

UNDAMPED OSCILLATIONS OF PYRIDINE NUCLEOTIDE AND OXYGEN TENSION IN CHEMOSTAT CULTURES OF *KLEBSIELLA AEROGENES*

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ABSTRACT

Klebsiella aerogenes was grown in chemostat culture with the pH controlled to ± 0.01 and temperature to $\pm 0.1^\circ\text{C}$. The oxygen tension of the culture was regulated by changing the partial pressure of oxygen in the gas phase and recorded by means of an oxygen electrode. Reduced pyridine nucleotide was monitored continuously in the culture by means of direct fluorimetry. On applying an anaerobic shock to the culture, damped oscillations in pyridine nucleotide fluorescence were obtained. Further anaerobic shocks decreased the damping and eventually gave rise to undamped oscillations of a 2-3 min period which continued for several days. These oscillations were paralleled by oscillations of the same frequency in respiration rate. The amplitude of the oscillations in the respiration rate was equivalent to only 1% of the total steady-state respiration, whereas that of pyridine nucleotide oscillations was equivalent to 10% of the total aerobic/anaerobic fluorescence response. The oscillations ceased on interrupting the glucose feed but restarted on adding excess glucose to the culture. Addition of succinate also restarted the oscillations so that they appear not to be of glycolytic origin. The frequency of oscillations varied with growth rate and conditions. Oscillations of much lower frequency were obtained under limited-oxygen and anaerobic conditions than under fully aerobic conditions. Under glucose-limited conditions, fluctuations were found in adenosine triphosphate (ATP) content which were in phase with the pyridine nucleotide oscillations, but under nitrogen-limited growth conditions no such fluctuations in ATP were observed. The primary oscillating pathway could not be identified but the mechanism would appear to be quite different from that involved in oscillations observed in yeast cells. The synchronization of oscillations and observations of negative damping could be explained by a syntalysis effect.

INTRODUCTION

Biochemical oscillations are of great interest for the information they can provide on metabolic control systems. For instance, in recent years extensive studies of oscillations in reduced pyridine nucleotide concentrations in yeast have con-

tributed a great deal to the understanding of the regulation of glycolysis (Ghosh and Chance, 1964; Betz and Chance, 1964; Pye, 1969).

A chemostat is a means of maintaining a steady state of growth of microorganisms (Herbert,

Elsworth, and Telling, 1956) and, provided the growth parameters of the cell are constant, oscillations would not be expected (Koga and Humphrey, 1967). However, there have been several reports of oscillations occurring in chemostat cultures of microorganisms which do not seem to be explicable in terms of an oscillating environment (Sikyta and Slezak, 1965; Günther, Knorre, and Bergter, 1966), but must be a reflection of the regulatory system of the organism. Harrison and Pirt (1967) found that the respiration rate of *Klebsiella aerogenes* oscillated at low oxygen tensions. A model has been proposed by Degn and Harrison (1968) to explain the mechanisms of these oscillations, based on an increased respiration rate at low oxygen tensions, and the biochemical mechanism has been recently studied (Harrison and Maitra, 1969). The oscillations discussed in the present paper differ from other oscillations reported in chemostat cultures, in being of relatively high frequency and appear to be of different origin. They resemble oscillations found in resting yeast cells (Betz and Chance, 1964), in that the oscillations are detected in the level of reduced pyridine nucleotide; but they differ from oscillations found in yeast in that they are obtained under aerobic conditions.

The monitoring of pyridine nucleotide reduction by means of fluorescence is a well-established technique. In recent years this technique has been widely used for following changes in pyridine nucleotide of resting cell suspensions (Chance, 1964; Maitra and Estabrook, 1967). The modification of a simple metabolite fluorimeter has made it possible for direct fluorescence measurements to be made in a chemostat culture (Harrison and Chance, 1970).

Preliminary reports of these oscillations have been published (Harrison, MacLennan, and Pirt, 1969; Harrison and Degn, 1969).

