REFERENCES

- Brady, R. O. & Barker, H. A. (1961). *Biochem. biophys. Res. Commun.* **4**, 464.
- Buchanan, M. J., Larrabee, A. R., Cathou, R. E. & Rosenthal, S. (1961). *Abstr. Pap. Amer. chem. Soc., 140th Meet.*, p. 8c.
- Foster, M. A., Dilworth, M. J. & Woods, D. D. (1964a). *Nature, Lond.*, **201**, 39.
- Foster, M. A., Jones, K. M. & Woods, D. D. (1961). *Biochem. J.* **80**, 519.
- Foster, M. A., Tejerina, G., Guest, J. R. & Woods, D. D. (1964b). *Biochem. J.* **92**, 476.
- Gibson, F. & Woods, D. D. (1960). *Biochem. J.* **74**, 160.
- Guest, J. R. (1960). D.Phil. Thesis: University of Oxford.
- Guest, J. R., Foster, M. A. & Woods, D. D. (1964a). *Biochem. J.* **92**, 488.
- Guest, J. R., Friedman, S., Dilworth, M. J. & Woods, D. D. (1964b). *Ann. N.Y. Acad. Sci.* **112**, 774.
- Guest, J. R., Friedman, S. & Foster, M. A. (1962). *Biochem. J.* **84**, 93r.
- Guest, J. R., Friedman, S., Woods, D. D. & Smith, E. L. (1962). *Nature, Lond.*, **195**, 340.
- Guest, J. R., Helleiner, C. W., Cross, M. J. & Woods, D. D. (1960). *Biochem. J.* **76**, 396.
- Kisliuk, R. L. & Woods, D. D. (1960). *Biochem. J.* **75**, 467.
- Mangum, J. H. & Scrimgeour, K. G. (1962). *Fed. Proc.* **21**, 242.
- Peterkofsky, A., Redfield, B. & Weissbach, H. (1961). *Biochem. biophys. Res. Commun.* **5**, 213.
- Rohrbaugh, P. (1962). *Fed. Proc.* **21**, 4.
- Rooze, V., Sakami, W. & Blair, D. (1962). *Fed. Proc.* **21**, 4.
- Rosenthal, S. & Buchanan, M. J. (1962). *Fed. Proc.* **21**, 470.
- Sakami, W. & Uktins, I. (1961). *J. biol. Chem.* **236**, rc50.

Biochem. J. (1964), **92**, 504

Effect of Cysteine on Respiration and Catalase Synthesis by *Saccharomyces cerevisiae*

By C. BHUVANESWARAN* AND A. SREENIVASAN*

Central Food Technological Research Institute, Mysore

AND D. V. REGE

University Department of Chemical Technology, Bombay, India

(Received 16 September 1963)

Catalase synthesis can be induced in anaerobically grown cells of *Saccharomyces cerevisiae* by aeration (Chantrenne, 1954). In a study of the effect of various nitrogenous substances on the ability of anaerobically grown yeast cells to synthesize catalase, it was found that cysteine inhibited the catalase induction severely (Bhuvaneshwaran, Rege & Sreenivasan, 1961). In the present paper, the effect of cysteine on catalase synthesis and on respiration is examined in more detail.

MATERIALS AND METHODS

Yeast cultures and preparation of suspensions. The organism used was a brewing strain of *Saccharomyces cerevisiae* which was maintained by bi-weekly transfer on agar slants consisting of 2% (w/v) glucose, 1% (w/v) Bactopeptone and 0.3% yeast extract with 2% (w/v) agar.

For experimental purposes the organism was grown in a broth of the same composition but with the omission of agar for 20 hr. at 30° in stationary flasks. Practically anaerobic conditions were maintained by using 500 ml. conical flasks filled to the neck. Most of the yeast cells were at the bottom of the flask at the end of the incubation

period. The cells were harvested and washed twice with ice-cold distilled water by suspension and centrifuging at 10° and used at once.

The organism also grew well on a synthetic medium consisting (w/v) of NH₄Cl (1%), glucose (1%), KH₂PO₄ (1%), MgSO₄·7H₂O (0.01%) and CaCl₂ (0.01%). This medium after the omission of NH₄Cl served as the non-growth medium in aeration experiments; the pH was adjusted to 6.8.

