ACTION OF CYCLOHEXIMIDE ON ZYGOSACCHAROMYCES SOJA

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ABSTRACT

TSUKADA, YOJI (Kyoto University, Kyoto, Japan), TSUNETAKE SUGIMORI, KAZUTAMI IMAI, AND HIDEO KATAGIRI. Action of cycloheximide on Zygosaccharomyces soja. J. Bacteriol. 83:70-75. 1962.—Cycloheximide is known to inhibit the growth of some species of Saccharomyces and other fungi. A new type of action of this antibiotic, with Zygosaccharomyces soja as the test organism, is described in this paper. Growth of Z. soja was completely inhibited under aerobic conditions by cycloheximide in concentrations of more than 5 μg per ml of culture solution. Continued shaking of the culture for more than 96 hr in the presence of an inhibitory amount of cycloheximide restored growth, accompanied by the excretion of riboflavin to the extent of 30 μg per ml of culture solution. In the cells recovering from cycloheximide inhibition, the type of glucose metabolism differed from that in the mother strain; a respiratory quotient of 4 with the mother strain fell to 1 in the progeny. Glucose metabolism (O2 uptake and CO2 evolution) by resting cells was investigated, and it was concluded that inhibition of glycolysis was not significant. This was verified by comparing various dehydrogenase activities in the cell-free extract. There were essentially no differences between riboflavin-forming and nonriboflavin-forming cells. The presence of the hexose monophosphate shunt was deduced from the fact that glucose-6-phosphate dehydrogenase activity was the strongest of the various dehydrogenase activities.

Commonly, most kinds of microorganisms have riboflavin-synthesizing ability. With yeasts, Pett (1935) found that 30 μg riboflavin per g of yeast was accumulated in the cells of brewer's and baker's yeast. Burkholder (1943) reported riboflavin accumulation of 75 μg per ml of extract with Candida guilliermondii. Tanner, Vojnovich, and Lanen (1945) and Levine et al. (1949), using the same yeast, investigated the industrialization of riboflavin production. Nagamichi and Ano (1949) found riboflavin formation by yeast belonging to the genus Debaryomyces, and Mitra (1949) obtained the same results with top yeast.

Recently, we have found riboflavin excretion of about 30 μg per ml of culture medium by Zygosaccharomyces soja. Riboflavin production in the Zygosaccharomyces group of yeasts has not been previously reported.

Whiffen, Bohonos, and Emerson (1946) discovered a physiologically active by-product of streptomycin production by Streptomyces griseus. After the structure of this substance had been determined, it was given the generic name cycloheximide.

Cycloheximide has been studied by Greig, Walk, and Gibbons (1958) and by many other investigators. It has been apparent from their experiments that cycloheximide is commonly antagonistic to yeasts and fungi, but there are still many unclarified points in its activity. When cycloheximide was added to a growing culture of Z. soja, under aerobic conditions, growth inhibition soon became evident. But, after a period of time, a rapid recovery of growth occurred, accompanied by excretion of large amounts of riboflavin. Furthermore, it became apparent that the route of glucose assimilation by riboflavin-forming cells was converted to aerobic respiration, in place of the fermentation typical of normal cells. In this study, we have attempted to clarify the mechanism of action of cycloheximide and the inter-relationship between growth recovery and riboflavin formation.

MATERIALS AND METHODS

Organism. The organism used in this study was a strain of Z. soja isolated from soy sauce mash.

Growth medium. The growth medium was composed of glucose, 7%; (NH4)2SO4, 1%; KH2PO4, 0.5%; MgSO4·7H2O, 0.1%; KCl, 0.1%; ZnCl2,
7 \times 10^{-5} \, \text{M}; \text{Ca-pantothenate, inositol, and thiamine, all } 1 \times 10^{-5} \, \text{M}; \text{and biotin, } 1 \times 10^{-4} \, \text{M}; \text{the pH was adjusted to 5.0. The organism was cultured in the medium, with shaking, at 30 C. An inoculum of } 10^4 \text{ cells per ml was employed.}

**Measurement of growth.** Turbidity was measured at 500 nm with a Hitachi E-type photometer. Dry weight of the cells was estimated from the weight-turbidity curve previously calibrated for this organism.

**Measurement of sugar.** The amount of reducing sugar was measured by the Bertrand (1906) method.

**Cycloheximide.** Cycloheximide was supplied through the courtesy of the Tanabe Pharmaceuticals Co., Ltd., Tokyo, Japan.

**Identification of riboflavin.** Riboflavin in the culture medium was extracted by Crammer's (1948) method, and was identified by paper chromatography, paper ionophoresis, absorption spectrum, and absorption ratio.

