CONTINUOUS OSCILLATIONS IN A CELL-FREE EXTRACT OF S. CARLSBERGENSIS[1]

by Benno Hess and Karl Brand[2]

Max-Planck-Institut für Ernährungsphysiologie, Dortmund

and

Kendall Pye

Johnson Research Foundation, University of Pennsylvania,
Philadelphia, Pennsylvania

Received March 9, 1966

In previous communications a cell-free system of S. carlsbergensis showing glycolytic oscillations has been described with respect to its general properties (Chance et al., 1964; Hess et al., 1964) and its specific response to titration with glycolytic intermediates (Chance et al., 1965) and enzymes (Hess et al., 1965; Hess and Brand, 1965). These studies indicate the adenine nucleotides as control metabolites and the phosphotransferases phosphofructokinase, phosphoglycerate kinase and pyruvate kinase as control units of the oscillatory operation. Whereas so far oscillations have only been maintained over a short time interval because of stronger damping activities recently continuous oscillations could be observed over a time interval of 22 hours (Hess and Brand, 1966) and will be described in this report.

Preparations and methods. S. carlsbergensis (ATCC 9080) is grown on a medium as previously described (Ghosh et al., 1960) and in an incubator (Kleinfermenter of CHEMAP A. G., Mennedorf, Zürich) in 5 l batches at 32° C

1 This work was supported by Deutsche Forschungsgemeinschaft, Bad Godesberg bei Bonn and PHS CA 06393 - 01 and PHS US 12202.

2 Present address: Department of Molecular Biology, Albert Einstein College, Yeshiva University, Bronx, N. Y., U.S.A.

and a pH of 4.9-5.2 under aeration at constant pressure which maintains oxygen saturation as monitored by a platinum electrode. The yeast (275,000 cells per mm^3) is harvested in its stationary phase after 24 hours by cooling to 4° C under further aeration for 1 hour and by centrifugation at 6,000 r.p.m for 10 min. (all centrifugations are carried out at 4° C) to yield a hard pellet. The sediment is washed and resedimented twice (7,000 r.p.m. and 8,000 r.p.m. respectively 10 min.) in potassium phosphate buffer (0.1 M, pH 4,5), resuspended in the same buffer (1+4(w:v)) and aerated at room temperature for 3 hours, finally centrifuged at 9,000 r.p.m. for 10 min. to a sediment, which can be stored at 4° C for 3-4 days. For extraction this sediment is resuspended in potassium phosphate buffer (0.1 M, pH 6.5) containing 5 mM glucose (12 gr wet weight of yeast - 20 ml buffer-glucose solution) in the cold for 2 min. and again sedimented (8,000 r.p.m., 10 min.). The supernatant is carefully discarded, the sediment is resuspended in 2 ml of potassium phosphate buffer (0.1 M, pH 6.5) per 12 gr wet weight of yeast and sonified in a BRANSON sonifier at 7-8 ampères 12 x 30 sec. with 30 sec. interval. During sonication the temperature must be carefully controlled and should not rise over 12° C. The sample is then centrifuged for 30 min. at 12,000 r.p.m. The sediment is discarded and the supernatant again treated for 1 hour at 40,000 r.p.m. in a SPINCO centrifuge (model L 2, Rotor 50) yielding the first sediment (N I). The supernatant is centrifuged for a second time under the same conditions for one and a half hours yielding the final supernatant (S I) and the second sediment (N II). N I, N II, and S I are stored in a liquid nitrogen tank. Under this condition activity for continuous oscillation can be retained for at least 2 months. For incubation S I, N I, and N II are mixed in appropriate ratios. Sometimes S I alone was active. Usually S I and N I were mixed in a volume ratio of 6:1. However, in some preparations S I alone showed full activity.

Oscillations are recorded with a double beam spectrophotometer employing interference filters (345 mμ and 400 mμ) or with an EPPENDORF

fluorimeter (excitation 366 mμ, emission 400-3000 mμ) and a VARIAN G 14 recorder attached through a suitable potentiometer and a zero-supression device.

Enzyme activities in the samples were assayed using methods described by Bücher et al. (1964) with slight modifications. Trehalase was tested by enzymic coupling with hexokinase and glucose-6-phosphate dehydrogenase. Protein determination was carried out according to the Biuret procedure. Ethanol, glucose, and pyridine nucleotides were assayed by conventional enzymic tests (Bergmeyer, 1963).