Yeast cells (about 60–80 mg. dry wt.) were suspended in 10 ml. of this medium and were aerated in 50 ml. conical flasks by shaking in a reciprocating shaker at 60 oscillations/min. for 4 hr. A colorimeter calibrated in terms of dry weight facilitated the adjustment of yeast concentration. The cells were then separated by centrifugation, washed with distilled water and resuspended in 10 ml. of water. Catalase activity of the cells was determined after rupture by repeated freezing and thawing.

Assay of catalase. Catalase was assayed according to a procedure based on the titanium colour reaction for hydrogen peroxide (Patti & Bonnet Maury, 1953) and developed in this Laboratory by S. P. Manjrekar & A. Sreenivasan (unpublished work). The titanium reagent was prepared as follows: 1 g. of TiO₂ and 10 g. of K₂SO₄ were mixed and digested with 150 ml. of conc. H₂SO₄ for 5–6 hr. The digest was then cooled and diluted to 1.5 l. with distilled water. This served as the stock solution and could be stored at room temperature. The working solution was obtained by diluting the stock solution fivefold with 10% (v/v) H₂SO₄.

* Present address: Biochemistry and Food Technology Division, Atomic Energy Establishment (Trombay), Bcyulla, Bombay 8, India.

To 10 ml. of buffered (phosphate, pH 6.8) $1.25 \mu\text{M}$ -hydrogen peroxide was added 2 ml. of the disrupted cell suspension and the whole was maintained at $0-4^\circ$. Then 2 ml. portions were removed at intervals and added to test tubes containing 5 ml. of the titanium reagent. This served to stop the enzymic reaction and the stable yellow colour produced was measured in a Klett-Summerson photoelectric colorimeter with a 42 filter. Catalase activity was expressed in terms of Kat.f. (units/ml.), where:

$$\text{Kat.f.} = \frac{\ln(X_0/X_t)}{et} \times 100$$

in which X_0 and X_t are the Klett readings corresponding to the residual hydrogen peroxide concentration at 0 and t min. and e is the concentration of the enzyme source in the reaction mixture.

Measurement of oxygen uptake. Manometric measurement of respiration was by the direct Warburg method at 37° and was expressed as $\mu\text{l. of O}_2/\text{hr./flask}$. Yeast cells were suspended in an aeration medium, which had the same composition as the non-growth medium with the omission of glucose, and adjusted to give 3–5 mg. dry wt. of yeast in each manometer flask. The yeast concentration was adjusted by using the calibrated colorimeter. The total volume in each flask was 3.2 ml., including 0.2 ml. of 10% (w/v) KOH used in the centre well to absorb carbon dioxide. Glucose was added from the side arm to give a final concentration of 1% (w/v) in the medium. Cysteine and other additions were added to give final concentrations indicated in the reaction system.

Chemicals. The compounds used in these experiments were obtained from the Nutritional Biochemicals Corp., E. Merck A.-G., British Drug Houses Ltd. and the California Corp. for Biochemical Research.

RESULTS

Nature of oxygen uptake by unadapted cells. There was a lag period in the oxygen uptake when the anaerobically grown cells were transferred to a medium containing glucose and phosphate and incubated under aerobic conditions. The initial rate of oxygen uptake of the same cells in the absence of glucose (or endogenous uptake) was greater than that in the presence of the substrate (Fig. 1).

Nature of oxygen uptake in semi-adapted cells. It is evident from Fig. 1 that, up to a period of 90 min., the endogenous rate of respiration by the cells exceeds that in the presence of the substrate. Beyond 90 min. there occurs a sudden increase in the system where substrate is present.

It was sought to avoid this lag phase by incubating a washed suspension of anaerobically grown yeast for 90 min. with glucose aerobically in the 'aerating medium'. After such incubation the cells were harvested, washed and resuspended in fresh lots of the aerating medium, and the oxygen uptake was measured.

For all other work relating to oxygen uptake reported in the present paper, the 'semi-adapted' yeast was used. Unadapted cells were, however, used for studying the induced synthesis of catalase.

Effect of cysteine on yeast respiration and catalase synthesis. At a concentration of 0.01 M, cysteine produced a fall in the oxygen uptake (Fig. 2). The effect of cysteine is seen at all stages of respiration tested. A parallel inhibition by cysteine in catalase synthesis is also seen from Fig. 3.

Effect of compounds related to cysteine. Table 1 summarizes the effect of other compounds related to cysteine on their ability to affect yeast respiration and catalase synthesis. Of the compounds tested, cystine and methionine were not inhibitory, whereas homocysteine and reduced glutathione showed slight inhibitory effects on both yeast respiration and catalase synthesis.