The riboflavin was estimated photometrically with a Hitachi E-type photometer at 430 nm using a previously calibrated standard curve for authentic riboflavin.

**Manometric method.** Oxygen uptake and carbon dioxide evolution were measured in a Warburg flask at pH 6. A suspension of intact cells was used. Exponentially growing cells were harvested, washed several times with water, and kept at 5 C overnight. This washed cell preparation had no endogenous respiration. The dry weight of these resting cells was 100 mg per ml of suspension.

**Cell-free extract.** According to the method of Hochster and Quastel (1951), the cells were harvested in the logarithmic phase of growth, centrifuged at 3000 rev/min for 10 min, washed three times with 0.05 M phosphate buffer (pH 6), suspended in the same buffer, and again centrifuged at 3000 rev/min for 10 min. The washed cells were put into a mortar previously chilled with ice, and were vigorously ground with glass powder for 30 min. A small amount of 0.05 M phosphate buffer (pH 6) was added and allowed to act for 10 min with occasional shaking. The homogenized suspension was then centrifuged at approximately 3000 rev/min at 4 C for 10 min. The opalescent supernatant liquid thus obtained was dialyzed against cold water for 3 hr. This dialyzed preparation was used as the cell-free enzyme preparation.

**Dehydrogenase activity.** Decolorization time of methylene blue in the reaction mixture containing each substrate and enzyme preparation was measured under reduced pressure with Thunberg tubes.

**RESULTS**

**Effect of concentration of cycloheximide on growth of Z. soja.** Whiffen et al. (1946) pointed out that cycloheximide shows considerable selectivity in inhibition of growth of various genera of yeasts. Figure 1 shows the effect of cycloheximide on the growth of Z. soja. At concentrations of 5 \mu g or more per ml of medium, growth of this strain was completely inhibited.

**Recovery from cycloheximide inhibition.** From Fig. 1, it is apparent that exponentially growing cells were affected by 1 \mu g per ml cycloheximide, and that the growth of this strain was completely inhibited by 5 \mu g per ml. When shaking was continued for about 70 hr in the presence of cycloheximide, this strain suddenly recovered from the inhibitory effect (Fig. 2) and at the same time comparatively large amounts of a yellow pigment were excreted.

**Identification of the yellow pigment.** Prior to the investigation of relationships between the recovery from growth inhibition and the excretion of a yellow pigment, the identification of this pigment was carried out. Under ultraviolet light, this pigment revealed characteristic fluorescence resembling riboflavin. Identification was carried out to find whether it was, indeed, riboflavin. Paper chromatographic studies in-

![FIG. 1. Effect of concentration of cycloheximide (CHI) on the growth of Zygosaccharomyces soja.](http://jb.asm.org/)

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dicated that the R_f values of the pigment were in good agreement with those of authentic riboflavin in various solvent systems (Table 1). Paper ionophoretic behavior of the yellow pigment was coincident with that of riboflavin (Table 2). The absorption spectra and absorption ratios (Table 3) indicate that the pigment agrees exactly with riboflavin.

Addition of a small portion of diethyl ether to the solution of yellow pigment (eluted with water from a paper chromatogram), followed by addition of sodium hydrosulfite, caused both the yellow color and the fluorescence to disappear. Hydrogen peroxide was added to the reduced pigment solution, and both the yellow color and the fluorescence were restored.

The pigment was insoluble in chloroform, but was soluble in phenol and p-cresol.

An alkaline solution (1 N NaOH) of the isolated yellow pigment was exposed to daylight for about 6 hr. Lumiflavin was verified as the photolysis product.

**Table 1.** R_f values in various solvent systems

<table>
<thead>
<tr>
<th>Pigment</th>
<th>B-A-W</th>
<th>W-isoA</th>
<th>B-P-W</th>
<th>Ph-B-W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow pigment</td>
<td>0.26</td>
<td>0.45</td>
<td>0.48</td>
<td>0.77</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.26</td>
<td>0.45</td>
<td>0.48</td>
<td>0.77</td>
</tr>
<tr>
<td>Riboflavin phosphate</td>
<td>0.01</td>
<td>0.96</td>
<td>0.01</td>
<td>0.18</td>
</tr>
<tr>
<td>Riboflavinylglucoside</td>
<td>0.15</td>
<td>0.56</td>
<td>0.43</td>
<td>0.61</td>
</tr>
</tbody>
</table>

* Butanol-acetic acid-water (4:1:1).
* Water saturated with isoamyl alcohol.
* Butanol-pyridine-water (6:4:3).
* Phenol-butanol-water (16:3:4).

