DAMPED SINUSOIDAL OSCILLATIONS OF CYTOPLASMIC REDUCED PYRIDINE NUCLEOTIDE IN YEAST CELLS*

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The most obvious periodicities of biological systems are the "clock phenomena."[1] An example of periodic fluctuations at the biochemical level is provided by the luminescence of Gonyaulax.[2] In this case, at least the intracellular localization of the biological system involved is known,[3] but the nature of the oscillator driving such clocks is unknown at either the physiological or the biochemical level.

While the transition from aerobiosis to anaerobiosis causes changes of cytochrome oxidation-reduction that are monotonic,[4] the concentration of intracellular reduced pyridine nucleotide (RPN) in yeast was observed to fluctuate widely in a cyclic fashion on the first occasion that direct optical method was applied to intact cell suspensions.[4, 5] Various explanations for these phenomena were considered, involving the possibility of binding of the reduced[4] or the oxidized[6, 7] form of pyridine nucleotide, particularly to glyceraldehyde-3-phosphate dehydrogenase. In 1957, Duysens and Amesz,[8] using fluorescence detection of reduced pyridine in yeast cell suspensions, observed a related and more extensive fluctuation in the fluorescence intensity. Estabrook *et al.*,[9] also employing the fluorometric method, observed cyclic fluctuations of cytoplasmic reduced pyridine nucleotide in suspensions of *E. coli.* In experiments with an inositol-requiring strain of *Saccharomyces carlsbergensis* (ATCC-4228), the generation of damped sinusoidal wavetrain of over 12 full cycles of oscillations was observed in 1963.[9, 10] These results were confirmed[10] through the use of the direct optical method; the oscillation was found to involve changes in the oxidation-reduction state of RPN rather than changes of its binding.

Metabolite assays[11] of the cell suspension of the oscillation indicated fluctuation of the glycolytic intermediates showing a crossover point at phosphofructokinase. The assays further show the fluctuations in fructose diphosphate concentration to be out of phase with glucose-6-phosphate and fructose-6-phosphate, leading to a simplified mechanism involving phosphofructokinase as the oscillatory element in the enzymatic sequence.[12] In 1955, Wilson and Calvin[12a] found brief oscillations of diphosphoglycerate and ribulose diphosphate upon illumination of *Scenedesmus.*

An example of such oscillation is provided by one of the first recordings of reduced pyridine nucleotide kinetics in yeast cells (Fig. 1) by the double beam spectrophotometer.[4, 5] 335 mμ and 350 mμ were employed as measuring and reference wavelengths, respectively, in view of the slight shift of the reduced pyridine nucleotide band of yeast cells to shorter wavelengths. Addition of 33 mM sucrose (which is rapidly converted to glucose in the yeast cells by invertase) causes the reduced pyridine nucleotide concentration to rise abruptly to a peak in 10 sec as indicated by the downward deflection of the trace. An approximately full cycle of oscillation ensues thereafter in which a second peak is reached in the course of slightly over a minute. Inhibitor studies identified the oscillation with the glycolytic rather than the oxidative enzymes and with glyceraldehyde-3-phosphate dehydrogenase. This cyclic response has been observed in ascites tumor cells[13] and more recently in

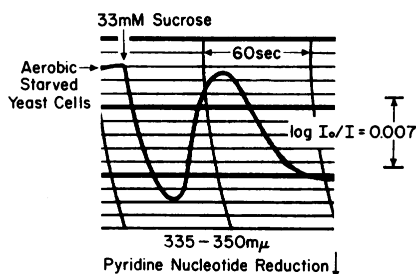

FIG. 1.—A cyclic response of DPNH caused by the addition of sucrose to a suspension of baker's yeast cells. Baker's yeast cells starved by 12 hr bubbling with air at a concentration of 30%, diluted to a concentration of 4% in an air-saturated 0.1 *M* phosphate buffer, pH 7.15, at a temperature of 25°. Spectroscopic observations made with the measuring wavelength of 335 mμ and the reference wavelength of 350 mμ in a 1-cm optical path. 33 mM sucrose added at the point marked by the arrow. The period of the oscillatory response of DPNH is approximately 1 min at 25° (expt. 864 D, series I).

