THE EFFECT OF GLYCINE AND PROTOPORPHYRIN ON A CYTOCHROME DEFICIENT YEAST

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A strain of yeast showing no respiratory activity and no absorption bands corresponding to cytochrome components on spectroscopic examination was isolated by Caroline Raut (1953) after ultraviolet irradiation of a haploid culture of Saccharomyces cerevisiae. Through the kindness of Dr. Raut, a segregant (strain 1914) of the original deficient isolate (W-1) was made available for further investigation. The work reported here was designed to investigate the nature of the cytochrome deficiency in this mutant.

MATERIALS AND METHODS

Growth media of the following composition were employed: Complete medium: 1.0 per cent bacto-peptone, 1.0 per cent yeast extract, and 2.0 per cent glucose, made to volume with tap water.

Wickerham’s medium: A commercial powdered preparation (bacto yeast nitrogen base) from Difco Laboratories which, when made up to volume with distilled water, is stated to contain the following: Trace elements, vitamins, amino acids, and salts per liter. H2BO3, 500 μg; CuSO4·5H2O, 40 μg; KI, 100 μg; FeCl3·6H2O, 200 μg; MnSO4·H2O, 400 μg; Na2MoO4·2H2O, 200 μg; ZnSO4·7H2O, 400 μg; biotin, 2 μg; Ca panthotenate, 400 μg; folic acid, 2 μg; inositol, 2,000 μg; niacin, 400 μg; p-aminobenzoic acid, 200 μg; pyridoxine hydrochloride, 400 μg; riboflavin, 200 μg; thiamin hydrochloride, 400 μg; L-histidine monohydrochloride H2O, 10 mg; dl-methionine, 20 mg; dl-tryptophan, 20 mg; KH2PO4, 1.0 g; MgSO4·7H2O, 0.5 g; NaCl, 0.1 g; CaCl2·2H2O, 0.1 g; and (NH4)2SO4, 5.0 g.

Minimal medium: The minimal medium used in this study contained the following: KH2PO4, 0.1 percent; K2HPO4, 0.01 percent; MgSO4·7H2O, 0.05 percent; CaCl2, 0.01 percent; NaCl, 0.01 percent; (NH4)2SO4, 0.2 percent; thiamin, 40 μg per cent; pyridoxine, 40 μg per cent; biotin, 0.2 μg per cent; L-tryptophan, 1.0 mg per cent; L-histidine, 1.0 mg per cent; DL-methionine, 5 μg per cent; B(OH)3, 5.7 μg per L; CuSO4, 350 μg per L; FeCl3, 580 μg per L; MnSO4, 54 μg per L; (NH4)2MoO4, 40 μg per L; ZnSO4, 8,800 μg per L; and glucose, 2 per cent. The minimal medium was made up with distilled water.

The yeast was examined spectroscopically, after reduction with hydrosulfite, as 40 to 50 per cent suspensions of washed cells. A Zeiss hand spectrocope and cuvettes of 1 cm thickness were employed. With this instrument, the position of the absorption bands can be determined only approximately. In the text, the cytochrome c band refers to the thermostable band at 550 mμ, and cytochromes a and b to thermolabile bands at 603 and 564 mμ, respectively. Cytochrome b1 (Ephrussi and Slonimski, 1950) refers to the diffuse thermolable band, at approximately 550 to 560 mμ, present in anaerobically grown yeast.

The presence of pyridine hemochromogen was demonstrated by the appearance of an absorption band at 555 mμ after addition of alkali, hydrosulfite, and pyridine to yeast suspensions which had been heated previously to destroy the thermolabile cytochrome components (Ephrussi and Slonimski, 1950). No method was available for the quantitative estimation of the cytochromes in the intact cells.

Cytochrome c was extracted and determined by the procedure of Borei and Sjoden (1943) utilizing a Beckman spectrophotometer.

Catalase was determined by the method of Feinstein (1949) using perborate as substrate at pH 6.8 and an incubation time of 5 minutes. Dilutions of washed cells were used which gave catalase reading proportional to yeast concentration. Readings were corrected for the blank...
due to reduction of permanganate by the cells. The activity of catalase is expressed as ml of 0.01 N permanganate reduced per mg dry weight of yeast.

Dry weights of yeast were determined by drying aliquots of cell suspensions in distilled water on tared watch glasses at 110 C for two hours and then weighing.

Growth was measured as turbidity using a Klett colorimeter with a red filter.