**Table 2.** Distance of movement during paper ionophoresis

<table>
<thead>
<tr>
<th>Pigment</th>
<th>0.05 M Phosphate buffer (pH 7.0)</th>
<th>0.05 M Acetate buffer (pH 3.2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow pigment</td>
<td>-30 (mm)</td>
<td>+32 (mm)</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>-30</td>
<td>+32</td>
</tr>
<tr>
<td>Riboflavinylglucoside</td>
<td>-40</td>
<td></td>
</tr>
</tbody>
</table>

* Voltage constant: 500 v; time: 2 hr.
* Voltage constant: 500 v; time: 5 hr.

**Table 3.** Absorption spectra and absorption ratios

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Absorption spectrum</th>
<th>Absorption ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max.</td>
<td>Min.</td>
</tr>
<tr>
<td></td>
<td>260:450</td>
<td>375:450</td>
</tr>
<tr>
<td>Yellow pigment</td>
<td>2.30</td>
<td>0.88</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>2.27</td>
<td>0.87</td>
</tr>
</tbody>
</table>

From these results, it was concluded that the yellow pigment was identical with riboflavin.

**Relationship between growth recovery and riboflavin excretion.** In control cultures, both growth and sugar consumption proceeded at parallel rates during 148 hr of incubation. Throughout this period, alcoholic fermentation was very active. The yeast used always reached the stationary phase of growth within a comparatively short time (when the negative logarithm of the turbidity had reached about 3), but with prolonged incubation, growth resumed after 120 hr (Fig. 3). This phenomenon may be the result of the formation of adaptive enzymes under aerobic conditions.

Novelli and Lipmann (1950) found that fully grown cells of Saccharomyces cerevisiae completely oxidized both glucose and acetate. Eaton and Klein (1954) found that ethyl alcohol was oxidized by the same mechanism. With Z. soja, ethyl alcohol previously produced during the first logarithmic phase of growth gradually disappeared from the medium during the second logarithmic growth phase. Cells in the second
logarithmic growth phase might assimilate ethyl alcohol by the same mechanism described by Novelli and Lipmann (1950) and Eaton and Klein (1954). We conclude that the Embden-Meyerhof-Parnas (EMP) pathway is the main pathway of glucose metabolism during the first logarithmic phase. After the induction period, however, oxidation becomes the main route of glucose, ethyl alcohol, and acetate utilization. When cycloheximide was added to the culture, a distinct inhibition of both growth and sugar consumption occurred. When the second logarithmic growth phase was reached, the yeast suddenly began to consume glucose and to grow rapidly, and the amount of riboflavin excreted in the medium was much larger than in the control (Fig. 4).

Glucose assimilation by normal and cycloheximide-treated cells. Glucose metabolism of resting cells harvested from normal and from cycloheximide-treated cultures was investigated by Warburg manometric methods (Table 4).

Cells forming no riboflavin fermented glucose mainly to ethyl alcohol, so the EMP pathway must be their main route of glucose metabolism. On the other hand, the riboflavin-forming cells apparently utilized glucose by oxidative pathways.

Latuasan and Berend (1958) verified the inhibition of alcohol dehydrogenase activity by cycloheximide. If the EMP pathway were the main route of glucose metabolism in our yeast, obvious inhibition of glucose assimilation should result. But only 12% inhibition of O₂ uptake and 19% of CO₂ evolution were obtained (Table 4).

Growth was completely inhibited by 5 μg/ml of cycloheximide, as previously indicated (Fig. 1); this concentration was only one-twentieth of the concentration of cycloheximide used for the manometric experiment. These results show that in this yeast the site of inhibition of growth is different from the sites of enzyme activity involved in glucose fermentation or respiration.

Kielhöfer and Aumann (1957) also investigated the action of cycloheximide on yeast. From their results, it was verified that the zymase complex prepared from maceration juice of yeast was not affected by cycloheximide, and that with intact cells of wine yeast neither the endogenous fermentation nor fermentation in the presence of added substrate was affected. From those
results, they considered that growth inhibition under aerobic conditions was due to the inhibition of enzyme synthesis or cell synthesis; namely, anabolism inhibition.

Our findings may agree with their conclusions. In our manometric experiments (Table 4), resting cells were used as the "enzyme preparation," and only glucose was used as the substrate. Since no nitrogenous substance was added to the reaction mixture, it was not a situation involving so-called "multiplication." New synthesis of enzymes or proteins is ruled out under these conditions, so that glucose catabolism must be caused only by inherent enzymes in the resting cells. Therefore, it is considered that cycloheximide inhibition is not an inhibition of fermentation and respiration, but probably the inhibition of new synthesis of enzymes or proteins. Further studies on these problems are now in progress.

