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CONTROL OF THE WAVEFORM OF OSCILLATIONS OF THE REDUCED PYRIDINE NUCLEOTIDE LEVEL IN A CELL-FREE EXTRACT

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We have reported preliminary observations on damped sinusoidal oscillations of the level of reduced pyridine nucleotide (DPNH) in suspensions of yeast cells[1-3] and have found similar oscillations to take place in cell-free extracts containing the glycolytic system of these cells.[4] Here we report biophysical observations on the control of the waveform, phase, and period of the oscillations which are of significance to the possible biological function of this interesting reaction. Biochemical aspects of the metabolic control phenomena in the extract are to be reported in detail elsewhere.[5]

Preparations and Experimental Method.—The enzyme system consists of the soluble cytoplasmic constituents of *Saccharomyces carlsbergensis*, obtained by high-speed centrifugation of ruptured cells.[4] Microscopic examination shows no visible cell fragments, and the extract exhibits no measurable respiratory activity. Enzymatic activities of the glycolytic sequence are, however, present. The substrate and nucleotide levels are appropriate for the oscillatory reaction. The extracts prepared as described previously[4] are designated Type I; however, it has recently been found that the extracts may be prepared in the same way from cells which have been stored at 0° for several days. Preparations from these stored cells are designated Type II and are described in detail in a separate communication;[5] the number of hours that the cells have been stored at 0° is noted in parentheses in the figure legends.

Experimental Results.—Waveform of the oscillations: In previous reports, we have observed the waveform of the oscillations to be approximately sinusoidal.[1, 4] However, more recent detailed studies indicate interesting variations and interconversions of waveforms, a typical example of which is shown in Figure 1. The oscillations, followed at 340 mμ in the double-beam spectrophotometer, with a

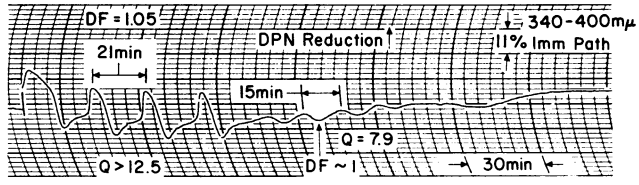

FIG. 1.—Waveforms of oscillations obtained in a yeast extract. Time and absorbancy scales indicated on the figure, time proceeding from left to right. The conversion from the nonsinusoidal, large-amplitude oscillations to the small-amplitude sinusoidal oscillations is spontaneous; no substances were added. Values of *DF* (damping factor) and *Q* (see text) are included on this and the following figures. Type II(18 hr). Double-beam spectrophotometer with Beckman monochromators, recording at 340–400 mμ. Optical path, 1 mm. Temperature, 25°C. 57.0 mg protein/ml. (Expt. 460 B-3.)

reference wavelength of 400 mμ, are in the form of a series of pulses with an over-all period of 21 min. However, over most of the period, the pyridine nucleotide is highly oxidized; the reduction lasts approximately 5 min. It appears that some product accumulates as the oxidation proceeds which must be expended before the cycle of reduction can occur. Of considerable interest is the fact that after 4 cycles of nonsinusoidal oscillations, the oscillations spontaneously become sinusoidal with a much lower amplitude and a period of 15 min. It is apparent from these data that more than one mode of oscillation exists and the waveform depends upon the levels of metabolite intermediates.

Control of the period of the oscillations: In intact cells, the period of the oscillations is approximately 0.6 min at 25°[1] and in cell-free extracts, approximately 7 min.[4] It is of interest to learn whether this system can be made to oscillate with the longer periods and ultimately to approach those of biological oscillations. Since the period depends upon the enzymatic activities, lowering the temperature affords a simple means of increasing the period; Figure 2 indicates the general effect of a 13° decrease of temperature on the waveform of the oscillations. First, the period is increased from 9.5 min to 41 min. Second, while the waveform of the oscillations is generally similar at the two temperatures, the nonsinusoidal waveform is more clearly seen at the lower temperature. The trend of the average DPNH level throughout the course of the oscillations is generally similar at the two temperatures, and characteristic of the oscillating system. The damping factor (computed as the ratio of the amplitude in one direction to the amplitude of the next deflection in the opposite direction) is 1.1 in both cases. The value of *Q* (defined as π times the number of oscillations required for the amplitude to fall to

