifurcation. Here, instantaneous addition of some species can make the oscillations stop temporarily provided the change of concentration by the addition and the phase at which it is made are chosen at unique values, determined by the kinetics of the system of reactions. Such perturbations (showing a characteristic dependence on the concentration change and phase) we refer to as quenchings.

The present Section provides some background from the theory of dynamical systems for understanding the perturbation experiments. It describes why quenching is a universal phenomenon associated with oscillations on a limit cycle.

Quenching can be understood in geometrical terms as described in [8]. A supercritical Hopf bifurcation is the change (with some external parameter) from a stable stationary state to limit cycle oscillations with amplitude growing continuously from zero as the parameter is changed from the bifurcation value.

There is still a stationary state, but beyond the bifurcation, it has become a saddle point, which is unstable in the plane of oscillations but is still stable ‘in other directions’.

For simplicity, we shall discuss the situation for a hypothetical 3D concentration space, but the results are similar in higher dimensions. In three dimensions, there is in fact a ‘stable curve’ through the saddle point such that any point on the curve moves towards it.

The perturbation experiments to be discussed try to shift the instantaneous state of the system from a point on the limit cycle (which it circulates during the oscillations) to a point on the stable curve. The perturbations are made by instantaneously adding a small amount of some selected species, resulting in a shift in the direction of the concentration of that species. If the new state after the shift (caused by the addition) is on the stable curve, then the oscillations stop. Subsequently, the system starts oscillating again with gradually growing amplitude (because the saddle point is unstable), and returns to the limit cycle oscillations that were seen before the perturbation.

Near the saddle point, the stable curve can be approximated by a straight line, its tangent in the saddle point, and it is sufficient to consider that line for perturbations from a small limit cycle. Close to a Hopf bifurcation, the limit cycle will be an ellipse (to a good approximation), so the geometry of an elliptic limit cycle and a straight stable line is very simple. As a result, it is possible to relate the experimental conditions where oscillations can be stopped (quenched) to the kinetics of the reaction. (More explicitly, the quenching data are simply related to eigenvectors of the Jacobi matrix at the saddle point.)

This feature allows one to use the data from quenching experiments for developing and optimizing models of an oscillatory reaction with very efficient, systematic methods [22,23]. Such systematic analysis is the goal of the perturbation study initiated in this paper. Unfortunately, we have not yet found viable small-amplitude oscillations experimentally, so we must defer the more fundamental analysis until we have improved our experiments.

For relaxation oscillations away from any Hopf bifurcation, the simple quantitative theory does not apply. Nevertheless, it is still possible, at least in principle, to use special perturbations of the same

character as quenchings for characterization of an oscillatory reaction. This fact is a consequence of a result suggested by Winfree [24] and subsequently proved by Guckenheimer [25]: the presence of a single limit cycle in any n -dimensional chemical system is invariably associated with an $n-2$ dimensional manifold of ‘phase singularity points’. A point near such ‘phaseless set’ will end on the limit cycle with some asymptotic phase as time goes to infinity. But arbitrary close to any point on the phaseless set, there are points of any asymptotic phase. (In three dimensions, the stable curve of a saddle point near the center of a limit cycle arising from a Hopf bifurcation is in fact a special example of phaseless set.) The fundamental ideas of this approach were developed by Winfree in 1968 [13].

Experimentally a phaseless set can be found by measuring asymptotic phases of a large number of perturbations from the limit cycle as a function of the initial phase, as shown by Winfree [24]. The method has been applied to a suspension of yeast cells using perturbation with O_2 [26]. However, the method is very laborious [27], and if quenching is possible, that method is preferable and provides equivalent results.

4.2. Results of perturbation experiments

Perturbations have been carried out on stable relaxation oscillations shown in Fig. 5. The flow rate and other parameters of these oscillations are described in the figure. They apply to all of the perturbation experiments.

We have made perturbations with adenosine monophosphate (AMP), adenosine diphosphate (ADP), ATP, uridine triphosphate (UTP), glucose-6-phosphate (G6P), fructose-1,6-bisphosphate (FBP), and phosphoenol pyruvate (PEP). The response of the system depends on the species added. For some of the substrates, namely ADP, ATP, UTP and FBP, we have been able to stop the oscillations with specific perturbations which may be interpreted as quenching.

