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17. First REM period: placebo, 17.5 ± 2.1 minutes; physostigmine, 20.7 ± 4.5. First REM period density: placebo, 1.59 ± 0.17; physostigmine, 1.62 ± 0.30.
18. A waking is scored if subject wakes within 15 minutes of infusion and remains awake for more than 3 minutes.
19. Total REM time: placebo, 93.8 ± 7.9 minutes; physostigmine, 93.4 ± 7.8 minutes. REM density: placebo, 1.57 ± 0.18; physostigmine, 1.65 ± 0.38. Total non-REM time: placebo, 280.1 ± 31.2; physostigmine, 285.2 ± 24.0. Total delta sleep: placebo, 26.5 ± 9.6; physostigmine, 27.5 ± 3.5.
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β-D-Galactosidase Activity in Single Yeast Cells During Cell Cycle of *Saccharomyces lactis*

Abstract. *Single* Saccharomyces lactis *cells taken from a random population were assayed for β-D-galactosidase activity under a microscope equipped for fluorogenic measurements. The cells were also photographed, and enzymatic activity was correlated to the size of cell buds. A periodic pattern of enzyme synthesis was found during the cell cycle.*

Although most enzymes are synthesized discontinuously at a particular stage of the yeast cell cycle, examples of continuous synthesis have been reported (*1, 2*). Previous methods that made it possible to observe and investigate these patterns of changes in enzymes levels are based either on the use of synchronously growing cultures (*2*), or on gradient centrifugation of a random population of yeast (*3*). These methods provide only a close approximation of events which occur at the single cell level. The present work is concerned with the measurement of the activity of β-D-galactosidase (E.C. 3.2.1.23) in single yeast cells of *Saccharomyces (kluyveromyces) lactis* taken from a random population. The enzymatic activity of individual cells was determined under a microscope equipped for fluorogenic assays (*4*). Photographs of the cells after measurements of enzymatic activity were used to obtain an index of the size of the cell buds and, from this information, to establish the stage of the growth cycle of individual yeast cells. Thus, it was possible to examine directly the correlation between levels of β-D-galactosidase in a given cell and stage of the growth cycle.

Cells of *S. lactis*, strain Y-14 (*5*), were grown aerobically at 25°C in 0.2 percent succinate synthetic medium (*3*) supplemented with 2 percent lactose and harvested during exponential growth. Cells were washed with 0.025*M* phosphate buffer (*p*H 7.2) containing $10^{-3}M$ $MnCl_2$ and then resuspended in the same buffer to a density of about 10^9 cells per milliliter.

The assay of β-D-galactosidase in single cells was performed as follows. The cell suspension was treated with 0.2 volume of isoamyl alcohol at 0°C for 10 minutes with occasional shaking. The suspension was then diluted 1 : 10 with 0.02*M* phosphate buffer, *p*H 7.2, and 0.05 ml was mixed with 0.05 ml of $4 \times 10^{-4}M$ fluorescein-di-(β-D-galactopyranoside) (purchased from Nortok Associates, Lexington, Massachusetts). The mixture then was dispersed into a microchamber filled with silicone oil [SF-96 (200) from General Electric] as previously described (*4*). The time of mixing with the substrate was taken as the zero time for the enzymatic reaction. Immediately after dispersion, the microchamber was centrifuged to increase the rate of settling of the microdroplets to the bottom of the slide. The microchamber was then placed on the stage of microscope equipped for quantitative measurements of single molecules of β-D-galactosidase by fluorogenic methods (*4*). Microdroplets smaller than 100 μm containing a single yeast cell were selected for assay. The course of the enzymatic reaction in a given microdroplet was followed by recording the fluorescence every 60 seconds for at least 5 minutes. The fluorescence of droplets without cells was used as blank correction.

