SHANMUGAM, Keelnatham T., 1941-
INDUCTION OF CATALASE IN THE
ATHIORHODACEAE.

University of Hawaii, Ph.D., 1969
Bacteriology

University Microfilms, Inc., Ann Arbor, Michigan
INDUCTION OF CATALASE IN THE Athiorhodaceae

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MICROBIOLOGY AUGUST 1969

By
Keelnatham T. Shanmugam

Dissertation Committee:
Leslie R. Berger, Chairman
Albert A. Benedict
Kaare R. Gundersen
John B. Hall
Kerry T. Yasonobu
ABSTRACT

Catalase induction was investigated in various organisms belonging to the family Athiorhodaceae. Catalase induction could be demonstrated only in *Rhodopseudomonas spheroides* and related locally-isolated organisms. Both RNA and protein synthesis were found to be necessary for the induced synthesis. Although H₂O₂, the added inducer, was decomposed in the first 5 min., m-RNA synthesis continued up to 30 min. In *Rh. spheroides*, maximum induction occurred during the early stationary phase, while in TL-1, TL-4 and Rps. D, the induction was during the logarithmic phase of growth. *Rh. spheroides* alone excreted porphyrins into the medium during the stationary phase. Inhibition of porphyrin synthesis by 8-Hydroxy quinoline (4 x 10⁻⁵M) also inhibited catalase induction. The induced synthesis of catalase has been interpreted to require porphyrin synthesis. Growing TL-4 at constant pH decreased the log phase level of induction. Inducibility under these conditions was maximum at pH 7.3. Growing either *Rh. spheroides* or TL-4 at constant pH had no effect on the stationary level of induction.

Induction of catalase was observed only in those organisms which can effect an oxygen dependent conversion of the carotenoid spheroidene to spheroidenone. Inhibition of this carotenoid conversion by inhibitors like diphenylamine (3 x 10⁻⁴M) or acridine (5 x 10⁻⁴M) also inhibited the catalase induction. Lower concentration (0.5 to 1.0 x 10⁻⁴M) of the inhibitors had a stimulatory effect. The data is discussed in terms of the involvement of carotenoids in the induction of catalase.
TABLE OF CONTENTS

ABSTRACT ................................................................. iii
LIST OF FIGURES ....................................................... v
LIST OF TABLES ......................................................... vii
LIST OF PLATES ......................................................... viii
INTRODUCTION .......................................................... 1
MATERIALS AND METHODS ............................................ 5
RESULTS AND DISCUSSION ............................................ 13
SUMMARY ................................................................. 69
APPENDIX A ............................................................. 70
APPENDIX B ............................................................. 73
APPENDIX C ............................................................. 75
BIBLIOGRAPHY .......................................................... 84
<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. He-train set-up for the cultivation of anaerobic cultures</td>
<td>8</td>
</tr>
<tr>
<td>2. Time course of catalase induction in <em>Rh. spheroides</em> with air as the inducer</td>
<td>16</td>
</tr>
<tr>
<td>3. Time course of catalase induction in <em>Rh. spheroides</em> with 40 μM H₂O₂ as the inducer</td>
<td>19</td>
</tr>
<tr>
<td>4. Effect of concentration of cells on catalase induction in <em>Rh. spheroides</em> with H₂O₂ (40 μM) as the inducer</td>
<td>21</td>
</tr>
<tr>
<td>5. Effect of the time of addition of acriflavine (20 μM) on catalase induction</td>
<td>24</td>
</tr>
<tr>
<td>7. Effect of the concentration of 8-hydroxy quinoline on catalase induction</td>
<td>31</td>
</tr>
<tr>
<td>8. Effect of 8-hydroxy quinoline on growth and catalase induction in <em>Rh. spheroides</em></td>
<td>34</td>
</tr>
<tr>
<td>9. Effect of 8-hydroxy quinoline on the growth and catalase induction in TL-1</td>
<td>36</td>
</tr>
<tr>
<td>10. Effect of growing TL-4 at constant pH on catalase induction</td>
<td>47</td>
</tr>
<tr>
<td>11. Effect of growing <em>Rh. spheroides</em> at constant pH on catalase induction</td>
<td>54</td>
</tr>
<tr>
<td>12. Oxygen dependent carotenoid conversion in <em>Rh. spheroides</em></td>
<td>56</td>
</tr>
<tr>
<td>13. Effect of the addition of di-phenylamine at various time periods after the initiation of induction</td>
<td>60</td>
</tr>
</tbody>
</table>
| 14. a. Effect of concentration of di-phenylamine on the induction of catalase and carotenoid interconversion in *Rh. spheroides*  
   b. Effect of concentration of Acridine on the induction of catalase in *Rh. spheroides* | 62 |
| 15. A suggested model for the mechanism of catalase induction in *Rh. spheroides* | 67 |
A-1. pH-stat apparatus ........................................ 74
A-2. Power supply and catalase assay system .............. 76
A-3. Power supply and catalase assay system .............. 77
A-4. Voltage-time curve for the decomposition of $H_2O_2$ .... 79
A-5. Effect of beef liver catalase concentration on reaction velocity ........................................ 83
LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Inducibility of Catalase in Athiorhodaceae</td>
<td>14</td>
</tr>
<tr>
<td>II Effect of Protein Synthesis Inhibitors on Catalase Induction</td>
<td>22</td>
</tr>
<tr>
<td>III The Relationship between the Bacteriochlorophyll and Catalase Induction</td>
<td>41</td>
</tr>
<tr>
<td>IV Amount of Catalase Induced During the Stationary Phase in TL-4 in the pH stat</td>
<td>49</td>
</tr>
</tbody>
</table>
## LIST OF PLATES