METHODS

The organism used was *Kl. aerogenes* (NIBC 8017), and the medium was that described by Harrison and Pirt (1967), except that the glucose concentration was increased to 10 g/liter.

The culture was grown in a 1 liter magnetically stirred chemostat vessel. The temperature of the vessel was controlled at $30 \pm 0.1^\circ\text{C}$ and pH at 6.0 ± 0.01 . Aeration of the culture was achieved by means of a vortex. The dissolved oxygen tension was regulated by mixing air and nitrogen in metered amounts to adjust the partial pressure of oxygen in the gas

supply to the culture. For steady-state conditions, the oxygen tension of the culture was constant for a constant oxygen partial pressure in the gas phase unless oscillatory phenomena occurred. The oxygen tension in the culture was monitored by means of a Mackereth electrode (Mackereth, 1964) (E. I. L. Richmond, Surrey, England). CO_2 production was measured by bubbling the effluent gas through bicarbonate buffer held at a constant temperature, and monitoring the pH of the buffer with an electrode. The cell dry weight concentration of the culture was 5.0 g/liter.

Samples (10 ml of 5 mg dry weight per ml.) for measurement of adenine nucleotides and metabolic intermediates were taken and analyzed as described by Harrison and Maitra (1969).

Pyridine nucleotide fluorescence was monitored by means of a modified Johnson Foundation metabolite fluorometer; the method is described in full elsewhere (Harrison and Chance, 1970. In press).

Briefly, light at $360 \mu\mu$ was shone through the pyrex glass wall of the vessel, and the light emitted at $460 \mu\mu$ was collected and metered by means of a phototube. Light source and phototube were at an angle of 60° to one another. The output from the phototube was recorded.

RESULTS

Oscillations in the pyridine nucleotide fluorescence were first observed after an anaerobic shock had been applied to a steady-state aerobic culture. When the air supply to the culture was restarted, the fluorescence fell and then underwent a few damped oscillations (Fig. 1). This phenomenon was found to be repeatable, and it was observed that after several such anaerobic shocks the damping became less and the number of oscillations increased until, eventually, undamped oscillations were obtained (Fig. 2). The precise number of anaerobic shocks required to produce continuing oscillations seemed to vary with the state of the culture, but generally about six shocks over a period of 24 hr produced continuing oscillations. When the culture was maintained in aerobic state, these oscillations continued for over 2 days without a change in amplitude or frequency. No changes in the oscillations of pyridine nucleotide or the mean fluorescence reading were detected when the oxygen tension was varied between 2 and 100 mm Hg, which is the range over which respiration rate and metabolism are independent of dissolved oxygen tension in *K. aerogenes* (Harrison and Pirt, 1967). This would seem to eliminate the possibility that the oscillations in pyridine

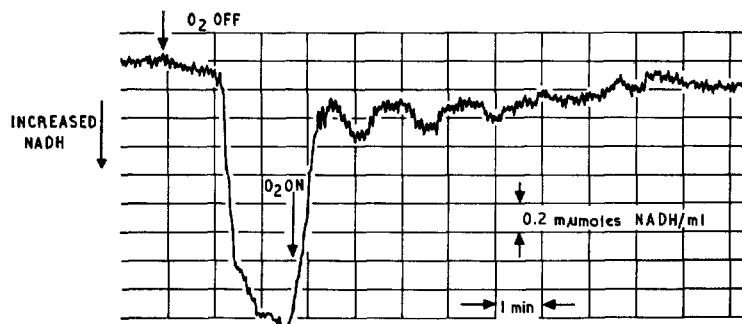


FIGURE 1 Damped oscillations in pyridine nucleotide fluorescence following an anaerobic shock to an aerobic chemostat culture of *K. aerogenes*. Calibration of fluorescence is in units of NADH per milliliter culture, based on enzymic assay. The delay between turning off the oxygen supply and the increase in fluorescence represents the time required for the culture to become anaerobic. Growth was glucose limited at a rate of 0.2 hr^{-1} . Steady-state oxygen tension, 43 mm Hg.

nucleotide were caused by small fluctuations in oxygen tension. At oxygen tensions below 1 mm Hg, when the oxygen supply becomes limited, the frequency and amplitude of oscillations were affected (Fig. 6).