At the end of the assay, two photographs were taken (Kodak Tri-X 35-mm film, 15-second exposure). One of the photographs was focused on the microdroplet and served to determine the diameter of the droplet. The other photograph, focused on the yeast cells, was used to measure the size of the bud. For this measurement, the photographs were enlarged tenfold and the cell images from the enlargements were

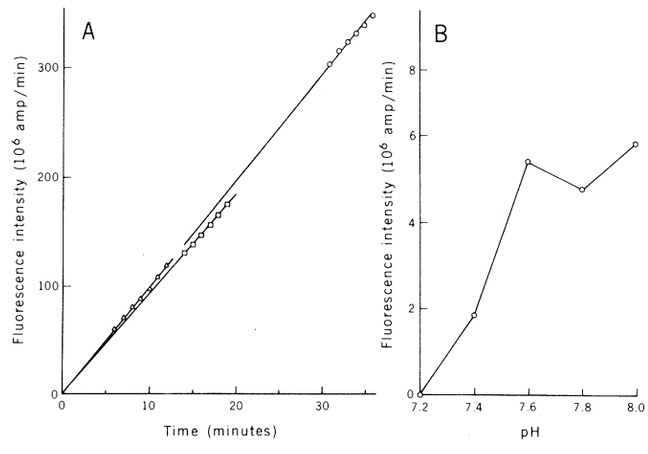

Fig. 1. (A) β-D-Galactosidase activity of single *S. lactis* cells in three different drops on the same slide. the activity of the budded cell in each drop was measured consecutively. A Zeiss Universal microscope equipped with a III-Z condenser was used. For photometry, the image from the objective was diverted to the monocular tube of the microscope where an Aminco photomultiplier microphotometer equipped with shutter and Wratten No. 12 filter, to exclude exciting light, was installed. The area measured by the photomultiplier (RCA 1P21) was reduced to about 100-μm diameter by placing a fixed pupil of 2-mm diameter near the point where the image of the object was focused. The amount of fluorescence was measured as the amperage recorded by the photomultiplier. (B) Effect of *p*H on β-D-galactosidase activity in single unbudded cells. The fluorescence of fluorescein increased 10 percent from *p*H 7 to 8.

copied on transparent graph paper. The size of the bud was obtained by cutting and weighing the paper images on an analytical balance. Cell size is expressed in arbitrary units related to the weight of the paper image.

Initial experiments showed that treatment of the yeast with isoamyl alcohol in standard buffer caused a rapid loss of β-D-galactosidase activity. High concentrations of Mn^{2+} [(1 to 5) × $10^{3}M$], which were about 100 times higher than those used for the measurement of enzyme activity in extracts (*5*), stabilized the activity for at least 3 hours. However, serum albumin (3 mg/ml) and 5 × $10^{-4}M$ dithiothreitol were required in addition to $10^{-3}M$ Mn^{2+} for enzyme stabilization in single cells.

Because of the high β-D-galactosidase activity in single yeast cells and the lapse of 3 to 5 minutes between mixing with substrate and the first measurement of enzyme activity, it was imperative to reduce the rate of the enzyme reaction and to shorten the time required for the assay. We found that addition of glycerol reduced the rate of β-D-galactosidase reaction; 5 percent glycerol gave a rate suitable for the time schedule of the assay. At this glycerol concentration the enzyme reaction was linear with time for at least 5 minutes (Fig. 1A). Under these conditions the assay procedure was shortened by consecutive assay of single cells in drops on the same slide. In each case, the rate of formation of fluorescent product in these cells could be extrapolated to zero time of the reaction.

Figure 1B shows the effect of *p*H on β-D-galactosidase activity in single cells. In the presence of 5 percent glycerol no activity was observed at *p*H 7.2, whereas there was substantial activity in its absence. This activity increased as the *p*H was raised to 7.6 and reached a plateau in the range of 7.6 to 8.0.