perfused rat heart as well,[10] where it is also associated with the glycolytic system. These data, therefore, confirm and extend the early observations that there exists an instability in metabolic control phenomena involving glyceraldehyde-3-phosphate-dehydrogenase in the cytoplasm of baker's yeast cells. The present results indicate that this instability can lead to a damped train of sinusoidal oscillations. (Information on the persistent fluctuations in *S. carlsbergensis* was communicated to F. Hommes, who was able to confirm our results on the fluctuations of fluorescence intensity and of the concentrations of glycolytic intermediates—F. Hommes, personal communication.) This paper describes the appropriate transient for starting the oscillations, the nature of the waveform, and the effect of temperature upon it. Appropriate parameters for quantitation of the sinusoidal waveform are presented.

Experimental Methods.—An inositol-requiring strain of *Saccharomyces carlsbergensis* (ATCC-4228) has been grown in an artificial medium containing glucose, citrate, inositol, vitamin mixture, casein hydrolysate, and salt mixture.[14] Since the condition of the cells is important with regard to the character and duration of the oscillatory response, the conditions appropriate to Figure 2 are described in detail. The cells are grown for 13 hr at 30°, giving a packed cell volume of 5.5 ml. These cells were washed 3 times in 30 ml 0.1 *M* KH_2PO_4 and resuspended at a concentration of 30 gm wet weight/100 ml solvent. The cells were aerated for 2 hr, centrifuged and again resuspended at 30 gm wet weight/100 ml solvent concentration, and were then aerated vigorously for $1^1/_2$ hr in the case of Figure 2. The cells were diluted 5 times in 0.1 *M* KH_2PO_4. The cell count for 1 gm wet weight/100 ml is 1.8×10^8 cells/ml. Pyridine nucleotide reduction is observed with either the double beam spectrophotometer[15] or the "Eppendorf" fluorometer. Metabolism is initiated by the addition of glucose to a final concentration of 3.3 mM. Thereafter, an interval of rapid respiration ensues, and the dissolved oxygen is expended in approximately $1^1/_2$ min, giving rise to the metabolic transient that sets the glycolytic system into a damped train of oscillations.

The yeast cells are found to have the usual cytochrome components a_3, a, b, c_1, and c, and in addition, a considerable amount of pigment absorbing at 557 mμ (observed at 77°K). Examination of the cytochromes shows that the respiratory carriers remain reduced during the oscillations of the cytoplasmic reduced pyridine nucleotide.

The cells have also been grown in an inositol-deficient medium, and under these conditions, the oscillations have not yet been found.

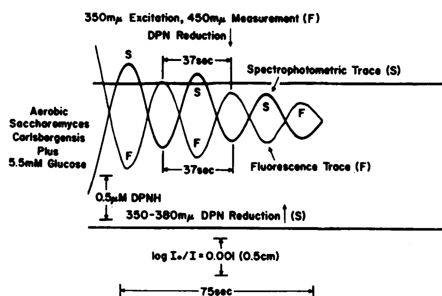

FIG. 2.—Validation of the fluorometric method for measuring the DPNH oscillation. Combined spectrophotometric (*S*) (350–380 mμ) and fluorometric recording (*F*) with 350 mμ excitation and 450 mμ measurement of yeast at 25° (expt. 100–III).