We show the dependence of the response on the phase of the perturbations by addition of UTP in Fig. 6. Here and in the following figures of perturbation experiments, time is shown in units of the period of oscillation, counted from the nearest previous maximum. (As period we use the time between that max-

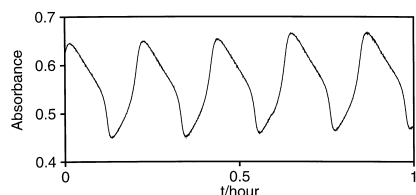


Fig. 5. Oscillations used for the perturbation experiments. Experimental time series showing the absorbance of light at 340 nm relative to that at 400 nm in glycolysis conducted in a CSTR at 30°C. Inflow of 100 mM glucose and of yeast extract having a protein concentration of 10.0 mg/ml, [ATP] = 3.3 mM, [NAD-H] = 0.5 mM and $[Mg^{2+}] = 10$ mM. The two inflows have equal rates, and the total specific flow rate is 2.4×10^{-2} /min. The period of oscillation is approximately 13 min.

imum and the previous one.) The instant of a perturbation is marked with an arrow, and the instant of perturbation may be read from a figure as a phase (which is a number between 0 and 1).

When the addition of UTP is made at a phase of 0.39 with a change of concentration of 1.47 mM, the oscillations almost stop. Subsequently, the amplitude grows and is back to normal size after approximately one and a half period, as shown in Fig. 6b. If the same amount of UTP is added at any other phase, the oscillations are much less affected by the perturbation. Fig. 6a,c illustrate this effect for additions made earlier (Fig. 6a) or later (Fig. 6c) than that of (Fig. 6b). In Fig. 6a, the phase is zero, i.e. the perturbation is made right at the maximum. In Fig. 6c, the phase is 0.65.

If the perturbation is made with a concentration change of UTP that is significantly different from 1.47 mM (used in Fig. 6b), the oscillations also are little affected, regardless of the phase of the addition. Such dependence of phase and concentration change is characteristic of a quenching. Only use of precisely the right amount of the substrate and addition at the right moment (the right phase) will stop the oscillations.

In the case of small-amplitude oscillations near a supercritical Hopf bifurcation, this uniqueness of the phase and concentration change of a quenching can be demonstrated theoretically and understood in terms of the geometry of the limit cycle and the associated saddle focus with its stable curve and unstable plane of oscillation [8]. In the present experiments the operating point is so far from a Hopf bifurcation that the simple theory does not necessarily apply. Even if the qualitative aspects of the theory apply, the oscillations

are so stiff that it may not be possible in practice to actually make a quenching. In any case, the fast return to the limit cycle oscillation is a consequence of the stiffness. (Close to a Hopf bifurcation, the return to limit cycle oscillations after a successful quenching usually takes several oscillations [7].)

Nevertheless, it was possible to get responses that seem to be almost quenchings with additions of ATP, ADP and FBP (as well as UTP), whereas any attempt to quench the oscillations with AMP, G6P or PEP were unsuccessful. The successful temporary stops of oscillations are shown in Fig. 7 for ATP (Fig. 7a), ADP (Fig. 7b,c) and for FBP (Fig. 7d). However, ADP showed a feature that would be impossible near a Hopf bifurcation: it was apparently possible to quench the oscillations at two distinct phases, at 0.13 with a change of concentration of 1.47 mM and at 0.60 with

