THE GLYCOLYTIC OSCILLATOR

By BENNO HESS

Max-Planck-Institut für Ernährungsphysiologie, 4600 Dortmund 1,
Federal Republic of Germany

SUMMARY

The glycolytic oscillator, mainly studied in yeast, is described with respect to its overall dynamic and biochemical properties and the kinetics of its master enzyme phosphofructokinase. Biochemical and kinetic analyses are complemented by analysis of mathematical models. In addition to the discussion of structure and function of the glycolytic oscillator under homogeneous spatial conditions, recent theoretical and biochemical experiments demonstrating spatial pattern formation are also discussed, followed by a presentation of physiological viewpoints.

I. INTRODUCTION

Glycolysis represents the prototype of sustained oscillation in a metabolic pathway. Its oscillating mechanism has been deeply investigated. Indeed, since its discovery some 20 years ago, the general phenomenon of oscillating chemical and biochemical reactions attracted the attention of many branches of science, and it is significant to observe that the work on oscillatory reactions was accompanied by the development of the theory of dissipative structures as well as the rediscovery of the feedback principle and the formulation of the allosteric theory of enzyme regulation in biochemical and biophysical processes.

Certainly, the general interest in glycolysis also arose from the fact that the glycolytic process is the most ancient and powerful bioenergetic process occurring uniquely and universally in living nature. Thus, the elucidation of an oscillatory state might well unravel some principles of its organization. The search for the mechanism of glycolytic oscillations had to meet a number of technical problems. In the last 50 years biochemical processes were generally investigated under conditions of arbitrary stationary states, and enzymology focused its interest and methodology fully upon equilibrium conditions.

However, oscillatory states occur under non-equilibrium conditions in specified dynamic domains, which are complex and not readily excessible experimentally if continuous read-out techniques to observe variables of the enzymic processes are not used. The application of such techniques as in conjunction with metabolic assay studies, reconstruction experiments, titration experiments in combination with mathematical analysis and computer simulation led to a good understanding of the biochemical requirement for glycolytic oscillation. In this presentation I would like to summarize some of the current views on the mechanism of glycolytic oscillation

with general reference to several reviews (Hess & Boiteux, 1971; Chance *et al.* 1973; Boiteux & Hess, 1974; Goldbeter & Caplan, 1976; Hess, Goldbeter & Lefever, 1978).

II. BIOCHEMICAL ANALYSIS UNDER HOMOGENEOUS CONDITIONS

The demonstration of oscillations in glycolysis of yeast cells and in cell-free extracts of yeast was the first indication that periodic activities of biological systems might be caused by the function of a single enzyme in a chemical pathway consisting of a sequence of enzymic reactions. It could be shown that in a given flux domain the concentrations of all glycolytic intermediates oscillate. The analysis of this pheno- menon was greatly facilitated by the use of spectrophotometric and potentiometric techniques allowing a continuous readout of components of the reaction pathway, namely reduced pyridinenucleotide, pH and CO_2 as indicators of the dynamic state of the process. Using a continuous substrate injection technique to induce dynamic states like those observed in classic stirred flow reactor systems it could be shown that the frequency and amplitude of the glycolytic NADH oscillation in yeast extracts are flux dependent, and that a critical flux range was established in which oscillations are possible. The range of this rate was found to extend over 1·5 orders of magnitude well within the physiological flux range (Hess, Boiteux & Krüger, 1969). The turnover of anaerobic fermentation given in the literature agrees with the flux rates in cell free extracts induced by injection of glucose or fructose. Using the $Q^N_{glucose}$ of 3·085 ($\mu mol \times h^{-1}$) per mg dry weight of baker's yeast, a flux of 308·5 ($\mu mol \times h^{-1}$) per 50 mg cellular protein equals 3 times the injection rate of 100 ($\mu mol \times h^{-1}$) per 50 mg extract protein in agreement with the dilution factor of three. Thus the saturation level during oscillating glycolysis represents the functional range of glycolysis under physiological conditions (Hess *et al.* 1969).