On stopping the glucose supply to a glucose-limited oscillating culture, the oscillations immediately ceased. Provided the medium supply was switched on again within 5 or 10 min, the oscillations restarted, but if the medium supply was interrupted for more than 15 min no oscillations were obtained on resuming the supply until another anaerobic shock was applied. Thus the oscillations could be started or stopped at will.

On a few occasions, after stopping the oscillations with a prolonged interruption of medium supply, the oscillations restarted spontaneously and with negative damping (Fig. 3). As this occurred only four times, the exact sequence of events which caused it are unknown.

Close attention was given to other parameters of the culture to see whether any of these also oscillated. No oscillations could be detected in acid production in the culture, although the pH recording was sensitive to changes of less than 0.01 unit. However, when the sensitivity of the oxygen tension—recording instrument was increased to 10 mm of Hg full scale, it was found that oscillations in oxygen tension of the same frequency as those of the pyridine nucleotide were occurring (Fig. 4). Also oscillations of the same frequency were detected in CO_2 production. The amplitude of these oscillations in oxygen tension and CO_2 production, however, represented an oscillation of only 1% of the total respiration rate of the

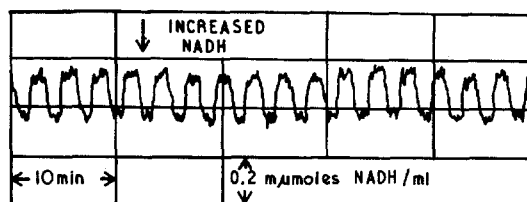


FIGURE 2 Continuing, undamped oscillations in pyridine nucleotide fluorescence obtained after repeated anaerobic shocks had been applied to an aerobic chemostat culture of *K. aerogenes*. Calibration of fluorescence is in units of NADH per milliliter culture, based on enzymic assay. Growth was glucose limited at a rate of 0.2 hr^{-1} . Steady-state oxygen tension, 43 mm Hg.

culture. The traces shown in Fig. 4 may be interpreted as follows: The sharp fall in fluorescence indicates a rapid partial oxidation of the pyridine nucleotide while the oxygen tension was at minimum, i.e., respiration rate was at a maximum. A few seconds later the oxygen tension began to rise, i.e. respiration rate fell, while the pyridine nucleotide remained oxidized. After about 70 sec the pyridine nucleotide trace showed a rapid shift back towards reduction and, about 30 sec later, while the pyridine nucleotide was still in the reduced phase, the oxygen tension began to fall, indicating an increase in respiration rate. This phase relationship was maintained. When comparing the phase relationships, it must be borne in mind that the oxygen probe had a much slower response time (90% in 30 sec) than the fluorimeter (0.5 sec) and that a further delay in oxygen tension response would be caused by the time required to

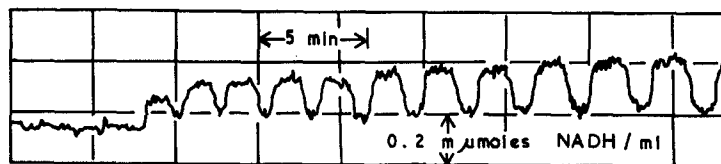


FIGURE 3 Spontaneous negative damping of oscillations in pyridine nucleotide fluorescence occurring in an aerobic glucose limited culture of *K. aerogenes*. Calibration of fluorescence is in units of NADH per milliliter culture, based on enzymic assay. The growth rate was 0.2 hr^{-1} and oxygen tension was 53 mm Hg.