The dimethyl ester of protoporphyrin IX was prepared from hemoglobin powder by the method of Grinstein (1947). It was recrystallized twice from chloroform-methanol and gave a mp of 224 to 225 C (corrected). The desired amount of ester was hydrolyzed in 25 per cent HCl as directed by Grinstein, and 0.33 ml of the solution of protoporphyrin in HCl was added directly to each 100 ml of autoclaved complete medium, bringing the pH to 3.5. Wickerham's and minimal media were buffered with 0.1 M succinate to pH 7.0, which fell to pH 5.4 after addition of the same quantity of protoporphyrin-HCl solution. Controls received the equivalent amount of HCl without protoporphyrin. In a few experiments the protoporphyrin was dried in vacuo over NaOH, taken up in 50 per cent ethanol 0.02 M in NaOH, and added to the autoclaved medium. It was found that changes in pH and addition of succinate buffer due to the above procedures had no significant effect on catalase production.

Yeast cultures were grown without shaking at 30 C. Shaken cultures were grown on a rotary shaker at room temperature (ca 25 C) using 250 ml of medium in 1 liter Erlenmeyer flasks. The rate of shaking gave sufficient aeration to induce formation of the complete cytochrome system in wild type yeast.

The methods of Lindegren (1949) were used in making matings and ascus dissections.

RESULTS

When grown in deep standing cultures of complete medium for 1 to 3 days, strain 1914 showed no cytochrome bands upon spectroscopic examination. No pyridine hemochromogen was detected upon addition of pyridine and alkali under reducing conditions, either before or after heating the cells. Catalase was present only in traces. Under the same conditions shaken cultures were found to show a faint cytochrome c band, would form a pyridine hemochromogen, and showed definite, although weak, catalase activity. In neither case did washed suspensions of the yeast show any uptake of oxygen with glucose as substrate.

In order to define the conditions needed for the production of cytochrome and catalase, the nutritional requirements of the mutant were investigated. It was found that the mutant would grow on a medium containing biotin, thiamin, pyridoxine, histidine, tryptophan, and either lysine, methionine, or valine. Growth on such a medium, here referred to as minimal medium, is rather poor. The cells grown with or without shaking were totally deficient in cytochromes and in substances forming pyridine hemochromogen. They showed only very slight catalase activity, and no oxygen uptake with glucose as substrate. The same results were obtained using Wickerham's medium which is essentially the same as minimal medium.

The above observations suggested that the mutant might be deficient in its ability to synthesize protoporphyrin. Addition of 5 μg per ml of protoporphyrin to standing cultures in complete medium resulted in the production of cells which had strong catalase activity and which showed the absorption bands of cytochrome c and b.

Since protoporphyrin has an absorption band which is close to that of cytochrome c, more conclusive proof of the presence of cytochrome c was desirable. Therefore, the mutant was grown in 6 L Erlenmeyer flasks with and without addition of 2 μg per ml of protoporphyrin in 4 liters of complete medium for 5 days, and extracted for cytochrome c. The absorption spectrum of the reduced and oxidized extracts was examined using the Beckman spectrophotometer. Up to 2.7 mg of cytochrome c per 100 g wet weight of yeast was found in cultures grown in the presence of protoporphyrin. Extracts of controls grown without protoporphyrin contained no cytochrome c.

Addition of protoporphyrin to Wickerham's medium also resulted in the production of cytochrome c and catalase. The absorption band of cytochrome c was more distinct in shaken cultures. The effect of varying amounts of protoporphyrin on catalase activity is shown in figure 1. Catalase activity was destroyed by heating
cells for 1 minute at 100 C and thus is presumably enzymatic and not due to the nonspecific activity of hematin.

Addition of protoporphyrin to both complete and Wickerham's media resulted in increased yields of yeast. When cultivated in complete medium plus protoporphyrin the cells showed a change in growth habit, growing clumped together as a layer on the bottom of the flask. This clumping was not evident in minimal medium plus protoporphyrin. Because of the clumping, growth could not be measured by turbidity reading. Instead, the cultures were centrifuged in graduated centrifuge tubes and the volumes noted. Table 1 shows the effect of protoporphyrin on growth in both media.

Since growth of strain 1914 is much poorer in minimal medium than in complete, the effect of added amino acids on growth in minimal medium was examined. Glycine was found to stimulate growth greatly (figure 2). Addition of glycine resulted also in the production of catalase although larger concentrations of glycine were needed for catalase production than for growth stimulation (figure 3). In view of the large

![Figure 1. Effect on catalase activity of protoporphyrin added to Wickerham's medium. Four days at 30 C, unshaken.](image)

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<thead>
<tr>
<th></th>
<th>COMPLETE</th>
<th>WICKERHAM'S</th>
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<tr>
<td>No addition</td>
<td>4.2</td>
<td>2.0</td>
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<tr>
<td>2 µg Protoporphyrin per ml</td>
<td>6.8</td>
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![Figure 2. Effect of glycine on growth in minimal medium. Four days at 30 C, unshaken.](image)

![Figure 3. Effect on catalase activity of glycine added to Wickerham's medium. Seven days at 30 C, unshaken cultures.](image)
amounts of glycine required for catalase production, the presence of some active impurity in the glycine could be suspected, but appears improbable, as the glycine used was a synthetic product and recrystallization did not change its activity.