The minimum structure of the glycolytic pathway required for oscillation was found by a demonstration that the substrate of phosphofructokinase, fructose-6- phosphate, does induce a glycolytic oscillation whereas an injection of fructose-1,6 bisphosphate or even phosphoenolpyruvate, the substrate of pyruvate kinase does not (Boiteux & Hess, 1974; Hess & Boiteux, 1968). This experiment demonstrated that the enzyme phosphofructokinase might be considered as the primary oscillophore and the enzymic basis of glycolytic oscillation. The term 'oscillophore' is used to designate enzymes in an enzymic process, which might be a primary source of the oscillation of the whole process, in contrast to other enzymes with kinetic properties insufficient to maintain an autonomous oscillatory state. The activity of the latter is mainly determined by the concentration pulses of metabolites produced by the periodic activity change of the primary oscillophore. The role of phosphofructokinase was furthermore stressed by the fact that its substrate fructose-6-phosphate clearly exhibits the relatively largest concentration amplitude of all oscillating glycolytic intermediates.

Furthermore, these experiments suggest that the minimum requirement for gly- colytic oscillation involves a first-order source step for the generation of a substrate for phosphofructokinase, which is experimentally simulated by the substrate injection technique. On the other hand, the elucidation of the nature of the sink step of the

phosphofructokinase reaction is more complicated because of the feedback recycling of the ADP-ATP system generating the second substrate of the phosphofructokinase reaction as well as the NAD-NADH recycling as part of the sink reaction pathway. Furthermore, the additional function of AMP equilibrating with ADP and ATP via the adenylate cyclase reaction is of importance because AMP reacts as an allosteric ligand of phosphofructokinase (Boiteux & Hess, 1974).

The influence of random perturbation of the glycolytic system is of interest with respect to general biological conditions. It was found that stochastic addition of the substrate of glycolysis led to periodic behaviour within a narrow range around the period length which was observed if a continuous rate of substrate supply was used. These experiments were accompanied by model analysis that showed that the reaction sequence acted as a narrow band-pass filter centred at the mean autonomous frequency, thus keeping the period stable in spite of short time variations of the source rate. We pointed to this property as of physiological significance and providing reliable timing mechanisms at the cellular level (Boiteux, Goldbeter & Hess, 1975).

It was also shown that by periodic addition of substrate the glycolytic system can be readily entrained with the oscillation period of glycolysis synchronizing with a period of substrate supply. In addition, synchronization to a subharmonic of the driving frequency of the periodic substrate supply was recorded. Again, in agreement with model experiments, domains of entrainment of fundamental frequency and two subharmonics could be drawn showing that subharmonic synchronization occurs if the driving frequency is approximately an integer multiple of the frequency recorded when a continuous rate of substrate supply is used. This phenomenon is also known as subharmonic resonance or frequency division. It is an important feature of circadian rhythms. Also it should be noted that the observation of subharmonic synchronization proves the nonlinear nature of the primary oscillophore phosphofructokinase, since this behaviour cannot be obtained in a linear system (Boiteux *et al.* 1975; Hastings & Schweiger, 1975).

The analysis of glycolytic intermediates during oscillation revealed concentration changes in the range of 10^{-5} to 10^{-3} M. In addition, the pulse production of protons and of carbon dioxide was recorded. A comparison of the normalized concentration changes per unit of time showed that concentrations of the various intermediates oscillate with equal frequency, but different phase angles relative to each other. This observation allowed classification of the oscillating glycolytic intermediates into two groups in which maxima and minima of concentrations coincide in time. The two groups differ by a phase angle, which depends on the experimental conditions. The phase-angle analysis of the concentration changes allowed, furthermore, the location of the enzymic steps controlling the oscillatory state. The phase shift of 180° between fructose-6-phosphate and fructose-1,6 bisphosphate as well as between phosphoenolpyruvate and pyruvate pointed to the enzymes phosphofructokinase and pyruvate kinase as the essential control points of the phenomenon. In further experiments it could be shown that a periodic activity change of phosphofructokinase generates pulses of ADP and fructose-1,6 bisphosphate (Boiteux & Hess, 1974; Hess *et al.* 1969). This periodic activity change is propagated along the glycolytic pathways through the adenine nucleotide system, which also effects the enzymes phosphoglycerate kinase

