# SUSTAINED SINUSOIDAL OSCILLATIONS OF REDUCED PYRIDINE NUCLEOTIDE IN A CELL-FREE EXTRACT OF *SACCHAROMYCES CARLSBERGENSIS**

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*Communicated February 4, 1966*

It has been postulated that even in complex organisms the basic control for the timing mechanisms of biological clocks must be at the cellular level.[1,2] The simplest type of mechanism would appear to be a periodic process occurring at the level of enzymic reactions. Within recent years many workers have observed a cyclic or damped oscillatory behavior of certain metabolic parameters in various biological systems[3-8] which indicates that periodic processes do occur at this level.

In particular, the oscillations in the level of reduced pyridine nucleotide observed in yeast[9-12] have been extensively investigated. Metabolite studies carried out on the whole cell[9] and on the cell-free extract,[16] together with related analogue computer studies,[13] have indicated that a prime site of the oscillations is the enzyme phosphofructokinase. It has already been suggested that such oscillations may well be the basis for biological clocks, since waveforms, period, amplitude, and phase relationships are under control.[14] However, a major objection to this hypothesis is that all metabolic oscillations reported so far have had damping factors greater than 1.0 and have been observable for not more than about 12 cycles after the transition which created them. A vital characteristic of a master oscillator would be that it is capable of essentially continuous oscillations. We are now able to demonstrate that a biological system, a cell-free extract of *Saccharomyces carlsbergensis*, is in fact capable of sustained oscillations, an essential characteristic of a timing mechanism for biological clocks.

*Experimental Methods.*—A strain of *Saccharomyces carlsbergensis* (ATCC 9080) was maintained and grown on a medium essentially similar to that described by Ghosh *et al.*[21] The yeast was harvested in the very early stationary phase after growth for approximately 21 hr with continuous aeration at 32°C. The cells were prepared, disrupted using high-intensity ultrasound, and the extract was produced in a similar manner to that previously described.[12] The extract, which normally contains 80–120 mg protein per ml, can be stored at −20°C for at least 2 months without noticeable loss of activity, but progressively loses its ability to show spontaneous oscillations if maintained at 0°C. This latter fact can be explained by the observation that such extracts will display oscillations even at 0°C, the period at this temperature being approximately 90 min.[14]

In these studies the level of DPNH in the undiluted extract was monitored continuously in a double-beam spectrophotometer employing interference filters and fitted with a 1-mm optical path cuvette. The wavelengths used were 345 and 400 mμ. The percentage absorbancy was recorded using an Esterline Angus Recti Graph recorder. Previous studies[15,16] on similar extracts have indicated that the changes observed in this manner represent the changes in DPNH concentration and have also shown that oscillations in the concentration of other glycolytic inter-

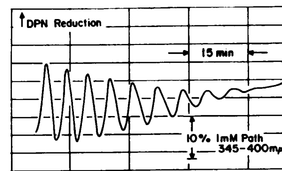


FIG. 1.—Typical oscillatory response of DPNH observed when the yeast extract is allowed to warm rapidly from 0°C to room temperature in the cuvette. Time proceeds from left to right. Absorbancy and time scales are indicated on the figure. At the midpoint of the train, the damping factor is 1.09 and the $Q$ value is 16. Trace obtained using filter double-beam spectrophotometer. Optical path 1 mm. Temperature 25 ± 1.5°C.

mediates occur simultaneously but not necessarily in phase with the oscillations in DPNH.

Glycolytic intermediates in deproteinized samples of the extract were measured fluorometrically using specific enzymic assays.[17]

Trehalose and glycogen were estimated as described by Wyatt and Kalf.[18]

In describing the oscillations we use the term "damping factor," which refers to the ratio of the amplitude in one direction to the amplitude of the next deflection in the opposite direction. $Q$ is defined as $\pi$ times the number of cycles required for the amplitude to fall to $1/e$ of its initial value,[24] where $e = 2.7$.