The effect of glycine on cytochrome formation in Wickerham's medium was also examined. Cultures were grown on a shaker with and without glycine (6.6 mM) and under atmospheres of air and O₂-free nitrogen. Glycine containing cultures showed the cytochrome c band (550 nm, stable to boiling) when grown under air, and the b₁ band (555 to 560 nm, destroyed by boiling) when

![Graph](Image)

**Figure 4.** Effect of nitrogen source on growth in minimal medium, sole nitrogen source glycine + (NH₄)₂SO₄. Total nitrogen equivalent to 27 mM glycine. Four days at 30°C, unshaken.

grown under nitrogen. Control cultures without added glycine showed no trace of any hematin compounds. As would be expected from the absence of cytochromes a and b, none of the cultures showed any oxygen uptake with glucose as substrate.

Glycine alone is a poor nitrogen source (figure 4). A functioning glycine oxidase system could not be demonstrated with intact cells.

The effect of glycine on catalase and cytochrome formation appears to be specific. Twenty amino acids were tested singly at a concentration of 0.01 M in minimal medium for their effect on the growth and catalase production of strain 1914. This high concentration was used because glycine affects catalase at comparable concentrations. As expected, addition of certain amino acids had considerable effect on the growth of the mutant. The following amino acids were tested (the number after the amino acid refers to the turbidity of the culture after five days' growth in colorimeter tubes at 30°C): control (minimal medium), 37; DL-threonine, 92; L-phenylalanine, 0; DL-methionine, 34; L-leucine, 44; L-hydroxyproline, 29; DL-valine, 49; L-lysine, 81; L-arginine, 72; L-cysteine, 3; L-glutamic acid, 22; L-histidine, 74; L-norleucine, 11; DL-serine, 49; DL-norvaline, 3; glycine, 153; DL-alanine, 51; DL-isoleucine, 67; L-tryptophan, 15; L-aspartic acid, 51; L-tyrosine (saturated solution), 0. Parallel cultures grown in unshaken flasks under the same conditions were titrated for catalase activity where the amount of growth was sufficient for such a determination. Lysine, arginine, hydroxyproline, methionine, alanine, isoleucine, leucine, threonine, histidine, valine, serine, and aspartic acid produced no appreciable catalase activity, glycine alone being active in this respect. Neither did addition to minimal medium of folic acid, vitamin B₁₂, acetate, formate, yeast nucleic acid, yeast nucleic acid hydrolyzate, adenine, guanine, thymine, uracil, nor cytosine result in the production of catalase activity although formate, adenine, and yeast nucleic acid hydrolyzate stimulated growth.

Although the above results show that addition of glycine and protoporphyrin to the medium in which strain 1914 is grown results in formation of catalase and cytochrome c, such additions do not make it physiologically equivalent to normal yeast. Cytochromes a and b are not formed and the cells do not respire. This suggests that a further deficiency not repairable by addition of glycine and protoporphyrin is present.

The absence of cytochromes a and b suggested a similarity to the "petite colonie" strains described by Ephrussi, Hottinguer, and Tavlitzki (1949) and Slonimski and Ephrussi (1949). Studies by these authors have led them to conclude that the petite strains lack an autoreproducible cytoplasmic factor which is needed for the formation of cytochromes a and b on the basis, in part, of the following observations. Fusion of petite and normal cells of opposite mating type produces zygotes with a normal cytochrome system. Sporulation of such zygotes gives spores, all of which
have the normal cytochrome system. Fusion of two petites produces zygotes with the petite phenotype. Further genetic analysis of such zygotes is not possible unfortunately as they do not sporulate, presumably because of the respiratory deficiency.

To determine whether strain 1914 is deficient in cytoplasmic factor, several crosses were made between it and petite strains. The zygotes were grown in complete medium and proved to be of the petite phenotype, with a strong cytochrome c band, strong catalase activity, and no cytochromes a and b. These results indicate that strain 1914 is indeed deficient in the cytoplasmic factor postulated by Ephrussi and his co-workers and thus explain the absence of respiration and of cytochromes a and b in this strain.

The deficiency repaired by addition of glycine and protoporphyrin shows a normal Mendelian segregation. Eleven four-spore asci obtained by crossing strain 1914 to normal strains were dissected. The progeny of these crosses was examined for the cytochrome deficiency as well as for the segregation of the following markers: mating type, fermentation of sucrose and galactose, and growth requirements for histidine and tryptophan. For examination of the cytochrome spectrum the segregates were grown on a shaker in complete medium for 24 to 48 hours. Catalase activity was qualitatively determined by suspending standard amounts of yeast into 3 per cent H₂O₂ and observing the evolution of oxygen.