Results.—Simultaneous spectrophotometric and fluorometric recording of the oscillation: Since our early results suggested that a bound form of pyridine nucleotide was involved in the oscillatory intracellular fluctuations[4, 5] and since the intensity of fluorescence of DPNH depends not only upon the concentration of DPNH but also upon its degree of binding to proteins,[16] we have recorded the oscillations of reduced pyridine nucleotide by absorption and fluorescence simultaneously. The double beam spectrophotometer is readily adapted for this purpose.[17] The measuring beam is set at 350 mμ, a wavelength at which binding changes would not alter the absorption, and the reference wavelength is set at 380 mμ. A guard filter protects the photomultiplier from fluorescence emission at 450 mμ. A second photomultiplier records the intensity of the 350-mμ light through a filter that absorbs the fluorescence emission at 450 mμ. In the recording of Figure 2, the spectrophotometric and fluorometric records are plotted 180° out of phase so that the traces can be more readily compared. It is apparent from this diagram that the amplitude and phase of the spectrophotometric and fluorometric records are proportional throughout the recorded period of the oscillation period. This record then validates the method of fluorometric recording by indicating that whatever the state of binding of reduced pyridine nucleotide may be in the cytoplasm of the yeast cell, it does not change sufficiently to cause an error of fluorescence recording of the kinetics of the oscillation. Since the fluorescence recording is more convenient, it will be employed in the majority of the traces which follow. The optical recording allows a precise determination of the concentration of DPNH involved in the oscillation. The extinction coefficient of free DPNH at 350–380 mμ is 2.4 $[(0.5 \text{ cm})^{-1} \times \text{mM}^{-1}]$ and indicates that roughly 1 μM DPNH is involved in the oscillation. The yeast cell concentration is 6 gm wet weight/100 ml solvent, corresponding to 16 mμmoles per gram of yeast cells.

Figure 3 illustrates in three traces variations of experimental conditions leading to various modes of oscillation. The experimental procedure is also illustrated by these recordings. The diluted cells are observed in this case by the Eppendorf fluorometer. Increased fluorescence is indicated by an upward deflection. Addi-

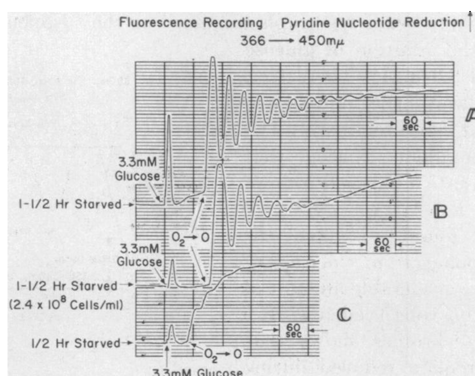

FIG. 3.—Forms of RPN oscillation in *S. carlsbergensis* recorded by the fluorescence method. (*A*) A recording of the oscillations initiated by the aerobic-anaerobic transition (oxygen → 0) under optimal conditions (7/2/63). (*B*) Oscillations in a yeast cell suspension aerated for 1$^1/_2$ hr (7/2/63). (*C*) Oscillations aerated for $^1/_2$ hr pror to the addition of glucose; 2% yeast 25° (7/2/63).

tion of glucose, marked by the arrow, causes the usual cycle of DPN reduction followed by reoxidation. A steady state of rapid respiration occurs, which lasts for approximately a minute. Thereafter, the abrupt upward deflection of the trace indicates pyridine nucleotide reduction in the aerobic-anaerobic transition, and the reaction affords a trigger for a damped train of oscillations which are superimposed upon a mean level of increasing fluorescence in Figure 3*A*. The period of oscillations is close to 30 sec initially and has increased to about 45 sec over the period of 7 min.

The starvation of the yeast prior to the addition of glucose has a large effect upon the oscillations. Figure 3*B* represents an experiment on another culture of yeast which has been aerated for $1^1/_2$ hr. This result may be compared with Figure 3*C*, which represents the oscillation amplitude of yeast which had been aerated for only $^1/_2$ hr. It is seen in the latter case that a small oscillation amplitude is superimposed upon the exponential reduction of DPN at the moment that oxygen falls to zero. Since the time interval between addition of glucose and anaerobiosis of the suspension in Figure 3*C* is about half that in Figure 3*B*, an appropriate buildup of the glycolytic intermediates is necessary to start oscillations optimally. An over-reduction of cytoplasmic pyridine nucleotide apparently inhibits the reaction. Also, the steady state appropriate to the oscillation lasts only for a short time.

