DPNH OSCILLATIONS IN GLYCOLYZING CELL FREE EXTRACTS FROM BEEF HEART*+

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The oscillatory responses of reduced pyridine nucleotide in Saccharomyces carlsbergensis have been the object of considerable attention (Chance et al., 1964a, 1964b). The oscillations, first observed in intact cell suspensions, have also been obtained in cell free extracts from the same yeast strain (Chance et al., 1964c, 1964d, 1965). The sensitivity of the oscillations to iodoacetic acid led to the conclusion that DPN, rather than TPN, was the nucleotide involved. (Chance et al., 1964c). The assay of glycolytic intermediates and adenine nucleotides in the oscillating cells has also demonstrated oscillations in these intermediates, each having a definite phase relationship with reduced pyridine nucleotide (Ghosh and Chance 1964; Betz and Chance, 1965a, 1965b).

This communication reports some results that have been obtained with glycolyzing cell free extracts from beef heart. These preparations have shown cyclic oscillations of reduced pyridine nucleotide in a manner similar to that observed in yeast extracts.

MATERIALS AND METHODS. Beef hearts were obtained at a local slaughterhouse immediately after sacrifice and packed in ice until arrival at the laboratory. All subsequent operations were carried out at 4-6° C. After removal of adipose and connective tissues, the ventricular muscle was ground in a meat grinder and washed three times with approximately 5 volumes of an ice cold solution containing 150 mM KCl, 10 mM potassium phosphate (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA (pH 7.4), and 1 mM 2-mercaptoethanol. After the last wash, the ground muscle was packed by centrifugation at 4,000 x g for 5 minutes and homogenized for 5 minutes in a Waring Blender with 0.5 ml of the same solution per gram

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of muscle. The resulting paste was then centrifuged for 2 hours at 78,000 x g. The supernatant was decanted through glass wool and frozen immediately in 5 ml aliquots.

The changes in the level of the reduced pyridine nucleotide were monitored fluorometrically in an Eppendorf fluorometer modified as described by Estabrook and Maitra (1962). Changes in fluorescence equivalent to 0.05 mμmoles of DPNH in 2 ml of solution could be observed with this experimental arrangement. The measurement of fluorescence rather than absorption was preferred due to the high myoglobin and hemoglobin content of the extract. In the experiments where the control of temperature was important, a modified sample holder providing water circulation was used. The same holder was employed for obtaining samples during the oscillations. A maximum of 5 cuvettes could be placed simultaneously inside the fluorometer chamber and samples withdrawn at the desired times by means of a syringe-pipette. The fluorescence changes were monitored continuously in one of the cuvettes. One half ml of sample was withdrawn from the cuvettes and added to 2.0 ml of 6 percent perchloric acid which was being stirred vigorously. After removal of the precipitated protein by centrifugation, the extracts were neutralized with 3 N K_2CO_3, the $KClO_4$ centrifuged off, and the samples stored at -20° C until the assays were done.

Glycolytic intermediates and adenine nucleotides were measured enzymatically in the perchloric acid extracts by means of fluorometric techniques (Maitra and Estabrook, 1964; Williamson, 1965).

RESULTS. The changes in fluorescence of a 2 ml sample of heart extract are shown in Figure 1. The figure shows a train of oscillations induced by the temperature transition from 0° C to 28° C, which was the temperature in the sample compartment of the fluorometer.

Some extracts did not show oscillations unless small amounts of

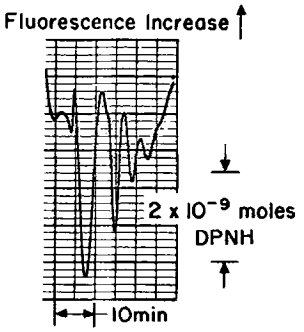

Figure 1. - Oscillations of reduced pyridine nucleotide in beef heart extract. Temperature in sample compartment: 28° C. Protein concentration: 14.7 mg/ml (Expt. 2-7).

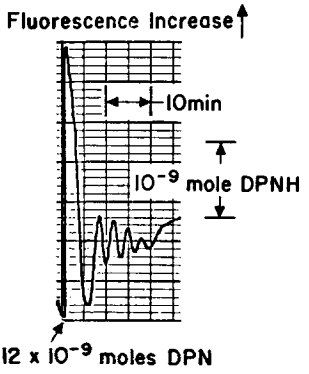

Figure 2. - Oscillations of reduced pyridine nucleotide in beef heart extract. At the point indicated by the arrow, 12 mμmoles of DPN were added to 2 ml of extract that had been equilibrated at 31° for 5 minutes. Protein concentration: 21.9 mg/ml (Expt. 2-8).

DPN were added. Such a case is illustrated in Figure 2. This particular extract did not show any oscillatory responses after the temperature transition. The addition of 12 mμmoles of DPN to 2 ml of extract results in an immediate increase in fluorescence followed by a train of oscillations.