To verify that the permeability barrier of most cells was broken by the isoamyl alcohol treatment, individual cells from a treated culture were allowed to accumulate fluorescein by a process known as fluorochromasia (*6*). Cell suspensions previously incubated with fluorescein diacetate (Nortok Associates) were examined under a fluorescence microscope. No cells with intact membranes were found after treatment of about 10^{7} cells with isoamyl alcohol. A similar test with lyophilized yeast cells revealed less than ten fluorescent cells per 10^{7} cells in each slide.

To follow the pattern of β-D-galactosidase synthesis during the cell cycle, a random population of yeast cells was dispersed into the microchamber, examined for enzymatic activity, and photographed. Droplets of similar size (about 100 μm diameter) were selected for the assay; relatively small variations in droplet size did not affect our results since the rate of the increase of fluoresence of the whole droplet was measured. Cells without buds and cells with intermediate-size buds had similar levels of enzyme (Fig. 2). In contrast, cells with full-size or nearly full-size buds contained about twice this level of enzyme. No intermediate levels of enzyme were found. The doubling of the level of enzyme is periodic and occurs near the end of the cell cycle period. These results, which are in agreement with those obtained previously by the use of synchronous cultures (*7, 8*), show that it is possible to study directly the changes of enzyme level in yeast on individual cells taken from a random population. This procedure also avoids artifacts resulting from induced synchrony.

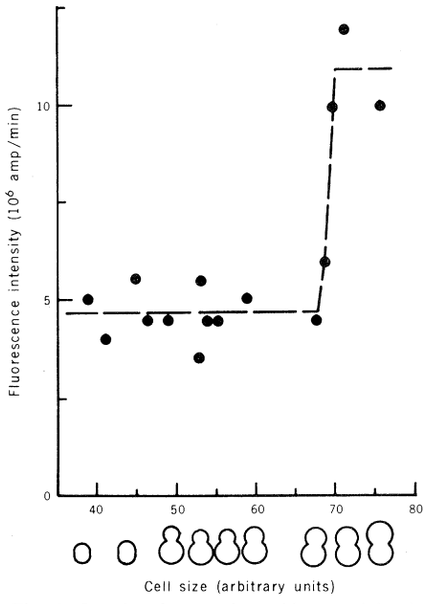

Fig. 2. Levels of β-D-galactosidase activity in single *S. lactis* cells during the cell cycle. The shape of the cells is drawn diagrammatically. The arbitrary units are described in the text.

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Duffy Blood Group and Malaria

Implications from the study of Miller *et al.* (*1*) that Fy(a-b-) erythrocytes are resistant to *Plasmodium vivax* raises some questions concerning selection of the *FyFy* genotype in zones endemic for malaria.

In testing Saudi Arabs for the sickle cell trait and Duffy antigens, we found 31 out of 37 sickle cell trait carriers (AS) to be *FyFy*. Of 106 normal homozygotes (AA), 46 were *FyFy*. The significant excess of *FyFy* among sickle trait carriers ($P < .01$) could be due to continuous African gene flow into Saudi Arabia, assortative mating (*AS FyFy* × *AA FyFy*), linkage between the hemoglobin beta-chain and Fy structural loci, or additive resistance against *P. falciparum* and *P. vivax* malaria in the presence of hemoglobin S and Fy(a-b-).

P. falciparum apparently maintains the AA,AS polymorphism in endemic areas; *P. falciparum* malaria was holoendemic, and *P. vivax* mesoendemic in the oases of eastern Saudi Arabia until 1948 (*2*), and sickle trait carriers average 25 percent in these oases. Accordingly, we favor the proposal that at least among Saudi Arabs, the *AS FyFy* genotype may have been selected by concurrence of endemic *falciparum* and *vivax* malaria, and that this genotype provides a greater survival advantage than either AS or FyFy alone in mixed *falciparum–vivax* infection. Alternatively, we propose that Fy(a-b-) erythrocytes may have partial resistance to *P. falciparum*, comparable to that conferred by hemoglobin S.

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