EXPERIMENTAL RESULTS

Continuous oscillation. When the sediment (N I) and supernatant (S I) are mixed, continuous oscillation can usually be recorded without any addition over a time of 10-20 periods until the cycles subside because of an apparent lack of substrate. Since the system does not contain glucose in appropriate amounts ($< 10^{-4}$ M), it was obvious that polymeric carbohydrates are the endogenous substrates for oscillatory glycolysis. Indeed, earlier experiments have shown that the extract contains oligosaccharide-material in the order of 7 mM glucose-equivalents (Hess et al., 1964). Trehalose, an endogenous substrate known to accumulate in large quantities in yeast (Trevelyan and Harrison, 1952) was tested for its effect on the oscillations, and it was observed that sustained oscillations were produced (Pye, 1965; Pye and Chance, 1966). Only maltose and sucrose have been tested apart from trehalose. Both compounds gave no oscillations by themselves. Both fractions (S I and N I) contain trehalase activity capable of hydrolyzing 30 nanomoles of trehalose per min. per mg. Other glycolytic enzymes are present in excess of recorded glycolytic flux. Complete enzyme patterns will be published elsewhere.

Additions of 150 mM trehalose immediately or after a time interval corresponding to one or two periods leads to continuous oscillations over 4 hours with a frequency of 0.20 min^{-1} and a damping factor over the middle region of 1.01 (see fig. 1). The system moves toward a more oxidized state

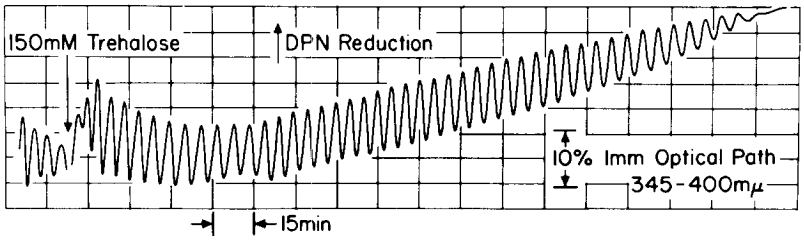

Fig. 1. Double-beam spectrophotometric recording of the sinusoidal oscillations of DPNH in a cell-free extract of S. carlsbergensis, showing 46 complete cycles after addition of 150 mM trehalose.

displaying maximum amplitude and then slowly rises to a more reduced state with constant frequency and damping until the oscillations subside. It probably stops because it has run out of trehalose. A further addition of trehalose gives more oscillations.

In separate experiments it was found that the trehalose content drops from 26 mM at the start of the oscillation and reaches a negligible level at the point when the oscillations ceased 90 min. later. The glycogen content on the other hand dropped only fractionally from 16 mM to 13 mM glucose equivalents during the same period. This seems to be fairly good evidence that trehalose is the natural substrate for the spontaneous oscillations (Pye and Chance, 1966).

Figure 2 indicates continuous almost sinusoidal oscillations over 60 periods with a constant frequency of 0.34 min^{-1} and an amplitude modulation of roughly 30% until strong damping activities develop for the last 4 cycles.

Figure 3 demonstrates a section of continuous oscillation lasting for more than 20 hours with a constant frequency of 0.166 min^{-1}, a Q-value of 200, an amplitude of 0.19 mM DPNH and a rate of 7.2 nmoles ethanol per mg protein per min. according to the DPNH and ethanol assays and corresponding roughly to the product of frequency and amplitude ($2\pi f \times \Delta$ moles DPNH (per amplitude) x min^{-1} x mg^{-1} (see Hess et al., 1964)). The second time derivative of DPNH is 7.8 moles DPNH per mg protein per min^2. During the experi-

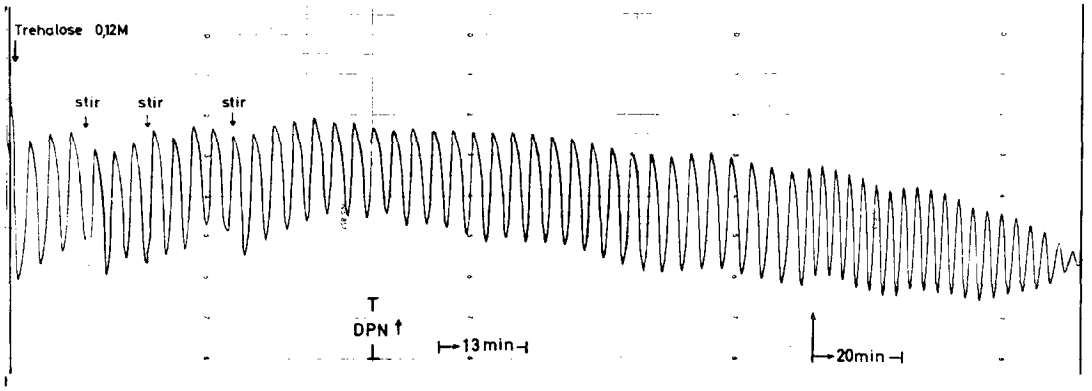

Fig. 2. Fluorometric recording of the oscillation of DPNH in a cell-free extract of S. carlsbergensis. DPNH scale given in arbitrary units. In the latter part of the recording the chart speed has been decreased: protein 50 mgr/ml, 180 vol. S I + 2 vol. N I (exp. no. 9/12).