*Experimental Results.*—Figure 1 shows the typical oscillatory response of DPNH observed after the cell-free extract is allowed to warm rapidly from 0°C to room temperature in the cuvette. A train of nine sinusoidal oscillations was initiated which at the midpoint had a damping factor of 1.09 and had a $Q$ value of 16. The damping factor increased from 1.07 for the first full cycle to 1.4 for the seventh cycle, indicating that the amplitude modulation was not exponential. Analysis of samples of the extract taken during and after such an oscillatory region showed that a sharp decrease in the concentration of both glucose-6-phosphate and ATP occurred at the time the oscillations ceased. This result could be interpreted in terms of a depletion of the substrate for glycolysis, with the possibility that the oscillations could be maintained if additional substrate were provided.

*Oscillations maintained by trehalose:* It was observed that when trehalose, a disaccharide known to accumulate in large quantities in yeast,[19] was added at a point just before the oscillations stopped, a long train of sinusoidal oscillations was produced. Figure 2 shows a typical case in which addition of 0.14 $M$ trehalose to the extract produced a train of 42 oscillations which, over the central region, exhibited an average damping factor of 1.01, a $Q$ value of 100, and showed negligible variation in frequency. A further addition of trehalose, after these oscillations had subsided, produced another train of oscillations.

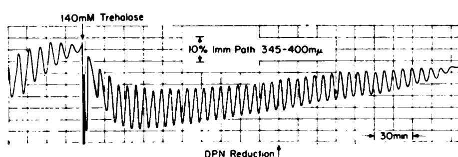


FIG. 2.—Sustained oscillations produced on the addition of trehalose to a yeast extract just before the spontaneous oscillations ceased. Over the central region of the train, the average damping factor is 1.01 and the average $Q$ value is 100. Other conditions are the same as in Fig. 1.

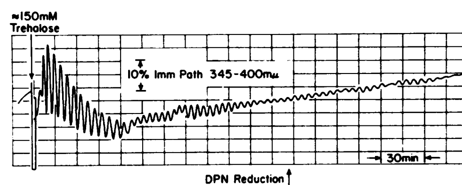


FIG. 3.—An example of the type of sustained oscillations occasionally observed on addition of trehalose to certain preparations of the yeast extract. The amplitude modulation which occurs during the latter portion of the trace may possibly be signs of a beat frequency. Conditions are the same as in Fig. 1.

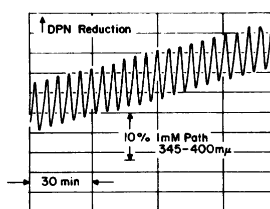


FIG. 4.—A region of a train of oscillations produced on addition of trehalose to the yeast extract. This region is essentially undamped, displaying an average damping factor at 1.0 and a $Q$ value $\gg$ 100. Conditions are the same as in Fig. 1.

In recent experiments we have obtained a continuous train of over 90 cycles with a single addition of trehalose and well over 100 cycles with multiple additions of trehalose. These particular oscillations displayed highly variable damping factors and $Q$ values, and had a period of approximately 7 min at 25°C. These experiments show that the enzyme system is stable for over 11 hr at this temperature.

Not all preparations would oscillate in exactly the same manner as that shown in Figure 2, although all extracts yielded sustained oscillations on addition of trehalose. Figure 3 shows such a preparation in which trehalose addition produced large-amplitude sinusoidal oscillations which became rapidly damped to give a train of small-amplitude oscillations showing distinct signs of a "beat frequency." In this instance the period was approximately 4.5 min and more than 60 oscillations were produced after the addition of trehalose. It is assumed that the differences observed in the wave patterns from one extract to another are due to slight differences in the constitution of each extract.

*Undamped oscillations:* Although Figures 2 and 3 exhibit some degree of damping, certain extracts would yield a region of essentially undamped oscillations. Figure 4 shows a case in which trehalose addition yielded a series of oscillations displaying an average damping factor of 1.00 and a $Q$ value $\gg$ 100.