It was found that the cytochrome and catalase deficiency segregated in all eleven asci in the ratio of two deficiencies to two normals. The deficiencies showed only slight catalase activity, a reduced rate of growth, and only a weak or no cytochrome c band. The growth of all cytochrome deficiencies in Wickerham's medium was stimulated by 0.01 M glycine. The normal progeny was either unaffected by glycine or showed varying degrees of inhibition, except for one strain which was slightly stimulated. The cytochrome deficiency segregated independently of the other markers present.

George Ridgeway has reexamined the above segregants after a lapse of several months. He has kindly informed us that most of the segregants originally found to be cytochrome deficient, while still showing a reduced rate of growth, now show a weak to moderately strong cytochrome spectrum of all three components. Stimulation by glycine can no longer be demonstrated clearly. These results would seem to indicate a reversion, presumably genetic, toward normal, but the exact nature of the phenomenon requires further study.

**DISCUSSION**

Since strain 1914 is deficient in cytochromes and catalase when grown in minimal medium and since both catalase and cytochrome c are produced if protoporphyrin is added, some metabolic block involving the synthesis of protoporphyrin is indicated. The very marked stimulation of growth by glycine and the parallel genetic segregation of the cytochrome and catalase deficiency and glycine requirement indicate that the mutant is deficient in its ability to synthesize glycine. The glycine deficiency is only partial since some growth occurs in the absence of added glycine. Glycine is known to be a source of the nitrogen of protoporphyrins (Shemin and Rittenberg, 1945). Therefore, the catalase and cytochrome deficiency is due most probably to lack of substrate for porphyrin synthesis. An examination of figures 2 and 3 shows that more glycine is required for the production of catalase (and presumably protoporphyrin) than for growth stimulation. It appears that the small amount of glycine synthesized by the mutant is used preferentially for purposes other than protoporphyrin synthesis, and that protoporphyrin is produced only when an excess of glycine is available. Which step in glycine synthesis is deficient cannot be determined from the available data. However, it is suggestive that serine shows no glycine-like activity. Serine and glycine are known to be interconvertible in other systems (Rittenberg, 1948).

If the above interpretation of the nature of the metabolic block is correct, addition of any intermediate between glycine and protoporphyrin should result in catalase production providing, of course, that it can penetrate into the cells. Through the kindness of C. Rimington of the University of London, a sample of coproporphyrin III was obtained. This compound has been suggested often as a precursor of protoporphyrin. When added to cultures of strain 1914 under the same conditions as protoporphyrin it produced no catalase activity.

There is no explanation at the moment for the effect of shaking on the production of catalase and cytochrome c in complete medium. As men-
tioned in the experimental section, unshaken cultures in complete medium show no trace of any hematin compound, but if shaken, cytochrome c, catalase, and a substance giving a hemochromogen with pyridine are produced. Presumably the production of porphyrin derivatives is due to the glycine content of complete medium. Shaking, however, is not necessary for the production of catalase in minimal medium with added glycine.

The result obtained by crossing strain 1914 with a petite shows that strain 1914 also lacks the cytoplasmic factor (Ephrussi, Hottinguer, and Tavlitzi, 1949) that is needed for the production of cytochromes a and b adaptively in the presence of oxygen (Slonimski, 1950). The results obtained by growing the mutant under air and nitrogen in the presence of glycine are in agreement with this interpretation. Under nitrogen, the b, component of the cytochromes is produced, and under air, cytochrome c. The same phenomenon has been demonstrated with the petite type of yeast (Slonimski, 1950). The fact that no oxygen uptake can be observed with strain 1914 under any condition so far tried thus is explained readily by the fact that strain 1914 is deficient in the cytoplasmic factor.

ACKNOWLEDGMENTS

We wish to thank Dr. Herschel Roman and Dr. Howard Douglas for advice and assistance. Dr. Boris Ephrussi kindly informed us of his own genetic results which indicated that strain 1914 was lacking in cytoplasmic factor before we had obtained similar results.

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SUMMARY

The cytochrome deficient mutant of Saccharomyces cerevisiae isolated by Raut was found to be deficient in catalase, cytochrome c, and hematin when grown in minimal media. Addition of either protoporphyrin IX or glycine resulted in formation of catalase, cytochrome c, and hematin. The quantitative response of growth and catalase production on addition of glycine indicates that the mutant is primarily a slow glycine synthesizer, and that the deficiency of catalase and cytochrome is a secondary result of an insufficiency of glycine which is known to be a precursor of protoporphyrin. Genetic results indicate that the deficiency is due to a single gene. Absence of cytochromes a and b under any conditions so far tested is due to the fact that the mutant is deficient for a cytoplasmic factor required for the synthesis of these components.

REFERENCES


Raut, C. 1953 Experimental cell research, to be published.