and pyruvate kinase. Both these latter enzymes generate the product ATP, which feeds back to the enzyme phosphofructokinase.

Phosphofructokinase not only operates in a feedback cycle via the adenine nucleotide system but also through an intrinsic feedback structure supplied by its oligomeric composition and non-linear allosteric function (Boiteux & Hess, 1974; Tamaki & Hess, 1975). The regulatory mechanism of the enzyme phosphofructokinase itself has autocatalytic features that are of primary importance for generating oscillatory activity.

From these data it is expected that the glycolytic oscillation should be sensitive to the controlling function of the ADP-ATP-AMP system, depending on time. The function of these nucleotides can be demonstrated by phase-shift experiments (Boiteux & Hess, 1974; Chance, Schoener & Elsaesser, 1964). The addition of ADP at the NAD minimum has no influence on the oscillation whereas addition of ADP at the NAD maximum corresponding to the lowest level of ADP shifts the phase of the oscillation by 180°. Because of the rapid acceleration of the reactions catalysed by phosphoglycerate kinase and pyruvate kinase, both enzymes are not saturated at this time and, thus, sensitive to ADP addition. ATP and AMP can also shift the phase of glycolytic oscillation if added at the appropriate time when the enzyme phosphofructokinase is sensitive to a change in the concentration of both nucleotides.

Phosphofructokinase of yeast was isolated in a highly purified form. Its molecular weight was found to be 720000 daltons. The enzyme is composed of at least four protomers associated into eight sub-units of two different types with molecular weights around 90000 daltons. The kinetics of the enzyme follow an allosteric model with the homotropic effectors fructose-6-phosphate and ATP as substrates and a strong heterotropic activator AMP. The Hill coefficients up to 4·9 were found to be pH-dependent (Boiteux & Hess, 1974; Tamaki & Hess, 1975). It is interesting to realize that the pH dependency of the allosteric properties of this enzyme coincides with the pH dependency of the glycolytic oscillation (Boiteux & Hess, 1974). The detailed analysis of this enzyme is still under investigation. The kinetics of the enzyme in the yeast extract under conditions observed during glycolytic oscillations show a strong dependency on the state of the adenine nucleotide system as well as the concentration of the substrate fructose-6-phosphate. It was found that the activity of phosphofructokinase under oscillating conditions rises to approximately 70% of its maximum activity *in vitro* with a minimum activity during the cycle of 1% and a mean activity in the order of 16% during one oscillatory cycle (Boiteux & Hess, 1974; Hess *et al.* 1969).

The function of the allosteric enzyme pyruvate kinase cannot be discussed in detail. Under conditions prevailing in the oscillating yeast extract its kinetics can be described by a Michaelian saturation function. However, conditions have been found where the allosteric properties of the enzyme come into play. It has been suggested, mainly from model studies, that these latter conditions are responsible for the observation of double periodicities of the glycolytic oscillation (Dynnik & Sel'kov, 1973; Hess & Plesser, 1978), which have been observed in biochemical experiments both spontaneously (see Fig. 5 in Hess & Boiteux, 1968) as well as under special conditions of entrainment (see Fig. 7 in Boiteux *et al.* 1975). The term 'double periodicities'

designates the observation of wave forms consisting of a set of two pulses of equal amplitude being generated periodically such as those observed in electronics by a square wave-amplitude modulated periodic function. Here the square wave has a selector function. However, it should be noted that such wave forms also can be observed by frequency modulation or for instance a separation of an autonomous frequency displayed separately by an input frequency (Boiteux *et al.* 1975).