*Amplitude modulation:* During the course of this work, amplitude modulation and double periodicity were observed on many occasions. Figure 5 shows different cases of amplitude modulation, while Figure 6 displays an example of double periodicity. Three separate cycles of the waveform displayed in Figure 6 were submitted to a 12-point Fourier analysis for which the fundamental period was regarded as the time between two large-amplitude waves. Figure 7 shows the magnitude plot which indicates that the magnitude of the second harmonic is considerably greater than that of the fundamental frequency and the third and fourth harmonics. The phase angle plot derived from this analysis is also given in Figure 7.

In order to observe amplitude modulation over a longer period, the normalized amplitude of each peak was plotted against time (Fig. 8). The broken line $A$ shows the relationship between the amplitude of successive peaks and clearly indicates the double periodic nature of the original waveform (Fig. 6). The upper line $B$ shows

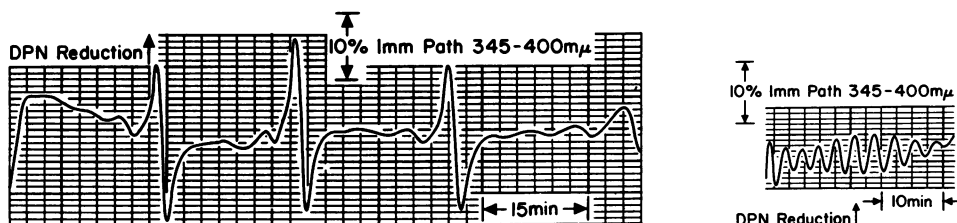


FIG. 5.—Different types of amplitude modulation observed during this study. The particular examples shown were observed in yeast extracts not supplemented with trehalose. Both examples appear to provide evidence for a beat frequency phenomenon. Conditions are the same as in Fig. 1.

the envelope of the large-amplitude waves, while the lower line $C$ represents the envelope of the small-amplitude waves, both of which show modulations which might well be interpreted as additional oscillations or beat frequencies.

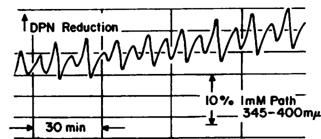


FIG. 6.—An example of a region of oscillations showing double periodicity. In this case the yeast extract was supplemented with approximately 70 mM trehalose. Conditions are the same as in Fig. 1.

*Waves of increasing amplitude:* Some proposed mechanisms for the oscillations can only apply to trains of damped oscillations. It is therefore of particular importance to the theory of the mechanism of the oscillations that wave trains displaying increasing amplitude have now been observed. Such a case is shown in Figure 9 in which the amplitude of the oscillations increases and appears to approach a limit cycle. The limit-cycle phenomenon is predicted on theoretical grounds.[22]

*Effect of other substrates for glycolysis:* In order to confirm that trehalose is the substrate for the glycolytic oscillations, samples were taken from a spontaneously oscillating extract and assayed for trehalose and glycogen. The trehalose concentration decreased in an apparently monotonic fashion from 26 mM at the start of the oscillations and reached a negligible level at the point when the oscillations ceased, 90 min later. This rate is consistent with the known rate of ethanol production in these extracts. During the same period the glycogen concentration, which was low compared to the initial trehalose concentration, decreased only fractionally from 16- to 13-mM glucose equivalents. It was not surprising, therefore, to find that glycogen addition did not support sustained oscillations but gave rise to a few highly damped oscillations. The addition of a large concentration (0.1 $M$) of glucose would not support sustained oscillations either, but simply yielded one large cycle and then a slow monotonic rise in the DPNH level.