In contrast to the normal cells, riboflavin-forming cells of this yeast metabolized glucose

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cycloheximide</th>
<th>Nonriboflavin-forming cell, de-colorizing time</th>
<th>Riboflavin-forming cell, de-colorizing time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate</td>
<td>-</td>
<td>18.20</td>
<td>18.00</td>
</tr>
<tr>
<td>Ethanol</td>
<td>+</td>
<td>19.00</td>
<td>23.00</td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
<td>25.00</td>
<td>49.00</td>
</tr>
<tr>
<td>Succeedate</td>
<td>+</td>
<td>42.00</td>
<td>62.00</td>
</tr>
<tr>
<td>Hexose-di-phosphate</td>
<td>+</td>
<td>29.00</td>
<td>50.00</td>
</tr>
<tr>
<td>Malate</td>
<td>-</td>
<td>37.00</td>
<td>56.00</td>
</tr>
<tr>
<td>Citrate</td>
<td>-</td>
<td>60.00</td>
<td>55.00</td>
</tr>
</tbody>
</table>

a Thunberg tubes contained the following final concentrations of reactants: glucose, 10 mM; methylene blue, 100 μM; cycloheximide, 350 μM; 0.02 mM phosphate buffer (12 μmole of Mg, Mn, and diphenophosphate nucleotide were present in 10 ml of buffer); cell-free extract, 0.5 ml. Total volume of mixture was 3.0 ml. The pH was 6.0, the temperature was 30 C, and the reaction was carried out under reduced pressure.

through oxidative pathways. This observation corresponds to the fact that the respiratory yeast was not affected by cycloheximide (Table 4).

Various dehydrogenase activities in cell-free extracts. Since cycloheximide inhibition of thezymase complex system was not detected, various dehydrogenase activities of this yeast in the presence or absence of cycloheximide were investigated. The results are shown in Table 5.

Essential differences between riboflavin-forming cells and cells not forming riboflavin were not detected. Thus, the finding of Latuasan and Berend (1958) that cycloheximide inhibits dehydrogenase is contrary to our observation.

In both riboflavin-forming cells and cells not forming riboflavin, the activity of glucose-6-phosphate dehydrogenase was the strongest of the dehydrogenases examined. This fact indicates that the hexose-monophosphate shunt (HMP shunt) operated extensively in this yeast. Dehydrogenases related to the tricarboxylic acid cycle intermediates were weak in both riboflavin-forming cells and in cells not forming riboflavin; only succinate was dehydrogenated to any extent.

DISCUSSION

Sisler and Marshall (1957) reported that respiration was inhibited by cycloheximide in experiments using young cells of S. pastorianus. Walker and Smith (1952) also obtained the same results. However, inhibition of respiration could scarcely be recognized from our results. Then the question arose concerning the point of inhibition of a growing culture of Z. soja by cycloheximide.

McCallan, Miller, and Weed (1954) investigated the action of cycloheximide upon spore germination and respiration, and upon mycelial growth and respiration of various molds, and found that cycloheximide affects growth rather than respiration. Lardy, Johnson, and McMurray (1958) also found, from their experiment with mitochondria of rat liver, that cycloheximide did not inhibit respiration or oxidative phosphorylation. Our findings seem to support these results.

Kerridge (1958) demonstrated that cycloheximide at concentrations of 0.5 to 1.0 μg per ml inhibited both protein synthesis and DNA synthesis in S. carlsbergensis. When we consider this finding in connection with the findings of
Kielhöfer and Aumann (1957), cycloheximide action on *Z. soja* may be an inhibition of anabolism.

The remarkable inhibition by cycloheximide of the growth of *Z. soja* can not be explained by the slight depression of glycolysis and respiration which occur. The problem is to find a relationship that applies to recovery from growth inhibition, conversion of the mode of glucose metabolism, and riboflavin formation.

Multiplication of organisms represents anabolism and requires a supply of energy. But, as shown in Fig. 3, this strain gradually consumed glucose to some extent, even after immediate inhibition of growth by cycloheximide occurred. It appears that the glucose-metabolizing systems, for instance glycolysis or the HMP shunt, might be operating even though to a lesser extent, and it is excluded that growth inhibition was caused merely by the inhibition of such systems. It is considered that cycloheximide uncouples ATP formation or blocks ATP uptake in cell synthesis (nucleic acid or protein synthesis).

The next problems are riboflavin formation and the conversion of glucose metabolism. The fact that the conversion of glucose assimilation from glycolysis to respiration is accompanied by the excretion of riboflavin suggests the presence of a relationship. The physiological meaning of riboflavin excretion and the pathway of riboflavin biosynthesis in this yeast are under investigations.

LITERATURE CITED