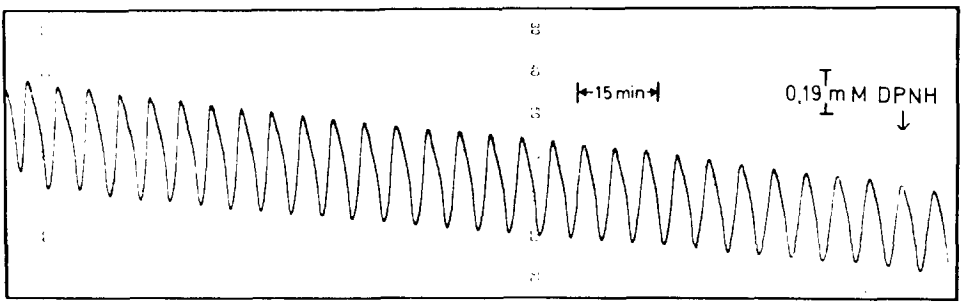

Fig. 3. Fluorometric recording of the oscillation of DPNH in a cell-free extract of S. carlsbergensis; protein 73 mgr/ml, 200 vol. S I (exp. no. 105/10).

ment the sample was stirred and fortified by addition of (2 times) 120 mM and (2 times) 60 mM trehalose consecutively. During stirring an appreciable amount of CO_2 escaped from the sample. A final level of 340 mM ethanol was observed after 20 hours corresponding to one-fourth of the added trehalose being glycolysed.

Whereas the frequency remained constant during the total period of observation the amplitude did not remain so constant as shown in the section of figure 3, but was modulated during later sections of the experiment

almost 6-fold from minimum to maximum of the scale value. Also, when the system runs short of trehalose, the amplitude is diminished. Furthermore, the mean state of reduction varied slightly as can be seen from figure 3, where a trend to reduction is recorded. However, it stayed constant later in the experiment. In the first 4 hours the waveform was slightly non-sinusoidal, later sinusoidal. During the last 2 hours of the experiment the oscillations died slowly and could not be restored either by the addition of trehalose and/or cyclic AMP.

In separate experiments oscillations of constant frequency and a Q-value of 1000 in the range of electronic oscillators have been recorded over 22 hours with a final ethanol concentration of 0.78 mM. Occasionally, higher harmonics of this frequency have been observed in a ratio of 1:2:4:8, however, with strong damping. The frequency-temperature dependency was semilogarithmic up to 33° C (see table I), and an activation energy of 19 $\left[\text{kcal} \times \text{Mol}^{-1}\right]$ was computed. At 40° C precipitation of the

Table 1

(exp. 27/33/11/151)

Temperature °C	Frequency min^{-1}
6	0.022
12	0.042
15	0.071
20.5	0.120
25	0.204
30	0.294
35	0.555
40	precipitation

system stopped the oscillation. The constancy of the frequency at room temperature is remarkable. It points to the great stability of the enzymic sequence and is in contrast to the lability in the isolated state of some glycolytic enzymes like phosphofructokinase or pyruvate kinase of yeast. However, the final subsidence of the oscillation could be due to the beginning of denaturation by ethanol and/or the higher dilution of the system.

We have thus shown that the addition of trehalose to cell-free extracts of S. carlsbergensis permits continuous glycolytic oscillation to be maintained over a period of 20 hours and more, with constant frequency and amplitude of DPNH changes. The continuity of the oscillations described here displays the property of a true biochemical oscillator with Q-values up to 1000, whose general operation and mechanism will be discussed elsewhere.

REFERENCES

Bergmeyer, H. U., Methods of Enzymatic Analysis, Academic Press, New York, Verlag Chemie Weinheim (1963).

Bücher, T., Luh, W., and Pette, D., Einfacheund zusammengesetzte optische Tests mit Pyridinnucleotiden, in Handbuch der Physiolog. and Patholog. Chem. Analyse, 6. Band/Enzyme Teil A 1964, Seite 292, Springer Verlag, Berlin-Heidelberg-New York.

Chance, B., Hess, B., and Betz, A., Biochem. Biophys. Res. Commun. 16, 182 (1964).

Chance, B., Schoener, B., and Elsaesser, S., J. Biol. Chem. 240, 3170 (1965).

Ghosh, A., Charalampous, F., Sison, Y., and Borer, R., J. Biol. Chem. 235, 2522 (1960).

Hess, B., Chance, B., and Betz, A., Berichte der Bunsengesellschaft für physikal. Chemie 68, 768 (1964).

Hess, B., and Brand, K., Abst., Amer. Chem. Soc. 55, 27c (1965).

Hess, B., Brand, K., and Cassuto, Y., Federation Proc. 24, 537 (1965).

Hess, B., and Brand, K., Abst., 3rd Febs. Meeting, Warschau, F 108 (1966).

Pye, K., Commun. to the 150th meeting of the Amer. Chem. Soc. (1965).

Pye, K., and Chance, B., Proc. Natl. Acad. Sci. U.S., in press (1966).

Trevelyan, W. E., and Harrison, J. S., Biochem. J. 50, 298 (1952).