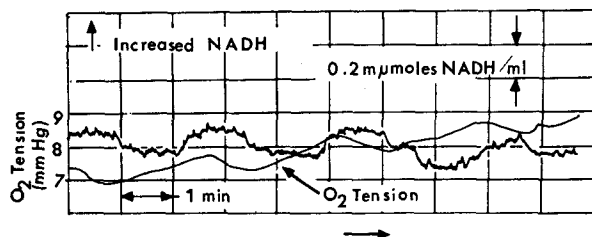


FIGURE 4 Oscillations in pyridine nucleotide fluorescence and corresponding oscillations in dissolved oxygen tension of a chemostat culture of *K. aerogenes*. Calibration of the fluorescence is in units of NADH per milliliter of culture, based on enzymic assay. The time scales are synchronized. Growth was glucose limited at a rate of 0.2 hr^{-1} .

equilibrate the gas and liquid phases in the culture vessel. Therefore, although the oscillations in oxygen tension and pyridine nucleotide appear to be 20–30 sec out of phase, the changes in respiration rate may coincide with the changes in the state of the pyridine nucleotide. That a shift to reduction of pyridine nucleotide should be followed by increased respiration rate and a shift to oxidation by a fall in respiration rate, would be consistent with control of respiration rate by substrate level, reduced pyridine nucleotide being the main substrate for respiration.

Although the frequency and amplitude of the oscillations were constant while the growth conditions of the culture were unchanged, they were found to vary when the culture was grown under different conditions. Fig. 5 shows the type of oscillations obtained in a nitrogen-limited culture with excess glucose present. Both the frequency and shape of the oscillations were modified. Although the oscillations in fluorescence were unaffected by changes in oxygen tension at values above 2 mm Hg, on reducing the oxygen tension to below 0.5 mm Hg (this was the minimum value that could be measured with the Mackereth electrode) a fall in the frequency of the oscillations occurred (Fig. 6). Previously, it has been shown

(Harrison and Pirt, 1967) that at such low oxygen tensions respiration is oxygen-limited, and profound changes in metabolism occur: the Q_{O_2} of the cells increases and fermentation products are formed. At oxygen tensions above 2 mm Hg, cell metabolism is independent of oxygen tension (Harrison and Pirt, 1967). Thus the dissolved oxygen concentration only affected the oscillations in pyridine nucleotide over the range where the cell metabolism is sensitive to dissolved oxygen. When a culture was allowed to remain anaerobic for long periods, the fluorescence trace showed oscillations of a very much reduced frequency (Fig. 7). These oscillations were also undamped and continuous. The shape and amplitude of these anaerobic oscillations were similar to those obtained in an aerobic culture, and it would seem likely that they have the same origin. This being so, the oscillator mechanism must function under aerobic, oxygen-limited, or anaerobic conditions, but presumably the feedback reactions involved in the oscillations are slower when the availability of oxygen is low. That the frequency of oscillations changes with the metabolic state of the cells indicates that the oscillations must have intracellular sources rather than being an artifact of the apparatus.

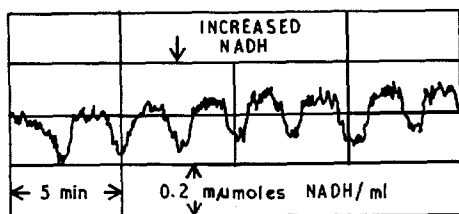


FIGURE 5 Continuing oscillations in pyridine nucleotide fluorescence obtained in a nitrogen-limited (glucose in excess) culture of *K. aerogenes*. Calibration of fluorescence is in units of NADH per milliliter of culture, based on enzymic assay. The growth rate of the culture was 0.2 hr^{-1} . Oxygen tension, 45 mm Hg.

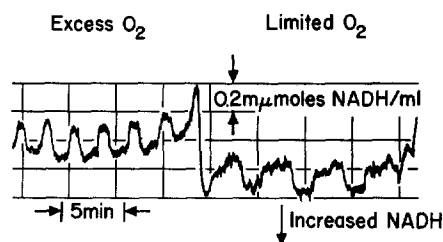


FIGURE 6 Effect of changing from excess to limited-oxygen conditions on oscillations in pyridine nucleotide fluorescence of a chemostat culture of *K. aerogenes*. Calibration of fluorescence is in units of NADH per milliliter culture, based on enzymic assays. Growth was glucose limited at a rate of 0.2 hr^{-1} . Oxygen tension changed from 54 to $< 1.0 \text{ mm Hg}$.