A number of other amino acids were tested for their ability to modify the effect of cysteine on catalase synthesis. These amino acids were added to the aeration medium, at the same concentration as that of cysteine, at the start of aeration. None of the amino acids tested showed any marked effect on catalase synthesis in the absence of cysteine. When they are present together with cysteine, they caused little or no decrease of the inhibition by cysteine.

Reversal of cysteine inhibition by Fe^{3+} ions. Ferric ammonium citrate at a concentration of 0.01 M- Fe^{3+} ion was added and reversed the cysteine inhibition of yeast respiration and catalase synthesis. No other metal ions (added as chlorides) tested were effective (Table 2).

Effect of ethanol on cysteine inhibition. Cysteine might also inhibit catalase activity by generating

Fig. 1. Uptake of oxygen in unadapted yeast. Cells grown anaerobically in glucose-peptone-yeast extract medium for 20 hr. were harvested and washed, and the oxygen uptake was measured. O, Endogenous; ●, with glucose (1%).

hydrogen peroxide by its autoxidation which then will combine with catalase to form the inactive catalase-hydrogen peroxide complex II (Chance, 1950). Alexander (1957) has shown that ethanol reverses this effect of cysteine by a coupled oxidation whereby it is oxidized to acetaldehyde. To study whether such coupled oxidation might prevent the inhibition of catalase synthesis, ethanol (0.01 or 0.02M) was added to the aeration medium at the start of aeration together with cysteine. No reversal of the inhibition was obtained.

DISCUSSION

The lag phase seen when anaerobically grown yeast is aerated (Fig. 1) may be due to the reorganization of metabolic pathways and the synthesis of new enzymes necessary to handle the substrate at higher concentration in an entirely different environment.

The suppression of yeast respiration and catalase synthesis by cysteine (Figs. 2 and 3) would mean that it tampers with the cell function in one or

Fig. 2. Effect of time of addition of cysteine on oxygen uptake by yeast. Cysteine (final concn. 0.01M) was added to aeration medium after: Δ , 0 min.; $\blacktriangle$, 10 min.; $\square$, 20 min.; $\blacksquare$, 30 min. $\bullet$, Endogenous; $\circ$, control without addition of cysteine.

Fig. 3. Effect of time of addition of cysteine on catalase synthesis. Cysteine (final concn. 0.01M) was added to the aeration medium after: $\bullet$, 0 hr.; Δ , 1 hr.; $\blacktriangle$, 2 hr.; $\square$, 3 hr. $\circ$, Control without addition of cysteine.

Table 1. *Effect of cysteine and related compounds on yeast respiration and catalase elaboration*

Cells obtained as described in the text were aerated for 4 hr. in the aeration medium. The final concentration of each added compound was 0.01M. The initial catalase activity was 27 ± 1.8 units. Each flask contained 10 ml. of yeast suspension (69 mg. dry wt.). The oxygen uptake was measured after adapting the cells for about 90 min. in the aeration medium. Each manometer flask contained 3 ml. of yeast suspension (4.95 mg. dry wt.).

Addition to aeration medium	O ₂ uptake (μ l./hr./flask)	Inhibition (%)	Catalase activity increase in 4 hr. (units/ml.)	Inhibition (%)
None	396 ± 4.1	—	252 ± 5.0	—
Cysteine	110 ± 3.2	72	6 ± 0.6	98
Cystine	399 ± 7.4	—	253 ± 5.5	—
Methionine	398 ± 4.3	—	249 ± 4.1	—
Homocysteine	276 ± 3.1	30	189 ± 4.3	25
Glutathione (reduced)	308 ± 4.4	22	218 ± 4.0	13

Table 2. *Reversal by metal ions of cysteine inhibition of yeast respiration and catalase synthesis*

The final concentration of each added compound was 0.01 M. The Fe^{3+} ions were added as ferric ammonium citrate. The other metal ions were added as the chlorides. The initial catalase activity was 28 ± 1.1 units. Each flask contained 10 ml. of yeast suspension (67 mg. dry wt.) and each manometer flask contained 3 ml. of yeast suspension (4.65 mg. dry wt.).