<table>
<thead>
<tr>
<th>PLATE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Phase contrast photomicrographs of <em>Rh. spheroides</em>, Rps. D., TL-1 &amp; TL-4</td>
<td>40</td>
</tr>
<tr>
<td>II Composite of phase contrast photomicrographs of TL-4 grown in the pH-stat</td>
<td>45</td>
</tr>
<tr>
<td>III Composite of phase contrast photomicrographs of <em>Rh. spheroides</em> grown in the pH stat</td>
<td>52</td>
</tr>
</tbody>
</table>
INTRODUCTION

Loew (1901) designated the enzyme systems of plant and animal tissues involved in the catalytic decomposition of hydrogen peroxide as catalases (Burris, 1960). He also emphasized the ubiquitous presence of the enzyme. Later work indicated that the enzyme is not found in obligate anaerobes and some of the facultative bacteria. The inability of these anaerobic organisms to grow or survive aerobically has been equated with the absence of catalase. The role of the enzyme in aerobic organisms is still obscure.

Catalase is the enzyme which accelerates the following reaction:

$$2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$$

Peroxidase, which also catalyzes the decomposition of hydrogen peroxide to water requires organic hydrogen donors.

$$\text{H}_2\text{O}_2 + \text{organic donor} \rightarrow \text{organic donor + 2 H}_2\text{O}$$

Although catalases have the ability to oxidize organic donors, using hydrogen peroxide, they have low affinity for the donors compared to peroxidases (from the same sources).

Two different kinds of catalases have been observed in biological system. The main and more widespread enzyme is a metalloporphyrin with protohematin IX as prosthetic group. The valence state of iron is three (Chance, 1951; 1954). Some facultative bacteria, eg. *Streptococcus*, *Lactobacillus* and *Pediococcus* have a catalase system which has no porphyrin as coenzyme moiety. This enzyme was termed non-heme catalase (Whittenbury, 1964; Johnston, 1965).
The family Athiorhodaceae is comprised of the non-sulfur purple photosynthetic bacteria. These bacteria utilize organic compounds as hydrogen donors for photosynthesis. With few exceptions, these organisms are capable of growing aerobically in the dark. During aerobic growth, the organic acids and other carbon compounds are oxidized to CO$_2$ and H$_2$O and simultaneously both energy and reducing power for growth are generated. Excellent reviews and articles cover various aspects of this group of organisms (Van Niel, 1944; Cohen-Bazire et al., 1957; Kamen and Gest, 1960; Jensen, 1962; Gest, 1965; Pfenning, 1967; Lascelles, 1968; Vernon, 1968).