The period and amplitude of the oscillations were little affected by the metabolic rate of the cells. Table I shows the effect of varying the growth rate of a glucose-limited culture on the period of oscillations. When the growth rate was reduced to 0.04 hr^{-1} , oscillations ceased. Oscillations were obtained at a growth rate of 0.1 hr^{-1} with a lower frequency than at 0.2 hr^{-1} . Increasing the growth rate from 0.2 to 0.36 hr^{-1} had little effect on the period of oscillation, although the respiration rate of the culture and Q_{glucose} increased by more than 50%. Also, on adding excess (20 g/liter) glucose to the culture after turning off the medium supply, the oscillations restarted with little change in frequency and amplitude, although it has been shown that under these conditions the rates of glucose uptake and respiration are increased on adding excess glucose (Harrison and Maitra, 1969). Eventually, presumably on exhaustion of the glucose, the oscillations ceased again.

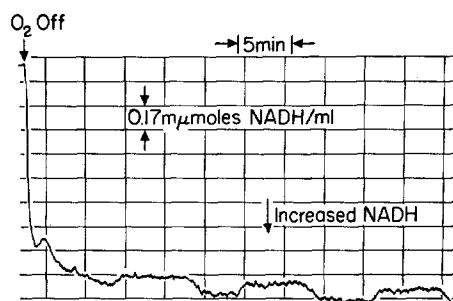


FIGURE 7 Low frequency oscillations in pyridine nucleotide fluorescence obtained under anaerobic conditions, in a chemostat culture of *K. aerogenes*. Calibration of fluorescence is in units of NADH per milliliter culture, based on enzymic assay. Growth was glucose limited at a rate of 0.2 hr^{-1} .

TABLE I

Effect of Growth Rate on the Period of Oscillations in Pyridine Nucleotide Fluorescence of an Aerobic Chemostat Culture of *K. Aerogenes*

Growth rate	Period of oscillations
hr^{-1}	min
0.04	∞
0.10	3.4
0.20	2.4
0.36	2.3

The culture was glucose limited.

Clearly, the continuous medium supply played no part in maintaining the oscillations.

When a pulse of succinate is added to a glucose-limited culture of *K. aerogenes*, it is oxidized, causing an increase in the respiration rate of the culture (Harrison and Maitra, 1969). The medium supply to an oscillating glucose-limited culture was switched off so that the oscillations ceased, and 5 mM succinate were added to the culture. As in the case of glucose addition, the oscillations restarted with similar period and amplitude as before. Thus the oscillations must rise in a pathway common to both glucose and succinate metabolism. This would seem to exclude a glycolytic intermediate as the main oscillator, and so the oscillations are not analogous to those reported in yeast cells (Betz and Chance, 1964).

A series of samples were taken from the culture while it was oscillating to see whether any oscillations in the metabolic intermediates could be

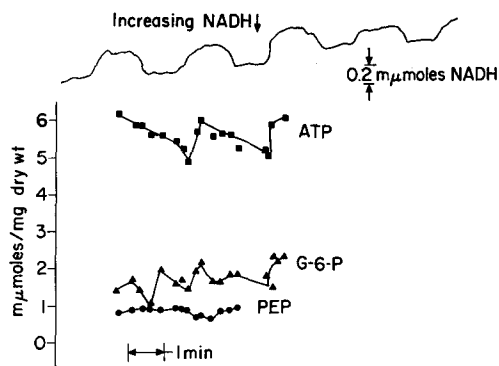


FIGURE 8 Changes in levels of intermediates during oscillations in pyridine nucleotide fluorescence of a chemostat culture of *K. aerogenes*. Calibration of fluorescence is in units of NADH per milliliter culture, based on enzymic assay. Other intermediates were determined enzymically on samples removed from the culture. Growth was glucose limited at a rate of 0.2 hr^{-1} . Oxygen tension, 46 mm Hg.