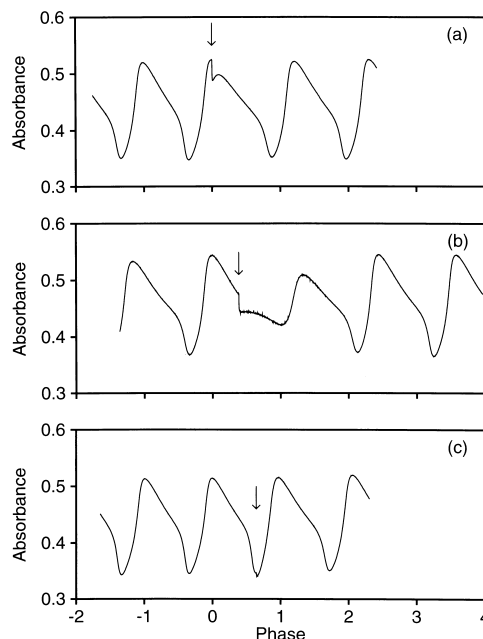


Fig. 6. Perturbations with UTP. Experimental time series showing the response of glycolytic oscillations to addition of UTP resulting in a change of concentration of UTP of 1.47 mM. The response depends very much on the phase at which the addition is made: (a) 0.00, (b) 0.39 and (c) 0.65. The perturbation at a phase of 0.39 almost makes the oscillations stop. Note that the following minima and maxima are not so deep or high (respectively) as for the undisturbed oscillations. This fact together with the characteristic phase dependence of the response make it reasonable to consider the event shown in (b) as a quenching. The operating conditions for the perturbation experiments are described in Fig. 5.

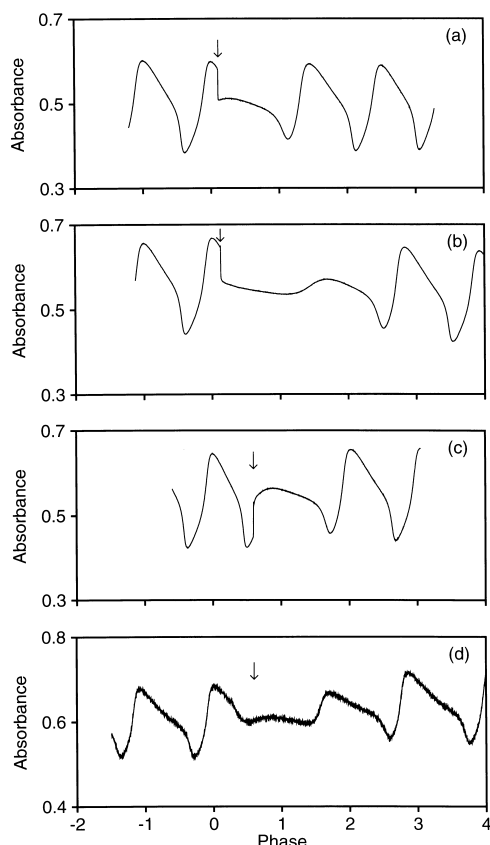


Fig. 7. Perturbations with ATP, ADP and FBP. Experimental time series showing the response of glycolytic oscillations to addition of (a) ATP, (b,c) ADP and (d) FBP each of which almost stops the oscillations. The change of concentrations by the additions and the phases at which they are made are as follows: (a) ATP 1.18 mM, 0.11, (b) ADP 1.47 mM, 0.13, (c) ADP 1.18 mM, 0.60 and (d) FBP 1.18 mM, 0.23. Note that perturbation with ADP can greatly reduce the amplitude of the oscillations at two almost opposite phases. Such response is different from quenching of small-amplitude oscillations, which is only possible at a unique phase.

1.18 mM change. The responses are shown in Fig. 7b,c, respectively.

For comparison, Fig. 8 shows three examples of unsuccessful attempts to quench the oscillations with AMP. In all three experiments the same amount of AMP was added, giving a concentration change of 0.59 mM. The AMP was added in three different phases, namely (a) 0.45, (b) 0.55 and (c) 0.79. Each of the perturbations caused a clear change of the oscillations but with no sign of quenching. We have tried additions of several different amounts of AMP in

many more phases. In all cases, the effect was much smaller than in Fig. 8. This may just mean that we have not tried close enough to the right combination of amount and phase to see an approximate quenching. Alternatively, it may indeed be impossible to quench the oscillations with AMP at the operating point used.

In any case, the responses (including lack of response) can be used to fit models of glycolysis, as we discuss in the following section.