III. MODELS

Following the phenomenological models of Higgins (1967) and Sel'kov (1968), an allosteric model for the oscillating phosphofructokinase reaction was developed, which takes the biochemical experiments fully into account (Boiteux *et al.* 1975; Hess & Plesser, 1978). A detailed study of this model showed that it is indeed important to represent the nonlinearity of the oscillophore by the autocatalytic-allosteric mechanism of the enzyme phosphofructokinase in order to achieve a representation of the dynamic properties of oscillating glycolysis.

The dynamic behaviour of this enzyme was described as an open system based on the allosteric model as described by Monod and his colleagues (see Boiteux *et al.* 1975). Neglecting diffusion, a set of ordinary differential equations permits the description of the time evolution of the metabolite and enzyme concentrations. The treatment of the concentrations of the various enzymic species as defined by their variable affinities to substrate and product of the reaction yields a total of 13 equations. This set of equations can be simplified by assuming a quasi steady state for the enzymic forms and is justified because of the observation that the concentration of metabolite exceeds that of the enzyme by several orders of magnitude.

The reduction procedure leads to two equations, which define the concentration changes of ATP and ADP as a function of source rate, of sink rate as well as the saturation function for the phosphofructokinase reaction. The dominant source of the non-linearity in this function is the allosteric behaviour of phosphofructokinase. On lowering the allosteric constant of phosphofructokinase, the allosteric property of the system vanishes and the function finally reduces to a simple Michaelis–Menten rate law. This mechanism illustrates the autocatalytic character of the function of the enzyme.

The term describing the activation of the enzyme by its product is essential for the destabilization of the steady state. It is important to note that the allosteric constant with the corresponding Hill coefficient, although a source of non-linearity, yields only the occurrence of a unique stable state but no limit cycle. Thus, the additional condition of the feedback term must be effective in order that oscillations occur (Hess, *et al.* 1978).

The stability analysis of the system demonstrates an excellent agreement between the dynamic behaviour of the allosteric model of glycolytic oscillation and biochemical experiments. These results justify the assumption that to a large extent the dynamic property of the system can be reduced to the properties of phosphofructokinase as the master enzyme within the set of glycolytic enzymes, and that it can be described by a global master equation (Boiteux *et al.* 1975). However, it should be recognized

that the full understanding of the overall dynamics of the glycolytic pathway under all experimental conditions can only be obtained by a quantitative comparison of model and experiments for conditions under which a two-substrate model is the basis of the rate law, and also other regulatory enzymes in the glycolytic sequence are analysed. For technical reasons, extensions of the simple model to date have been analysed only theoretically, and yielded results that cannot be discussed here because of limited space (Hess & Plesser, 1978).

The theoretical studies of process dynamics are commonly based on linear stability analysis and computer simulation. For nonlinear systems where the existence and stability of periodic solutions are of interest, these methods alone may lead to uncertain results. Therefore, proved mathematical theorems are necessary and only the stable periodic solutions guarantee the possibility of experimental observation. Recently the existence of an asymptotic stable limit cycle for the general enzyme catalysed reactions with positive feedback as given here has been proven mathematically by application of the Poincaré–Bendixson theorem (Hess & Plesser, 1978; Erle, Mayer & Plesser, 1979).