*Discussion.*—The demonstration that an enzymic system is capable of producing sustained oscillations of metabolic intermediates fortifies the hypothesis that such

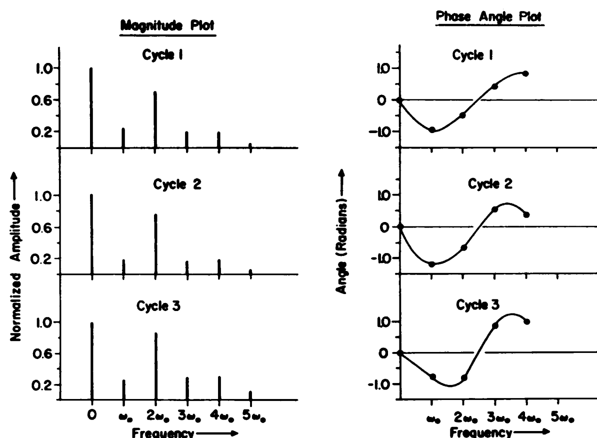


FIG. 7.—The magnitude and phase angle plots derived from a 12-point Fourier analysis of three separate cycles of the waveform shown in Fig. 6. For this analysis the fundamental period was regarded as the time between two large-amplitude waves. (Thanks are due to J. Meidel for help with this analysis.)

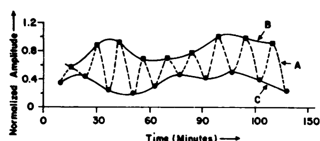


FIG. 8.—The normalized amplitude of each peak of the waveform shown in Fig. 6 is plotted at its respective time. The broken line *A* shows by its oscillation the double periodic nature of the original waveform. The lines *B* and *C* represent the envelopes of the large- and small-amplitude wave, respectively, both of which show distinct modulation.

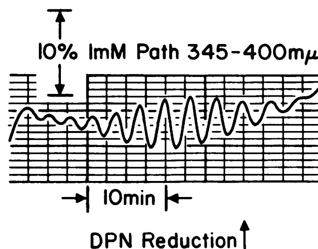


FIG. 9.—Oscillations showing a region of increasing amplitude and an approach to a limit cycle. This wave pattern was observed in an extract not supplemented with trehalose. Conditions are as in Fig. 1.

oscillations can be the basic regulator of biological clocks. Of almost equal importance is the apparent observation of "beats" (Figs. 3 and 5), since these could provide various frequencies for the regulation of functions controlled by such clocks. The finding that the oscillations in the cell-free extract are maintained by an *in vivo* endogenous substrate, a finding which also applies to the beef heart extract,[25] makes it tempting to suggest that sustained oscillations are also possible *in vivo*, especially in view of the previously reported close correlation between the oscillations in yeast cells and in the cell-free extract.[12] In fact, it is more probable that truly continuous oscillations will occur in whole cells in view of their homeostatic abilities.

*Mechanism of the oscillations:* Studies on the mechanism of the damped sinusoidal oscillations of DPNH in yeast cells,[9] cell-free extracts of yeast[16] and beef heart[7] have shown that oscillations of the glycolytic intermediates occur together with, but not necessarily in phase with, the oscillations in DPNH. They have also implicated PFK as the primary site of the oscillations. Computer studies[13] have offered several possible explanations based on the known activation of PFK by either FDP or ADP or both, in conjunction with the associated metabolite F6P. The studies of Chance *et al.*[12] have already shown that ADP exerts control during certain phases of the DPNH cycle. The apparent beat frequencies observed in this study would point to control by more than one mechanism. It may be that control is exerted at a single site by a number of metabolites or at different sites by different metabolites. Both possibilities exist, since it is known that PFK activity can be regulated by many substances including FDP, ADP, ATP, and AMP.[20] At the same time, ADP can interact with glycolysis at the 3-PGK and PK sites, as well as at PFK, and may therefore control glycolysis at any of these sites.