detected. Fig. 8 shows some of the results obtained with samples from an aerobic glucose-limited culture. There were no changes in glucose-6-phosphate (G-6-P), fructose diphosphate, triose-phosphates, or phosphoenolpyruvate which could be correlated with the oscillations in pyridine nucleotide. However, the adenosine triphosphate (ATP) level gave a sawtooth type of fluctuation with a frequency similar to that of the pyridine nucleotide oscillations. In a glucose-limited culture which did not demonstrate oscillations in pyridine nucleotide the ATP level showed no such fluctuations, and the standard deviation for ATP estimations on 20 samples was $\pm 0.4 \text{ m}\mu\text{mole/mg dry weight}$. Therefore the fluctuations obtained in ATP level in an oscillating culture would seem to be significant. Standard deviation for G-6-P measurements in a nonoscillating culture was $\pm 1.0 \text{ m}\mu\text{mole/mg dry weight}$, so that the fluctuations in G-6-P were within the error for the estimation. From Fig. 8 it can be seen that the sharp decrease in nicotinamide adenine dinucleotide (NADH) was accompanied by a sudden increase in the ATP level, indicating that the oxidation reactions involved in the oscillations may be accompanied by phosphorylation. A similar experiment was carried out on an oscillating culture which was nitrogen limited, with glucose in excess. In this case no oscillations in the ATP level were detected. Although oscillations in pyridine nucleotide may be accompanied by oscillations of the ATP level,

this does not appear to be an essential feature of the oscillations. Thus it would seem that in the case of glucose-limited cells, the ATP fluctuations are a result rather than a cause of the pyridine nucleotide oscillations.

DISCUSSION

The oscillations reported here appear to be the first high-frequency oscillations reported for growing cells. That the oscillations are a genuine metabolic feature and not an artifact of the apparatus is shown by the way in which they can be started and stopped at will by applying anaerobic shocks or starving the culture. Also, the oscillations may be modified by changing the metabolic state of the cells.

The oscillations involved only about 10–15% of the total pyridine nucleotide fluorescence and, because the analytical methods available were not sufficiently sensitive, it was not possible to use biochemical assays to confirm that the oscillations in fluorescence represented fluctuations in NADH level. Therefore, it is possible that fluorescence oscillations arose from changes in nicotinamide adenine dinucleotide phosphate (NADPH), or even that a change in the state of binding of NADH causes the changes in fluorescence intensity (Estabrook, 1962). Other workers (Chance et al., 1964) have shown, by simultaneously following changes in fluorescence intensity and absorption at $340 \text{ m}\mu$, that the oscillations in yeast cells are not caused by changes in binding of NADH but do in fact represent changes in NADH concentration. Absorption could not be measured directly in the chemostat culture, so this check could not be made. However, since the oscillations in fluorescence reported here were paralleled by oscillations in respiration rate, it is reasonable to assume, in the absence of any evidence that periodic changes of NADH binding may take place, that the fluorescence changes represent changes in concentration of NADH or NADPH.

Continuing oscillations in pyridine nucleotide which are similar in frequency to those reported here have been obtained in cell-free extracts of yeast (Pye and Chance, 1966). These were shown to be of glycolytic origin. However, the fact that the oscillations of pyridine nucleotide in chemostat cultures of *K. aerogenes* can be maintained with succinate as sole carbon supply would seem to exclude glycolysis as the source of the oscillations. Also, the oscillations were maintained under