*Rhodopseudomonas spheroides* has very low catalase activity during photosynthetic growth. The catalase level increases rapidly, when the cells are incubated under aerobic conditions, in the light. Catalase activity of the cells is very high when the cells grow under aerobic conditions, in the dark (Clayton, 1960). Most of the work on catalase in this organism has been done by Clayton and his co-workers (Clayton, 1959 a,b,c; 1960 a,b,c,d,e; 1961, 1962; Clayton and Smith, 1960; Clayton and Adler, 1962; Adler and Clayton, 1962). Clayton, while studying the kinetics of enzyme formation in an aerated culture in the light, observed no enzyme formation in the first seven minutes. Enzyme synthesis proceeded at a constant rate after this initial lag. Addition of H$_2$O$_2$ to an anaerobic culture also led to enzyme formation. The maximum amount of catalase induced could be altered by varying the concentration of hydrogen peroxide while maintaining the same rate of synthesis. Under these conditions, hydrogen peroxide acts as a better inducer than air. From this, Clayton concludes that during aeration
of a photosynthetic culture, peroxides formed 'in vivo', rather than oxygen act as true inducers.

Clayton observed that the inducibility of the cells increases to a maximum during the early stationary phase. Removal of acetate and glutamate from an acetate-glutamate-malate medium abolishes the low inducibility of the log phase culture (Clayton, 1960d). Induction can be completely abolished by inhibitors of protein synthesis and messenger RNA (m-RNA) production. Inhibitors of DNA replication have no effect (Kiguchi, 1965). Clayton also obtained evidence for 'de novo' synthesis of the protein during the induction period. Although a high level of enzyme is produced, Gray et al. (1964) were unable to isolate a m-RNA fraction, in an induced culture. Clayton, while studying the mechanism of induction found evidence suggesting that an internal inducer is formed in the early stages of induction. This inducer decays photochemically at a fixed rate in room temperature. Decay of the inducer could be prevented by maintaining the culture in the dark at 4°C.

Clayton studied the role of catalase in protection against damage by ionizing radiation. Certain carotenoidless mutants are killed by light in the presence of air (Sistrom et al., 1956). These mutants, like the wild type have bacteriochlorophyll and they can grow photosynthetically as well as aerobically in the dark. Similar results were observed with organisms in which carotenoid biosynthesis was specifically inhibited by diphenylamine (Cohen-Bazire et al., 1958). One of the explanations offered for this photodynamic kill is that peroxides are formed which are detrimental to growth. Based on this argument, Clayton
obtained carotenoidless mutants which are either constitutive or inducible for catalase. In the presence of small amounts of air, catalase protected the system, but it had no effect at higher levels of aeration. Catalase had no effect in protecting cells against UV- or X-radiation.

The uniqueness of the family Athiorhodaceae, in its ability to grow either aerobically or anaerobically and also to induce catalase lends itself to a study of the physiology of catalase induction. A comprehensive study of catalase induction in these organisms should lead to an understanding of the mechanisms of catalase induction and eventually of the role of the enzyme in biological systems. It is this view which led to the present study of the physiology of catalase induction in the Athiorhodaceae.
MATERIALS AND METHODS

Methods:

A. Organisms used in this study:

The following organisms were used in this study:

i. *Rhodopseudomonas capsulata*

ii. *Rh. gelatinosa*

iii. *Rh. palustris*

iv. *Rh. spheroides*

v. *Rhodospirillum molischianum*

vi. *R. rubrum*

A number of local isolates, belonging to the genus *Rhodopseudomonas* were also used.

B. Media used in this study for the cultivation of organisms are described in Appendix A.

C. Cultural conditions:

i. Stock cultures:

Stock cultures were maintained as stabs in the Stock-culture agar in screw cap tubes (13 x 100 mm or 16 x 150 mm). The cultures were transferred twice a year and maintained at room temperature, 1 to 2 feet from two 60 watt incandescent lamps for illumination.