Characteristics of oscillations: The waveform of the oscillations as indicated in Figures 2 and 3 closely approximates a damped sinusoid of the form derived for the "pulsed oscillator." Where E_0 is the portion of the initial voltage E across the inductance L in series with resistance R, ω is the frequency of the oscillations, t is time, and Q is $r\,C\,\omega$, where r is the resistance and C is the capacitance of L.[18]

$$E_0 = \frac{E}{R} L e^{-\omega t/2Q} \sin \omega t. \qquad (1)$$

TABLE 1

Summary of Properties of Oscillations of Reduced Pyridine Nucleotides in Yeast

Material	Exptl. no.	Yeast conc. (% wet weight)	Aeration interval (hr)	T (°C)	Transition	Time after glucose (min)	Freq. (min⁻¹) ω	Damping factor	Q
S. cervisiae	12/15/51 (864d-I)	4	12	25	33 mM sucrose	—	1.7	1.2	—
Baker's yeast	Duysens and Amesz[8]	2	24	(Room)	O_2–N_2	—	0.9	1.4	1.6
*S. carlsbergensis**	7/10/63	2.2	1.5	25	O_2–N_2	1.4	1.8	1.16	4.3
S. carlsbergensis	7/17/63	3	2	25	O_2–N_2	1.0	1.6	1.15	5.1
S. carlsbergensis	7/17/63	3	2	35	O_2–N_2	0.3	4.0	1.15	5.4
S. carlsbergensis	7/2/63	2	2	25	O_2–N_2	1.5	1.9	1.09	7.4
S. carlsbergensis	7/31/63	3	2	25	O_2–N_2	1.5	1.6	1.59	2.1
S. carlsbergensis	7/31/63	3	2	25	Na_2S	1.0	1.6	1.35	4.1
S. carlsbergensis	7/31/63	3	2	25	Na_2S	1.5	1.6	1.12	8.0
S. carlsbergensis	7/31/63	3	2	25	Glucose	0	—	3.4	—

* ATCC-4228.

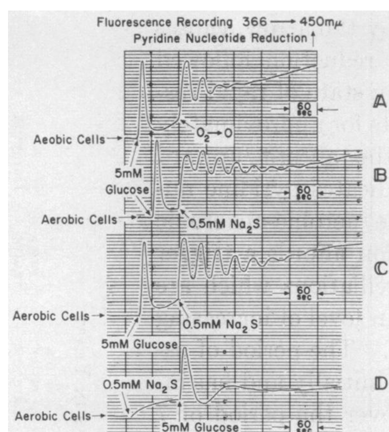

FIG. 4.—Dependence of the nature of oscillations upon the period after glucose addition. (*A*) Oscillations started by anaerobiosis marked by the point oxygen → 0. (*B*) Oscillations initiated by the addition of sulfide 1 min after addition of glucose. (*C*) Oscillations initiated by addition of sulfide $1^1/_2$ min after addition of glucose. (*D*) Oscillations initiated by adding glucose after sulfide; 3% yeast 25° (7/31/63).

Such oscillators generate a damped sinusoidal oscillation in which the rate of damping is such that the amplitude is reduced to $1/e$ of its initial value in Q/π cycles of oscillations. The frequency is in units of $\min^{-1}$. A summary of values of Q and ω for various conditions is provided in Table 1. Included in this table is also the value for the damping factor which is defined as the ratio of the amplitude of oscillation in one direction to the amplitude of the next oscillation in the opposite direction.[19] While this evaluation of the oscillatory reaction involves engineering terminology, it is perhaps the most concise and effective way of comparing the results obtained in one laboratory with those in another and is to be preferred to counting the number of oscillations. In Table 1, the initial experiment with the double beam spectrophotometer gives a frequency and a damping factor which is in reasonable agreement with those observed later with *S. carlsbergensis*. The experiment of Duysens and Amesz shows a lower frequency and a rather high damping factor together with a low Q. The experiments on *S. carlsbergensis* show an average frequency of 1.7 $\min^{-1}$ at 25°, which appears to be highly consistent. Under optimal conditions the damping factor falls to 1.1 and the Q rises to 8.