Certain mechanisms for the oscillations can be ruled out now that sustained oscillations and beat frequencies have been observed. Almost all metabolic oscillations reported so far have been trains of damped oscillations induced by a metabolic transition. One explanation could be that they represent an oscillatory movement from one metabolic steady state to another. Such an explanation must now be discarded in the case of the yeast extract, and it might be anticipated, in view of the apparently similar mechanisms involved, that this explanation will not apply to the yeast cell either. It should be pointed out that the observations of sustained oscil-

lations and oscillations of increasing amplitude are entirely consistent with the type of reaction mechanisms proposed by Higgins.[23]

The ability of trehalose to maintain the oscillations in the yeast extract, a fact recently confirmed by other workers (B. Hess, personal communication), provides a system more amenable to study than the damped sinusoidal oscillations observed in the nonsupplemented yeast extract. Long-term effects of the addition of various metabolites can now be observed.

At the present time we feel that trehalose sustains the oscillations in the yeast extract by providing, through the mediation of the specific hydrolytic enzyme trehalase, a regulated and continuous supply of glucose to glycolysis, thus allowing the system to operate in a steady state. Preliminary evidence in favor of this hypothesis is that oscillations can be induced in an extract by the addition of maltose and a regulated amount of maltase, a system which also provides a continuous supply of glucose. This theory would predict that glycogen could also maintain the oscillations if phosphorylase activity were of the correct order and if sufficient ATPase were added to the extract to compensate for the loss of the ADP-yielding hexokinase step. With trehalose as substrate, the metabolism of one hexose unit by the Embden-Meyerhof-Parnas pathway would yield a net gain of two ATP molecules as opposed to a net gain of three ATP molecules for the metabolism of one hexose unit from glycogen. Provided the glycolytic flux is the same in both cases, more ATPase would be required to maintain steady-state conditions during glycogen metabolism than during trehalose metabolism.

We consider that the slight variability observed between different extracts is mainly due to differences in the activity of ATPase and trehalase, both enzymes being predominantly particle-bound and therefore subject to greater variability during the preparation of the extract.

*Summary.*—A train of over 90 sinusoidal oscillations of the DPNH level has been observed in a cell-free extract of *S. carlsbergensis* supplemented with a single addition of trehalose. Such sustained oscillations frequently have an average damping factor of approximately 1.01 and an average $Q$ value in excess of 100. Trehalose is also the primary substrate for the spontaneous oscillations in the nonsupplemented extract. The observation of doubly periodic oscillations and oscillations showing beat frequencies indicates that glycolytic control can be exerted by at least two mechanisms in this system. These observations rule out certain mechanisms for the oscillations and suggest that sustained oscillations may exist *in vivo* where they could function as the basic regulators of biological clocks.

The authors would like to thank Barbara Bonte for her technical assistance.

* A preliminary report of this work was presented at the meeting of the American Chemical Society, Atlantic City, New Jersey, September 1965. This work was supported by U. S. Public Health Service grant 12202.

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# *OPTICAL ROTATORY DISPERSION OF A HEME PEPTIDE FROM CYTOCHROME C**

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*Communicated by Herman M. Kalckar, February 23, 1966*

The optical rotatory properties of horse heart cytochrome *c* have been reported recently,[1,2] and have suggested new approaches for relating the structure of this molecule to its function.[3–6] Since the prosthetic group of cytochrome *c* is covalently bonded to the protein through cysteinyl side chains, heme peptides consisting of only about 10 per cent of the total residues present in the parent molecule may be obtained by means of proteolytic digestion.[7] It seemed of interest to examine the spectropolarimetric characteristics of such a peptide in terms of its possible structure and as a guide for the interpretation of the origin of the optically active transitions observed in the intact cytochrome. The observations here reported suggest that the limited segment of the peptide chain to which the heme is bonded largely determines the magnitude and general form of the extrinsic Cotton effects in the native protein.

*Methods and Materials.*—Horse heart cytochrome *c*, Type III, was obtained commercially (Sigma Chemical Co.), as was twice-crystallized pepsin (Worthington Biochemical Co.). Heme peptide *c* was prepared by peptic digestion,[8] and its composition determined by amino acid analysis.[9] Oxidation and reduction of the peptide were performed as described previously for cytochrome *c*.[1] Optical rotatory