IV. BIOCHEMICAL ANALYSIS UNDER NON-HOMOGENEOUS CONDITIONS

Although the analysis of glycolytic oscillations so far has mainly been carried out under homogeneous experimental conditions, where transport processes can be neglected, it is expected that spatial structures occur whenever the evolution of enzymic processes is allowed to couple with transport via appropriate diffusion gradients. Under these conditions oscillations of concentrations of glycolytic intermediates should lead to spatial inhomogeneity and the development of spatial structures as a function of time. Theoretical analysis of simplified models for the oscillation of glycolysis predicted the boundary conditions necessary for the development of dissipative space structures (Goldbeter, 1973; Hess & Chance, unpublished results). Recently, the development of suitable analytical techniques allowed the experimental demonstration of time-dependent evolution and maintenance of oscillating spatial structures in the concentrations of pyridinenucleotides of oscillating glycolysing yeast extract. The experiments indicate that spontaneous spatial self-organization of a complex multienzyme system, such as glycolysis, can be observed under temporal oscillations. This is the first observation that a biochemical system in homogeneous phase might break spatial homogeneity and evolve to a spatio-temporal order (Hess *et al.* 1975; Boiteux & Hess, 1978).

V. PHYSIOLOGICAL VIEWPOINTS

The demonstration of stable periodic reactions in enzymic systems raises a number of questions with respect to its physiological significance. Although the physiological function of periodic biochemical reactions is clearly evident in the case of the slime mould *Dictyostelium discoideum* (Gerisch & Hess, 1974), in many other systems their function is not known. The search continues for direct evidence showing that simple enzymic reaction systems such as those observed in metabolic and epigenetic processes

displaying limit cycle behaviour might serve as a molecular clock for processes like the mitotic cycle differentiation, morphogenesis or pathway separation. Here the term 'pathway separation' has the following meaning: Different biochemical processes frequently share the same intermediate like nucleotides or other small molecules serving as substrates for enzymic conversions or as controlling ligands. These shared functions might lead to self-inhibition or destruction of one or the other processes, necessitating a separation of both processes in space or time. A spatial separation is commonly achieved by localization of two processes in different cellular compartments. A time separation means the separation of two processes by conditions, under which only one of two processes occurs at a time and an alternation of activity of the two processes in time is realized. A typical case in cell physiology is the separation of gluconeogenesis and glycolysis in one single cell, both processes occurring only one at a time. For oscillating processes such time separation might simply be achieved by alternating activation and/or inhibition of two processes during one period of the oscillation. Furthermore, a basis for circadian rhythms that are abundantly observed in living systems of higher order must still be found (Hastings & Schweiger, 1975). Certain components of the glycolytic pathway may be important in generating the rhythmical electrical activity seen in insulin-secreting β-cells (see Matthews & O'Connor, this volume) and in neuronal burster cells (see Chaplain, this volume).

With respect to glycolysis it should be remembered that glycolytic oscillations have been recorded in a single yeast cell and not only in large populations of yeast cells. Furthermore, it should be recognized that the change in concentration of the adenine-nucleotides during one period is large and well in the molar range to which many enzymes of metabolism respond. The observation of synchronization is relevant with respect to coordination of metabolic pathways. The oscillations are a predictable consequence of control mechanisms that involve gain and feedback. Since the oscillatory domain of glycolysis is well within the physiological range we might expect that this type of dynamic state is more general than presently expected.

Glycolysis in itself is an interesting example because it demonstrates that a primary function of phosphofructokinase is to generate an oscillation in its product that induces oscillations in other enzymes along the glycolytic pathway. Under many conditions the enzyme pyruvate kinase might simply follow passively the oscillating pace set by the enzyme phosphofructokinase, allowing perfect synchronization of the whole process. This type of synchronization is well established in the case of the slime mould discussed at this meeting.

Furthermore I would like to add that there is also an efficiency aspect which might be of significance. It has been suggested and shown that a positive free enthalpy change occurs for an oscillating reaction compared with the negative free enthalpy change of the same reaction proceeding under non-oscillatory conditions with the same average values of the chemical potential of reactants and products (Durup, 1979). This is of special interest and contrasts with the requirement of negative free enthalpy change for biochemical control and regulation (Hess, 1975). Thus, it might be significant and energetically advantageous for a system to run within the oscillatory domain.

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