Initiation of oscillation: In the records shown so far, the oscillation is initiated by the transition from aerobiosis to anaerobiosis; the addition of glucose under aerobic conditions gives only the one cycle of reduction and oxidation of DPNH as is indicated in Figure 1. The fact that the oscillations occur in the aerobic-anaerobic transition suggests that the mitochondrial control of the intracellular ADP level[9,13] prevents the glycolytic system from oscillating under aerobic conditions. Figure 4 illustrates conditions appropriate for initiating the oscillation. Figure 4*A* is a control study, and it is seen that the oscillation is highly damped, as is often the case with yeast that is inadequately starved prior to glucose addition. In trace *B*, the oscillation is started not by anaerobiosis but by blocking of the cellular respiration by the addition of sodium sulfide, 1 min after the addition of glucose to the aerobic cells. Under these conditions the decrement of the oscillation is much less, and the number of cycles is greatly increased (from 4 to approximately 8). If the metab-

olism of glucose is allowed to proceed for $1^1/_2$ min aerobically before the addition of sulfide, then the same number of cycles of oscillation is obtained, but they are of larger amplitude and of somewhat greater decrement. Lastly, in trace *C*, the cells are pretreated with sulfide so that respiration is not possible and glucose is added approximately 2 min thereafter. Here the oscillation consists only of the cycle of reduction and oxidation with an even smaller tendency to oscillate than is illustrated by Figure 3*A*. It is apparent that steady-state concentration of metabolites appropriate to the oscillation must be established by the metabolism of glucose and that the system must be shocked into oscillation by the inhibition of oxidative phosphorylation and the activation of glycolysis (the Pasteur reaction).

In order to shed some light on the type of reaction that is controlling the oscillation, we have investigated the effect of temperature. In this type of experiment, it is desirable to employ absorbancy measurements for DPNH concentrations since the efficiency of fluorescence emission will be temperature-dependent and will not accurately portray the concentration changes. The top trace of Figure 5 is a control experiment at 26° and shows a 37-sec period as in Figure 2. Below this trace is the recording obtained at 35°C. At 25° the frequency is 1.6/min, while at 35°, it is 4.0/min, a ratio of 2.5 for the 10° interval. Interestingly enough, the damping factor and Q values are almost independent of the temperature variation, suggesting that these parameters may be most useful in comparing oscillations of different cells under different conditions.

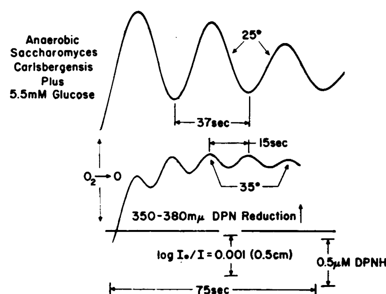

FIG. 5.—Effect of temperature upon oscillation amplitude and frequency. Spectrophotometric measurements at 350–380 mμ. Oscillation started with the aerobic-anaerobic transition. 6% yeast. Top trace 25°. Bottom trace 35° (expt. 100–IV).

Discussion.—The experimental data presented here greatly extend the original observations[4, 7] on fluctuation of RPN in cell suspensions. It is now apparent from these data that a fully developed damped sinusoidal oscillation can be executed by the reduced pyridine nucleotide component of these cells. Relatively low damping factors (1.1) and relatively high Q values (8) which appear to be relatively independent of temperature can be achieved. Rudimentary waveform analysis of the oscillator indicates a sinusoidal output. The period of the oscillation (1.7 min^{-1} at 25°) is sensitive to temperature in a manner to be expected of a chemical reaction mechanism. The metabolic state appropriate for the initiation of oscillations is apparently well defined with respect to the amount of glucose that must be metabolized to fill metabolic pools of glycolysis before an oscillation of appreciably small damping factor can be obtained.