Addition to aeration medium	O_2 uptake ($\mu\text{l./hr./flask}$)	Inhibition (%)	Catalase activity increase in 4 hr. (units/ml.)	Inhibition (%)
None	374 ± 4.3	—	227 ± 4.9	—
Cysteine	113 ± 3.5	70	0	100
Fe^{3+} ions	370 ± 5.1	—	218 ± 4.8	—
Mn^{2+} ions	374 ± 5.2	—	218 ± 5.0	—
Zn^{2+} ions	377 ± 6.3	—	224 ± 4.0	—
Mg^{2+} ions	375 ± 6.0	—	217 ± 5.6	—
Cysteine + Fe^{3+} ions	257 ± 4.9	31	138 ± 6.2	39
Cysteine + Mn^{2+} ions	115 ± 4.9	70	0	100
Cysteine + Zn^{2+} ions	117 ± 3.5	70	0	100
Cysteine + Mg^{2+} ions	107 ± 4.7	71	0	100

more of the following ways: (i) it inhibits the synthesis of the oxidative enzymes necessary to carry out aerobic metabolic processes; (ii) it blocks steps in carbohydrate metabolism; (iii) it might act by forming complexes with any essential metal ions necessary for various enzyme functions; and, (iv) in the case of catalase activity, it might convert the catalase produced into inactive catalase-hydrogen peroxide complex by generating hydrogen peroxide by its autoxidation.

There is as yet no evidence to support the first two of the above hypotheses. Warburg (1949) has, however, reported that cysteine is an effective inhibitor of yeast aldolase. That cysteine does act by forming complexes with metal ions has been demonstrated by Bhuvaneswaran *et al.* (1961). Albert (1952) has reported that cysteine has an exceptionally high affinity for metal ions. Further proof is derived from the observation that Fe^{3+} ions are able to reverse the inhibitory effect of cysteine on both catalase synthesis and yeast respiration (Table 2). Again, it is substantiated by the fact that cysteine is able to inhibit both respiration and catalase synthesis by yeast even when added at various stages of adaptation (Figs. 2 and 3).

That the thiol group of cysteine is responsible for this inhibitory effect is suggested by the finding that under similar conditions other substances related to cysteine prove ineffective (Table 1). Cysteine is the only inhibitory amino acid among those tested.

Similar effects of cysteine on respiration have also been reported by Massart & Horens (1953) in a strain of baker's yeast, by Maw (1961) in a strain of brewer's yeast and by Turner (1959) in *Ophiobolus graminis* var. *avenae* and *Aspergillus niger*.

The possibility that cysteine might act by converting the catalase produced into inactive catalase-hydrogen peroxide complex II by generating hydrogen peroxide by its autoxidation stems from the observation of Keilin & Hartree (1936, 1945), who first demonstrated the coupled oxidation of ethanol

to acetaldehyde by catalase in the presence of an enzymically generated (glucose oxidase) supply of hydrogen peroxide. Chance (1950) studied the spectra of such mixtures and observed the formation of active catalase-hydrogen peroxide complex I and inactive catalase-hydrogen peroxide complex II. The addition of ethanol caused complex II to disappear.

Dale & Russel (1956) and Boeri & Bonnichsen (1952) have shown that cysteine gives rise to hydrogen peroxide by autoxidation. Alexander (1957), in a study of the inhibition brought about by normal and neoplastic tissue extracts, has shown that the inhibition is due to the generation of hydrogen peroxide and the formation of catalase-hydrogen peroxide complex II. He also found that ethanol, by coupled oxidation, brought about a complete reversal of the inhibition of catalase.

In our study, however, no effect of ethanol is seen; on the contrary, ethanol appears to be slightly inhibitory. One possibility may be that, since whole cells were used, the ethanol may not have penetrated in sufficient amount to counteract the effects of cysteine.

The precise mode of action of cysteine remains to be established.

SUMMARY

1. Freshly harvested cells of *Saccharomyces cerevisiae* grown anaerobically exhibit a lag period before they are able to oxidize glucose under aerobic conditions.

2. This lag period can be avoided if the cells are first aerated in the presence of glucose.

3. L-Cysteine produced a marked inhibition of both yeast respiration and catalase synthesis.

4. Ferric ammonium citrate reversed this inhibition. Ethanol was ineffective.

5. Of compounds related to cysteine, cystine and methionine were non-inhibitory whereas homocysteine and glutathione were slightly inhibitory.