5. Modeling glycolysis in a CSTR

We have attempted to model the results of the experiments presented in the previous sections by extending earlier models [28,5] for simple oscillations in the glycolytic pathway to account explicitly for NAD and NADH and for the inflows and outflows. In addition to simple oscillations, the extended model

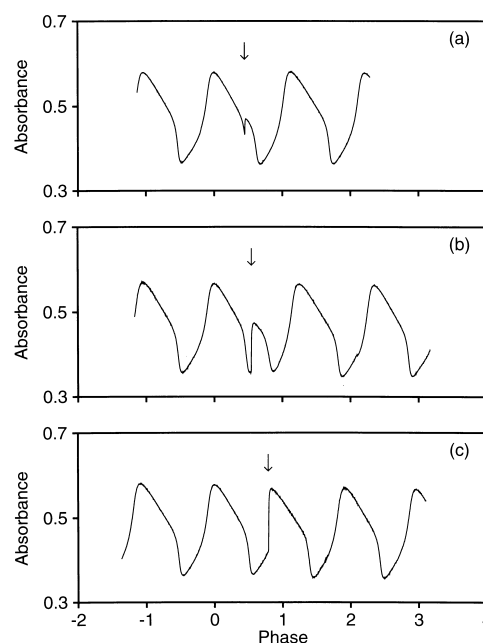


Fig. 8. Perturbations with AMP. Experimental time series showing the response of glycolytic oscillations to addition of AMP resulting in a change of concentration of AMP of 0.59 mM at three different phases (a) 0.45, (b) 0.55 and (c) 0.79. All attempts of finding a combination of amount of AMP added and phase of addition where the oscillations would stop, were unsuccessful.

Table 1

Model for simple and complex oscillations of glycolysis in a CSTR

	Reaction	Rate expression
1	$\text{GLC} + \text{ATP} \rightarrow \text{F6P} + \text{ADP}$	$\frac{V_1[\text{ATP}][\text{GLC}]}{(K_{1\text{GLC}} + [\text{GLC}])(K_{1\text{ATP}} + [\text{ATP}])}$
2	$\text{F6P} + \text{ATP} \rightarrow \text{FBP} + \text{ADP}$	$\frac{V_2[\text{F6P}]^2[\text{ATP}]}{(K_2(1 + k_2(\frac{[\text{ATP}]}{[\text{AMP}]})^2) + [\text{F6P}]^2)(K_{2\text{ATP}} + [\text{ATP}])}$
3	$\text{FBP} \rightleftharpoons 2\text{GAP}$	$v_{\rightarrow} = k_{3f}[\text{FBP}]$ $v_{\leftarrow} = k_{3b}[\text{GAP}]^2$
4	$\text{GAP} + \text{NAD} \rightarrow \text{DPG} + \text{NADH}$	$\frac{V_4[\text{GAP}][\text{NAD}]}{(K_{4\text{GAP}} + [\text{GAP}])(K_{4\text{NAD}} + [\text{NAD}])}$
5	$\text{DPG} + \text{ADP} \rightleftharpoons \text{PEP} + \text{ATP}$	$v_{\rightarrow} = k_{5f}[\text{DPG}][\text{ADP}]$ $v_{\leftarrow} = k_{5b}[\text{PEP}][\text{ATP}]$
6	$\text{PEP} + \text{ADP} \rightarrow \text{PYR} + \text{ATP}$	$\frac{V_6[\text{PEP}][\text{ADP}]}{(K_{6\text{PEP}} + [\text{PEP}])(K_{6\text{ADP}} + [\text{ADP}])}$
7	$\text{PYR} \rightarrow \text{ACA}$	$\frac{V_7[\text{PYR}]}{K_{7\text{PYR}} + [\text{PYR}]}$
8	$\text{ACA} + \text{NADH} \rightleftharpoons \text{EtOH} + \text{NAD}$	$v_{\rightarrow} = k_{8f}[\text{ACA}][\text{NADH}]$ $v_{\leftarrow} = k_{8b}[\text{EtOH}][\text{NAD}]$
9	$\text{AMP} + \text{ATP} \rightleftharpoons 2\text{ADP}$	$v_{\rightarrow} = k_{9f}[\text{AMP}][\text{ATP}]$ $v_{\leftarrow} = k_{9b}[\text{ADP}]^2$
10	$\text{F6P} \rightarrow \text{P}$	$k_{10}[\text{F6P}]$

GLC, glucose; ATP, adenosine triphosphate; F6P, fructose-6-phosphate; ADP, adenosine diphosphate; FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; NAD and NADH, nicotinamide adenine dinucleotides, DPG, 1,3-bisphosphoglycerate; PEP, phosphoenol pyruvate; PYR, pyruvate; ACA, acetaldehyde; EtOH, ethanol; AMP, adenosine monophosphate; P, inactive product.

is required to simulate also the complex and chaotic oscillations of Figs. 3 and 4 and the response to perturbation shown in Figs. 6, 7 and 8.