Effect of Temperature. In order to study the effect of temperature on the oscillations, 2 ml aliquots of the extract were placed in the sample holder designed to maintain constant temperature. After temperature equilibration had been achieved, 12 mμmoles of DPN were added to the cuvette. The results obtained at 4 different temperatures

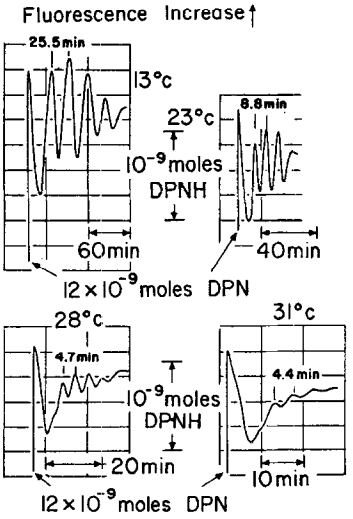

Figure 3. - Effect of temperature in the oscillations of reduced pyridine nucleotide in beef heart extract. The oscillations were started in each case by the addition of 12 mμmoles of DPN to 2 ml of extract after temperature equilibration. Protein concentration: 21.9 mg/ml (Expt. 2-8).

are shown in Figure 3. The period of the oscillations decreases progressively as the temperature is increased. At 13° C the period of one cycle, as measured at the reduction maxima, is 25.5 minutes. This value decreases to 8.1 minutes at 23° C, and to 4.4 minutes at 31° C. For the 10° interval between 13° C and 23° C a Q_{10} of 2.9 is obtained, which indicates a fairly high energy of activation. In order to estimate the value of this energy of activation for the oscillatory process, an Arrhenius plot of the reciprocal of the absolute temperature against the logarithm of the frequency was made, and the value calculated from the slope. The resulting energy of activation is 19 kcal.

Oscillations of Glycolytic Intermediates. In order to investigate the possible control mechanisms involved in the oscillations, samples were taken at different times during a series of oscillations and the glycolytic intermediates and adenine nucleotides measured.

The top part of Figure 4 shows the fluorescence trace as well as the points at which samples were taken for measurement of intermediates. Application of the crossover theorem at a maximum and minimum of fluorescence is indicated in Figure 4. At the point of minimum fluorescence there is a relative accumulation of G6P and F6P, while the remaining intermediates and ADP appear relatively depleted. The opposite is observed at the fluorescence maximum, where all the intermediates appear at higher concentrations, with the exception of G6P, F6P and ADP. These data indicate an apparent crossover at the level of phosphofructokinase. It is interesting that there appears to be no direct stoichiometric relationship between the changes in DPNH level and the simultaneous changes in the different intermediates. The overall change in DPNH between the maximum and minimum amounts to 0.095 mμmoles per ml of extract, while the smallest changes in intermediates, those observed in F6P, FDP, and GAP, are approximately 8 times larger. The largest absolute changes observed are those in ADP and ATP, showing a variation of 24 mμmoles per ml of extract, in opposite directions, during the same time interval.

DISCUSSION. The occurrence of cyclic oscillations of the reduced pyridine nucleotide in a mammalian cell free system indicates that the oscillations previously observed in yeast are not restricted to that species and that it is likely that such oscillatory responses are part of a general metabolic control mechanism. The nature of the oscillations in yeast is not completely resolved at the present time, but several

studies (Higgins, 1964; Chance et al., 1964b) have indicated that phosphofructokinase could be the basic oscillator due to its known inhibition by ATP and fructose diphosphate. These characteristics of phosphofructokinase result in the ability of the enzyme to turn itself on and off and in this manner originate pulsed fluxes (Garfinkel, 1965). These considerations do not exclude the possibility of other control sites contributing to the cyclic changes in the level of reduced pyridine

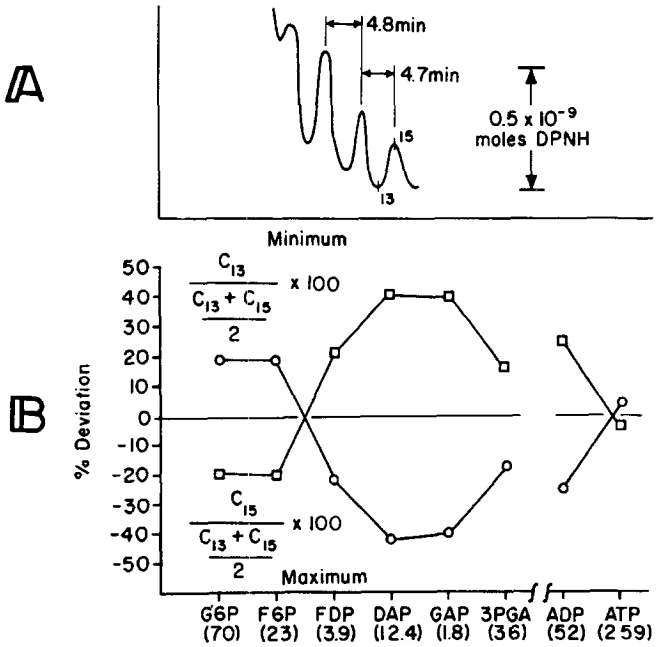

Figure 4. - Crossover plot of glycolytic intermediates. The abcissa indicates the glycolytic intermediates in sequence. The ordinate shows the percentage of each intermediate with respect to the average of their maximum and minimum concentrations. The upper part of the figure shows the fluorescence trace, indicating the points at which samples were taken. Values in parentheses indicate the average value of the different metabolites in mμmoles per ml of extract (Expt. 2-11).

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nucleotide in this glycolyzing system.

An interesting characteristic of the temperature studies, (Figure 3), is the increased amplitude of the oscillations at lower temperatures. A similar phenomenon is apparent in the observations made in yeast cells and extract (Chance et al., 1964a, 1964d), using a double beam spectrophotometer. This would indicate that the changes in amplitude are not due

to an enhancement of the DPNH fluorescence at lower temperatures, but rather to a different sensitivity of some of the enzymatic reactions responsible for the oscillatory responses.

The availability of a soluble system from a mammalian heart which is subject to oscillatory control offers the possibility of exploring in detail the more general characteristics of the control of glycolysis in this tissue. Work is currently in progress to establish the exact nature of the different control sites involved in the regulation of this oscillatory system.

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