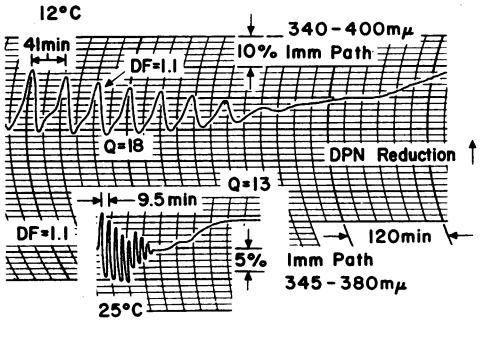

FIG. 2.—Comparison of the waveform of oscillation at 25° and 12°C. The record indicates the period, the damping factor, and the value of *Q*. The 12° record was made in the double-beam spectrophotometer with Beckman monochromators, and the 25° recording was run concurrently in the double-beam unit equipped with interference filters. Type II (72 hr). Optical path, 1 mm. 85 mg protein/ml. (Expt. 460 C-5,10.)

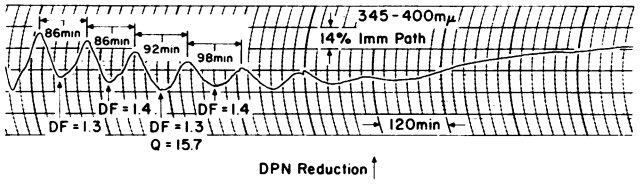

FIG. 3.—Long-period oscillations of the yeast extract. The periods of each oscillation are indicated, as are the damping factors. The total duration of the oscillations approaches a half day. Type I preparation. Double-beam spectrophotometer with Beckman monochromators. Optical path, 1 mm. Temperature, 0°. 121.5 mg protein/ml. (Expt. 462 A-7.)

$1/e$ of its initial value, where $e = 2.303$) is 13 at 25° and 18 at 12°.[1] The number of cycles that can readily be distinguished is 7 at 25° and 8 at 12°. It is apparent from these data that the oscillations proceed vigorously at the lower temperature with the longer period.

To determine whether an even longer period can be obtained, in Figure 3 we have decreased the temperature to 0°, and obtained periods over twice as long as those of Figure 1. The period varies from 86 min for the first cycle to 98 min for the last detectable oscillation. The damping factor (1.4 on the average) is somewhat larger than at 12° and the Q is 16. The oscillations have been observed to persist over an interval of approximately half a day.

This experiment indicates that the extract oscillates while it is stored in the ice bucket awaiting the withdrawal of successive samples for various experiments; the oscillations may perhaps start when the cells are ruptured. This would explain the recurrent observation that the start of the oscillations on warming the chilled extract to room temperature varies systematically; the direction in which the oscillations start would depend upon the phase of the oscillation which is occurring in the material stored at 0°.

In summary, the oscillations show no unexpected properties with respect to the variation of the enzymatic activities caused by a decrease in temperature, suggesting that the enzymatic mechanism operating in the cell extract could, theoretically, act with any desired period in accordance with the needs of systems involving biological clocks.

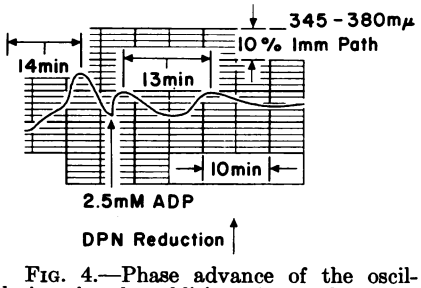

FIG. 4.—Phase advance of the oscillations by the addition of 2.5 mM ADP. Type II preparation (96 hr). Double-beam spectrophotometer with interference filters. Optical path, 1 mm. Temperature, 25°C. 69.1 mg protein/ml. (Expt. 462 D-5.)

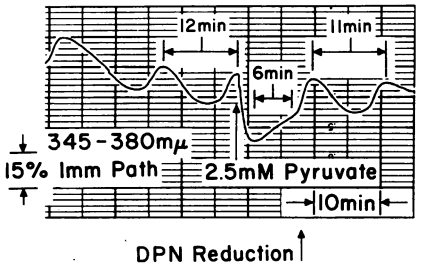

FIG. 5.—Phase retardation of the oscillations by the addition of pyruvate. Type II preparation (96 hr). Double-beam spectrophotometer with interference filters. Optical path, 1 mm. 69.1 mg protein/ml. (Expt. 462 D-3.)