REFERENCES

- Albert, A. (1952). *Biochem. J.* **50**, 690.
 Alexander, N. M. (1957). *J. biol. Chem.* **227**, 975.
 Bhuvaneshwaran, C., Rege, D. V. & Sreenivasan, A. (1961). *Proc. Symposium on Proteins*, p. 90. Mysore, India: Chemical Research Committee and Society of Biological Chemists.
 Boeri, E. & Bonnichsen, R. K. (1952). *Acta chem. scand.* **6**, 968.
 Chance, B. (1950). *Biochem. J.* **46**, 387.
 Chantrenne, H. (1954). *Biochim. biophys. Acta*, **14**, 397.
 Dale, W. M. & Russel, C. (1956). *Biochem. J.* **62**, 50.
 Keilin, D. & Hartree, E. F. (1936). *Proc. Roy. Soc. B*, **119**, 141.
 Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 293.
 Massart, L. & Horens, J. (1953). *Enzymologia*, **15**, 359.
 Maw, G. A. (1961). *J. Inst. Brew.* **67**, 57.
 Patti, F. & Bonnet Maury, P. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 1177.
 Turner, E. M. C. (1959). *Nature, Lond.*, **183**, 1130.
 Warburg, O. (1949). *Heavy Metal Prosthetic Groups and Enzyme Action*, p. 192. Oxford: Clarendon Press.

Biochem. J. (1964), **92**, 508

The Biosynthetic Origin of the Carboxyl Oxygen Atoms of the Carotenoid Pigment, Torularhodin

BY K. L. SIMPSON, T. O. M. NAKAYAMA AND C. O. CHICHESTER

Department of Food Science and Technology, University of California, Davis, Calif., U.S.A.

(Received 7 October 1963)

The structure of torularhodin was elucidated by Ruegg *et al.* (1958), and it was shown to contain a single carboxyl group at position 1'. The logical precursor to the carboxylated pigment would appear to be γ -carotene (Fig. 1). Kayser & Villoultreix (1960) studied an X-ray mutant of *Rhodotorula mucilaginosa* and found that the amounts of γ - and β -carotene decreased, whereas those of torulin and torularhodin increased, in the mutant. The authors therefore suggested that γ -carotene was the precursor of torulin and torularhodin.

Molecular oxygen is required in the formation of the xanthophylls (van Niel, 1947; Claes, 1959). Sapozhnikov, Maevskaya, Krasovskaya-Antropova, Prialgauskaite & Turchina (1959) and Yamamoto, Chichester & Nakayama (1962*a*) showed that molecular oxygen is incorporated directly into the leaf xanthophylls. In the latter paper it was shown that the biosynthetic origin of the oxygen in the hydroxyl group is the atmosphere, but it was further suggested that water furnished the epoxy oxygen.

Shneour (1962) has shown that molecular oxygen is utilized in the formation of the carbonyl group of the carotenoid spheroidenone in *Rhodopseudomonas spheroides*. The aim of the present investigation was to determine the biosynthetic origin of the oxygen atoms in torularhodin.

EXPERIMENTAL

Organism and growth conditions. A strain of *Rhodotorula rubra* var. *longa* was obtained from the Centraal Bureau voor Schimmelcultures, Yeast Division, Delft, Holland. The culture was grown in shasta flasks (flat whisky bottles of 473 ml. capacity) on the medium described by Nakayama, Mackinney & Phaff (1954), inoculated and harvested as described.

Extraction of pigment from cells. For a volume greater than 500 ml. a colloid mill (Gifford-Wood Co., Hudson, N.Y.; model QV 6) was used for the disruption of the yeast cells before solvent extraction. For volumes of cells of less than 100 ml. a modified French press was used for the disintegration of the yeast cells (Simpson, Wilson, Burton, Nakayama & Chichester, 1963).

Crystalline torularhodin from yeast was obtained by extracting the pigment from the cells harvested from 100 shasta flasks. The washed yeast suspension (1.25 l.) was added to the colloid mill together with 750 ml. of glass beads (average diameter 120 μ) (Minnesota Mining and Manufacturing Co., St Paul, Minn.) and 25 ml. of 0.2M-phosphate buffer at pH 7.0. Usually 1 hr. was required for an 80–90% disintegration of the yeast cells as estimated by microscopic examination.

The modified French press was used according to the procedure of French & Milner (1955) at 25 000 lb./in.². Usually two passages through the press were sufficient for a complete extraction of the pigment.

Fig. 1