There does not exist a generally accepted set of rate expressions and kinetic parameters for the enzymes active in yeast extract. Therefore we have extracted

Table 2

Kinetic constants used in the simulations

$V_1 = 0.50$ mM/s	[29]
$K_{1GLC} = 0.1$ mM	[29]
$K_{1ATP} = 0.063$ mM	[29]
$V_2 = 1.5$ mM/s	Estimated from [5]
$K_2 = 0.0016$ mM ²	[5]
$k_2 = 0.017$	adjusted from [5]
$K_{2ATP} = 0.01$ mM	Estimated [30]
$k_{3f} = 1/s$	Estimated
$k_{3b} = 50$ mM/s	$\frac{k_{3f}}{k_{3b}}$ Estimated from [10]
$V_4 = 20$ mM/s	Estimated
$K_{4GAP} = 1$ mM	Estimated
$K_{4NAD} = 1$ mM	Estimated
$k_{5f} = 1/mM/s$	Estimated
$k_{5b} = 0.5/mM/s$	Estimated
$V_6 = 10$ mM/s	Estimated from exp. values [31]
$K_{6PEP} = 0.2$ mM	Estimated from exp. values [31]
$K_{6ADP} = 0.3$ mM	Estimated from exp. values [31]
$V_7 = 2.0$ mM/s	Estimated
$K_{7PYR} = 0.3$ mM	Estimated
$k_{8f} = 1/mM/s$	Estimated
$k_{8b} = 1.43 \times 10^{-4}/mM/s$	Estimated from exp. values
$k_{9f} = 10/mM/s$	Estimated
$k_{9b} = 10/mM/s$	Estimated
$k_{10} = 0.05/s$	Estimated

data from various sources including data for enzymes from different organisms and values estimated from other experimental measurements. The reactions of the model (except for inflow and outflow) and the associated rate expressions are given in Table 1. The rate constants used in the simulations are shown in Table 2, and the mixed-flow concentrations of the model species contained in the inflow are shown in Table 3. The parameters of Table 3 are used for all the simulations even though they vary somewhat in the various experiments. Some of the model parameters have been adjusted to fit the experimental oscillations

Table 3

Characteristic concentrations of substrates in the simulations from input flows of extract and glucose

$[ATP]_0 = 3.5$ mM	Adjusted
$[ADP]_0 = 1.1$ mM	Adjusted
$[NADH]_0 = 0.24$ mM	Experimental value
$[NAD]_0 = 4.0$ mM	Adjusted
$[GLC]_0 = 50$ mM	Experimental value

as well as possible, particularly those shown in Fig. 5 which are simulated in Fig. 9d. The flow rate and other parameters for the inflow have been chosen as near the experimental values as possible for each pattern simulated.

The kinetic equations have been integrated with a fifth order Runge Kutta method described in [32], and the result has been checked with an independent, stiff integration method. The results of the simulations are exhibited in Figs. 9, 10 and 11. The oscillations of Figs. 9 and 10 and those before the perturbations of Fig. 11, show the oscillations after all transient effects of initial conditions have disappeared.

Fig. 9 shows how the pattern of oscillation depends on the flow rate. Going from the simple basic relaxation oscillations in Fig. 9d to lower flow rates, there first appears an interval of complex periodic oscilla-