The positive identification of this oscillation with the enzymatic reactions is now afforded by the recording of the damped sinusoidal waveform in a cell-free extract of the yeast.[20]

Relationship to biological clocks: Obviously the properties of the observed oscillations do not completely fulfill the requirement for a biological clock. While the oscillations are sinusoidal and of a relatively constant frequency (at a fixed temperature), the damping factor of the oscillations renders them unsuitable for timing over any extended interval. It is to be considered, however, that the damp-

ing factor of the oscillation is probably an indication of the departure from the steady-state conditions appropriate to optimal oscillation amplitude. It is very probable that the damping factor would be very small for this reaction in a homeostatic system such as a tissue. For example, the stirred flow reactor of Denbigh and Page[21] would provide a method for holding the average values of the intracellular metabolites constant. Thus, we see no fundamental objection to the reaction mechanism insofar as its damping factor is concerned.

The fact that the chemical system responds to a metabolic transient such as the aerobic-anaerobic transition or the addition of cyanide or sulfide identifies it as an oscillator of the "pulsed" or "synchronized" type. This phenomenon is not without parallel in the study of biological clocks where setting and resetting phenomena can be readily observed.[22]

The possibility of generating lower frequencies than the 1.7 min^{-1} value observed here has already been pointed out[23] and can be readily demonstrated with analogue computer solutions of the reaction mechanism.[12] It is apparent that the yeast oscillator is not compensated for temperature variation. However, the possibility of accomplishing this by product inhibition in enzyme reactions has already been pointed out.[22]

Summary.—The rapid reduction and reoxidation of cytoplasmic pyridine nucleotide observed upon addition of glucose or in the aerobic-anaerobic transition of baker's yeast studied over the past dozen years now appears to be the initial phase of a damped train of sinusoidal oscillations. Provided appropriate levels of glycolytic intermediates are present, the oscillations may be started when the mitochondrial control of the ADP and phosphate levels ceases due to inhibition of respiration. The oscillations are evaluated quantitatively on the basis of engineering criteria for a pulsed-sinusoidal oscillator, and show damping factors as low as 1.1 and Q values as high as 8. The oscillation frequency is 1.7 min^{-1} at 26° and 4.0 min^{-1} at 35°. The oscillations which were observed as fluctuations in the DPNH concentration in the cytoplasm are a consequence of variations of the glyceraldehyde-3-phosphate dehydrogenase activity in conjunction with other dehydrogenases of the cytoplasm. A possibility of generating continuous oscillations in this system and their relevance to biological clocks are discussed.

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*BIOCHEMICAL STUDIES ON ADENOVIRUS MULTIPLICATION, VI. PROPERTIES OF HIGHLY PURIFIED TUMORIGENIC HUMAN ADENOVIRUSES AND THEIR DNA's**

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Preparations containing adenovirus types 12[1, 2] and 18[2] have been found to induce tumors in newborn hamsters. We have previously studied the biochemical events associated with the replication of adenovirus type 2 in cell culture[3–8] and several of the chemical and physical properties of adenovirus type 2. In this paper, we present a comparison of some of the biological, chemical, and physical properties of highly purified tumorigenic adenovirus types 12 and 18 and their DNA's with those of nontumorigenic[9] adenovirus types 2 and 4. Preliminary reports of some of this work have appeared.[10–12]

Experimental Methods.—Cells, media, and virus: See Green and Piña for methodological details.[6] Seed cultures of the adenoviruses were generously supplied by Dr. R. J. Huebner. Stock virus was subsequently prepared by passage in KB cell monolayers. Plaque titration of the adenoviruses was performed by a modification of the procedure of Rouse, Bonifas, and Schlesinger.[13]

Virus purification: Adenovirus types 2 and 4 were cultivated, isolated, and purified as previously described.[6] In addition to the prototype strain of adenovirus type 12 (designated type 12A), three new strains kindly provided by Dr. Huebner were cultivated, purified, and studied (12B, 12C, and 12D). The following two modifications were employed in the purification of adenovirus types 12 and 18: (1) suspension cultures of KB cells were infected at 4–5 $\times$ 10^6 cells/ml and diluted to 3 $\times$ 10^5 cells/ml at two hr after the addition of virus; (2) infected cells were broken by sonic disruption for 5 min in the Raytheon 10-kc sonic oscillator.

The purified virus preparation (after the 2nd RbCl gradient) was made up to 1.0 ml with RbCl