anaerobic conditions, albeit with a much lower frequency, so that the main oscillator cannot be associated directly with the respiratory system. The fact that oscillations were observed under anaerobic conditions indicates that the oscillations in respiration rate were most probably an effect rather than a cause of oscillations in NADH. Although the precise phase relationship between the oscillations in fluorescence and the oscillations in oxygen tension could not be ascertained because of the much slower response of the monitoring system for oxygen, it would seem that they are consistent with substrate (NADH) control of respiration rate. This would be further indication that the oscillations in respiration rate are a secondary effect of oscillations in pyridine nucleotide. The amplitude of the oscillations in oxygen tension and carbon dioxide production were very small, representing only about 1% of the total respiration rate of the cell. Possibly, this represents a minor pathway for oxygen uptake which does not involve the normal electron-transport chain. Alternatively, the oscillations in oxygen uptake may reflect the dependency of the total respiration rate on the level of NADH which is the main substrate for respiration via the electron-transport chain. It would appear that the respiration rate is quite insensitive to NADH level in this range since a 10% change of NADH caused only a 1% change in respiration rate. The phase relationships between the oscillations in NADH and those in oxygen tension are consistent with the control of respiration rate by NADH level.

The availability of oxygen had a marked effect on the frequency of oscillations, in that under limited-oxygen and anaerobic conditions the frequency was much reduced. Therefore, the rate of reoxidation of NADH probably affects the feedback mechanism involved in the oscillations. Although the level of ATP was found to fluctuate with the same frequency as the NADH oscillations in a glucose-limited culture, this did not seem to be a constant feature of the system, since no correlating changes in ATP level were found in oscillating nitrogen-limited culture. Thus the oscillations in ATP level could also be an effect rather than a cause of the oscillations in other parameters. That neither amplitude nor frequency of oscillations was affected by an increase in the rate of respiration caused by adding extra substrate, suggests that the oscillating mechanism is not on the main pathway of substrate oxidation.

Positive identification of the primary oscillator reaction is not possible from the data so far obtained. Indeed, in such a complex system as a whole growing bacterial cell, it might be very difficult to decide which particular feedback reaction caused the oscillations since the observed manifestation of the oscillations may be quite remote from their origin. From the observations discussed above, it seems most probable that the primary oscillator reaction involves feedback from the NADH or NADPH level, and lies off the main pathway of glucose oxidation. The bacteria studied here were, of course, actively growing, and so it is possible that a synthetic pathway may be involved in the oscillations, although not directly linked with cell growth, because no simple correlation was found between growth-rate and frequency of oscillation.

The fact that oscillations may occur in a cell is not very remarkable, as oscillations are inherent in feedback systems, of which there are many in a cell. An example of undamped oscillations caused by feedback in the glycolysis pathway was demonstrated in cell-free extracts of yeast (Pye, 1969). What is more remarkable is that undamped oscillations should be detected in a culture of whole cells, for this requires that the individual cells oscillate in synchrony. Possibly the cells growing in the chemostat oscillate all the time, and the effect of successive anaerobic shocks is at first to put the cells into synchrony and, with further shocks, to induce a synchronizing mechanism. A mechanism which synchronizes oscillations in yeast cells has already been demonstrated by Pye and Chance (1968). Such synchronization may be explained by the theory of syntalysis proposed by Winfree (1967), which states that weak interactions between oscillating individual cells can bring about synchrony of a whole population. This would seem to provide the most plausible explanation of the observed negative damping.

The simplest form that a synchronizing mechanism could take would be one in which the extracellular concentration of a metabolic product controlled the cell metabolism. The oscillations reported here were obtained under such a wide variety of growth conditions that it would seem unlikely that a major metabolic product could have functioned as the synchronizer. Inorganic ions could possibly fulfill the role of synchronizer, but the addition to the culture of a mixture of all the basic salt ingredients of the medium had no

effect on the oscillations. The synchronizing mechanism may, however, involve a much more subtle form of cell communication, such as hormone-type secretions or charge transfer between cells.

Although the mechanism of these oscillations are far from being fully understood, such oscillations are of some theoretical significance, indicating the presence of complex feedback systems

and synchronizing mechanisms in growing populations of bacteria.

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