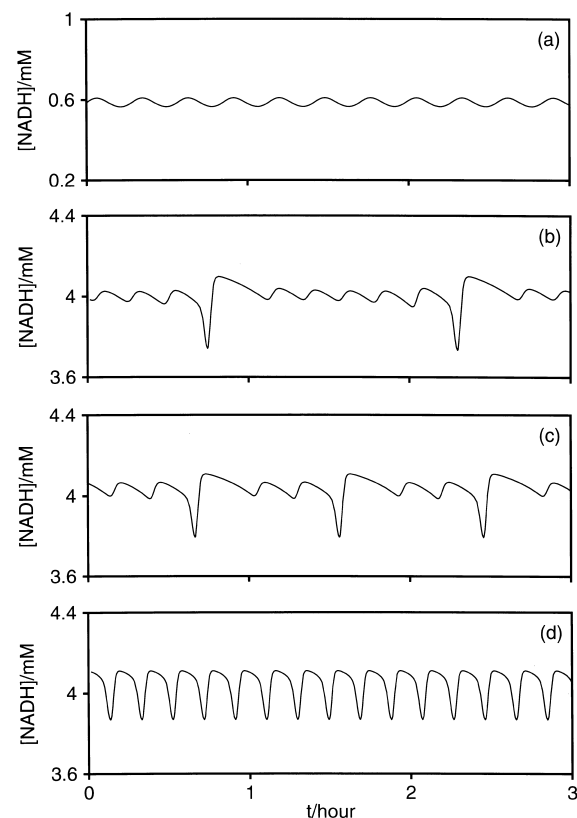


Fig. 9. Simulation of periodic oscillations in glycolysis. Simple and complex, periodic oscillations as predicted by the model described in Table 1 with the kinetic constants given in Table 2 and mixed inflow concentrations of Table 3. The total specific flow rates are (a) $1.0 \times 10^{-3}/min$, (b) $8.154 \times 10^{-3}/min$, (c) $8.25 \times 10^{-3}/min$ and (d) $1.1 \times 10^{-2}/min$.

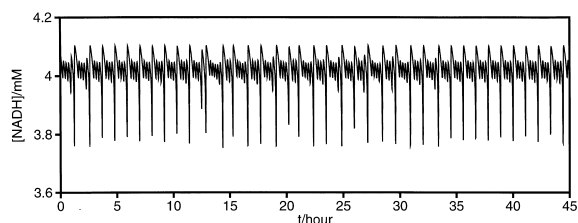


Fig. 10. Simulation of chaos in glycolysis. Chaotic oscillations predicted by the model described in Table 1 with the kinetic constants given in Table 2 and mixed inflow concentrations of Table 3. The total specific flow rate is $8.2 \times 10^{-3}/\text{min}$.

tions matching the experimental results shown in Fig. 3. Examples of these are shown in Fig. 9b,c which look quite similar to the experimental curves of Fig. 3 and appear in the right order; but the periods of oscillation and the flow rates at which they are found differ from the experimental values. At low flow rates, the complex oscillations are replaced by small-amplitude, nearly harmonic oscillations, shown in Fig. 9a, which eventually change to a stable stationary state at a supercritical Hopf bifurcation at still lower flow rates. However, the period of the oscillations is three times the experimental value. Between the Hopf point and the complex oscillations, the model also has a region with large simple oscillations which have not been seen in the experiments.

For flow rates near those of the complex periodic oscillations, there also appear aperiodic oscillations like in Fig. 10, akin to the chaotic oscillations of the experiment Fig. 4 (and Fig. 1c of Ref. [6]). Intervals of such chaotic oscillations are mingled with intervals of complex periodic oscillations, and are in fact dominant.

The perturbation experiments have been simulated at a flow rate of $3.0 \times 10^{-2}/\text{min}$, close to that of the experiments. We show only the result of a perturbation by addition of ADP which hits close to the phaseless set, Fig. 11b, and two other perturbations with the same change of concentration of ADP made at phases 2% smaller and larger, Fig. 11a,c, respectively. Note how much the response depends on the phase.

Interestingly, the phase at which addition of ADP can quench the oscillations in the model is very close to the experimental one of Fig. 7b. However, the model does not show any effect similar to that of Fig. 7c at the opposite phase. We have not succeeded in modeling the experimental quenching with ATP.

Perturbations are modeled by integrating the kinetic equations on the limit cycle until the phase at which an addition is made. The integration is then continued with an initial condition equal to the current value shifted appropriately in the direction of the concentration of the species added. The difficulty of reaching the phaseless set increases rapidly with the distance from the bifurcation point in the model as well as in the experiment. To see the effect more easily the model flow rate was therefore increased somewhat from the experimental value. (In the model, there is another supercritical Hopf bifurcation at large flow rates.)