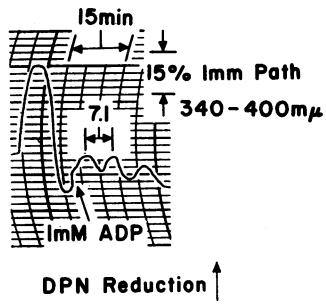

FIG. 6.—Control of waveform by addition of ADP. Type II preparation (18 hr). Double-beam spectrophotometer with Bausch and Lomb monochromators. Optical path, 1 mm. Temperature, 25°. 25.4 mg protein/ml. (Expt. 461 A-1, 4.)

Control of phase relationships—phase advance: One of the characteristic features of biological clocks is their property of shifting phase while retaining approximate frequency.[6] An example of this phenomenon is shown in Figure 4. The record shows a portion of the oscillation at a period of approximately 12 min with a nonsinusoidal waveform similar to that shown in Figure 1. As the trace swings from reduction toward oxidation, 2.5 mM ADP is added. There follows an abrupt reduction and reoxidation of pyridine nucleotide; thereafter, the oscillations bear a new phase relationship to the preceding trace. A similar effect is observed with 3′-5′ AMP at about 100-fold lower concentrations. Essentially, the addition of ADP has advanced the initiation of the reduction cycle by approximately 3.5 min, although the period of the remaining oscillations is but little changed (14 min versus 12 min). Thus this chart indicates that addition of a control chemical can advance the phase of the "clock" with little effect on the period.

Control of phase relationships—phase retardation: In the previous figure we illustrated an advance of the phase of the oscillations by the addition of ADP; here we describe a phase retardation. Figure 5 shows an approximately sinusoidal oscillation starting in the left-hand portion of the figure, and proceeding through one full cycle of oscillation with a period of 12 min. As the pyridine nucleotide level proceeds toward maximal reduction, 2.5 mM pyruvate is added, causing a rapid transition to the fully oxidized state. Instead of proceeding to the next cycle of DPN reduction, there is a plateau lasting approximately 7 min. Thereafter, the sinusoidal oscillation is resumed with an 11-min period and, as the record shows, with considerable phase retardation.

Control of the waveform: In addition to control of the time relationships of the oscillations illustrated in the preceding figure, it is possible to control the amplitude and waveform as well. Figure 6 shows the initiation, in the usual manner, of nonsinusoidal oscillations of large amplitude of the type shown in Figure 1. At the peak of the oxidation of pyridine nucleotide, the addition of 1 mM ADP causes an abrupt reduction to a new steady-state level. Thereafter, the oscillation proceeds as a small-amplitude sinusoid. The period of the modified oscillation is 7 min, compared with a period of approximately 20 min in the control experiment.

Discussion.—We can demonstrate by these experiments temperature control of the period and chemical control of the phase and amplitude relationships of oscillations of the pyridine nucleotide level in a system of glycolytic enzymes and their intermediates. These results indicate a great advance in obtaining long-period oscillations from the glycolytic system: periods reaching 1.5 hr have been obtained by decreasing enzymatic activities at lower temperatures. It is apparent that a system constituted of enzymes of even lower activity could readily approach the oscillation period required for the physiological function of biological clocks. Furthermore, the possibility of phase adjustment of the enzymatic system—

advancing the phase with ADP or retarding it with pyruvate without a large change in the period—may also be of interest.

Our observations now raise questions as to the nature of the "master oscillator" of the biological systems themselves. Although not all systems reveal the complete waveform of the oscillation, when such data are available (for example, for Gonyaulax), waveforms similar to both types exhibited by the enzymatic systems studied here are recorded.[7] In more complex systems[6–8] the nature of the basic waveform is more obscure, but in general it need not be vastly different from those observed here in our cell extract. Solutions to the problems of temperature compensation and of a stabilized biochemical environment involve complex speculations well beyond the scope of this paper. One can imagine, however, that chemical systems of the type described here, equipped in addition with temperature-compensating reactions and localized in the compartment of the cell where the external environment would have a minimum of influence, could serve some of the functions of a "master oscillator." Our data here are perhaps most useful in showing the plasticity of waveform generation in the multi-enzyme system of glycolysis.

Summary.—Experimental studies of the cell-free extract containing the glycolytic system of *Saccharomyces carlsbergensis* show oscillations with periods ranging from 13 sec *in vivo* at 37°[1] to 1.5 hr in a cell-free extract at 0°. The waveform of the oscillation may be sinusoidal or nonsinusoidal; in the latter case, the pyridine nucleotide remains oxidized for a longer time than it remains reduced. Addition of ADP advances the phase of the oscillations, and addition of pyruvate retards it.

The properties of this enzymatic system may shed some light on the function of biological clocks *in vivo*.

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