6. Conclusion

Glycolysis plays a crucial role in cell metabolism. This fact makes it important to know the dynamical

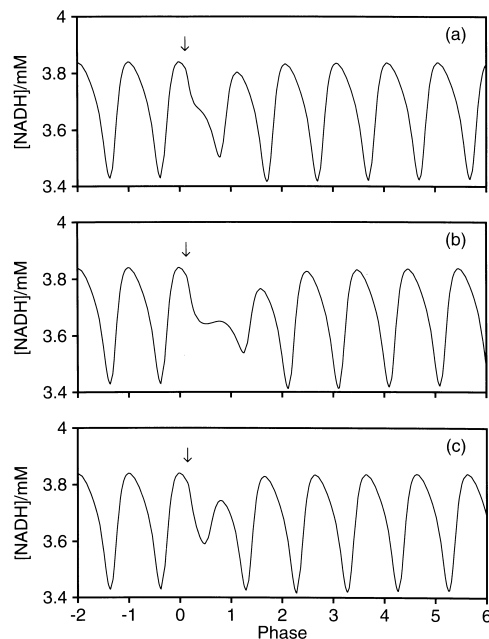


Fig. 11. Simulation of perturbations in glycolysis. Simulation of the response of perturbations by addition of ADP giving a change of concentration of 0.059 mM at phase (a) 0.140, (b) 0.158 and (c) 0.175. The model is defined by Tables 1 and 2, and the mixed flow concentrations of the species in the inflows are given in Table 3. The total specific flow rate is $3.0 \times 10^{-2}/\text{min}$. (b) A perturbation that almost quenches the oscillations is shown. (a,c) The fact that the response is very sensitive to the phase of the addition is demonstrated.

behavior that can occur, and to determine the kinetics of the complete system under conditions that are relevant to biology. The present paper contributes to the solution of these problems by showing how glycolysis can be conducted in a CSTR with inflow of glucose and yeast extract. In contrast to batch reactors or semi-open systems, a CSTR can sustain oscillations in a well defined dynamical state as long as desired.

By varying the external conditions, we have observed a wide range of simple and complex sustained oscillatory patterns. The most common patterns observed are relaxation oscillations with shape and period depending on conditions. By varying the flow rate and concentrations of reactants, we have obtained a succession of patterns from small sinusoidal oscillations through complex periodic and chaotic oscillations to relaxation oscillations. For high values of the specific flow rate the oscillations disappear (possibly through an infinite period bifurcation) to a stationary state with high level of NADH.

Sustained oscillations also make it possible to study the response of the glycolytic system to perturbations, which are potentially very useful for determining the kinetics. The paper reports perturbation experiments which demonstrate that it is possible to detect a phaseless set for limit cycle oscillations of relaxation type in glycolysis. This has been achieved by addition of any of ATP, ADP, UTP and FBP in such a way that the oscillations almost stop for a short time. Such response requires the addition of a specific amount of the species in a specific phase of the oscillations, depending on the particular species used, and it can be considered quenchings similar to those studied previously for small-amplitude oscillations [7]. Addition of AMP can shift the asymptotic phase but no quenching has been observed. Addition of G6P or PEP influences the oscillations but we found no quenching.

The experimental results have been compared with a model of glycolysis, developed from existing ones [28,5]. The model explains many of the experimental observations including the complex oscillations and chaos. It also accounts for the possibility of quenching the relaxation oscillations by addition of ADP as in the experiments, but some other features of the response to perturbations disagree. The experimental quenching with UTP, which is not part of the model, suggests that the predictive value of the model could be improved by including other parts of metabolism

such as the synthesis of polysaccharide [10]. The use of a more comprehensive model for glycolysis to obtain better agreement with the observations, will be left for future studies.

The characteristic response to perturbations, with its critical dependence on phase and amount, can be used in the search for better models. However, to reach the full power of the quenching method, it is necessary to work near a supercritical Hopf bifurcation. We hope to carry out such more complete and systematic analysis in a future paper. Meanwhile our results demonstrate encouragingly that it is possible to quench relaxation oscillations in experiments and simulations.

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