For experiments, an inoculum from the stock culture was grown in the appropriate liquid medium. The cultures were transferred after 24 hrs, into fresh medium, using a 5-10% inoculum. Cultures were grown in 13 x 100 mm screw cap tubes filled up to the top. Such cultures were used as inocula for the experiments.
ii. Growth of the organism:

Cultures were grown at 30°C under saturating light intensity under conditions which permitted periodic sampling while maintaining anaerobiosis (see Fig. 1). One hundred ml culture in a 4 oz bottle, capped with a serum stopper, was deaerated and filled with helium. The bottle with a sterile sinter filter in place was connected to the helium line, through a valve. Helium was passed through the line at a slow, steady rate. The valve was opened and the atmosphere above the liquid column was allowed to equilibrate with helium in the line. The medium was inoculated using a syringe. Samples were taken with syringes. Except for hydrogen, this system holds the gases evolved by the organism during growth, gases which may be used again in later stages of the culture. For example, CO₂ stays immediately over the liquid phase and layers below the helium phase due to its greater density.

Large cultures were grown in 16 oz bottles filled completely to the top.

Dark aerobic cultures were grown in erlenmeyer flasks. The liquid volume usually was 15% of the flask volume. The flasks were covered with a layer each of paper and aluminum foil (Kaiser, USA) to prevent entry of light. The cultures were aerated by shaking on a Gyrotory Shaker (New Brunswick Scientific Co. Model G-10). Transfer of these cultures was made under subdued light.

Cultures were maintained at constant pH during growth using a laboratory-made pH-stat (see Appendix B for apparatus and description).
Figure 1. He-train set-up for the cultivation of anaerobic cultures.
D. Measurements:

Growth of the organism was measured as increase in the absorbancy of the culture at 680 nm, using a Bausch & Lomb Spectronic 20 spectrophotometer. The pH was measured using a variety of laboratory pH-meters. Bacteriochlorophyll (Bchl), carotenoids and excreted porphyrins were estimated according to the method of Cohen-Bazire et al. (1957). Bchl and carotenoids were determined in an acetone:methanol:7:2 (v/v) extract of the cells. Using the extinction coefficient reported by these authors, the amount of the pigments present in the culture was computed in mg/100 ml culture. Protein was estimated according to the method of Lowry et al. (1951).

The cells were rendered permeable by shaking for 2 minutes with 1/10 volume of toluene. The catalase content of the tolenzized cells was estimated according to the method of Guildbault (1966) (see Appendix C for description of the apparatus). The assay medium contained in a final volume of 4.0 ml, 0.01 M of 2-hydroxymethyl, 2'-amino-1,3-propanediol (tris), pH 7.4, 2 mM of hydrogen peroxide and the appropriate amount of the enzyme preparation. The rate of the enzymatic reaction was computed from the linear part of the time vs voltage curve (see Fig. A-4). A standard curve was established using beef liver catalase (Fig. A-5). The amount of the enzyme present in the preparation could be estimated using this standard curve. The enzyme activity was expressed as volts/min/ml of culture.

E. Preparation of Cell-free extracts:

Induced cells (see section G) were washed 3 times with M/15 phosphate buffer, pH 6.8. A heavy suspension of the cells, in the same buffer was
sonicated for 5 min at 4C using a sonic disintegrator at full power (Branson Ultrasonic Corp. Model LS-75). The sonicated cell suspension was sedimented at 20,000 x g for 30 min, at 4C. The supernatant was centrifuged again at 100,000 x g for 2 hrs in a Beckman Model L-2 ultracentrifuge using a No. 40 head. The supernatant fluid was separated into a clear top layer and fluffy bottom layer. The sediment in each tube was resuspended in 2.5 ml phosphate buffer.

F. Gel electrophoresis:

Gel electrophoresis was done according to the method of Davis (1964), with minor modifications to suit the catalase system. The sample gel was omitted. The spacer (concentration) gel was polymerized over the 40% sucrose solution giving space for sample application before electrophoresis. To the separation gel mixture, a deaerated, soluble starch solution was added to a final concentration of 0.5%. The separation gel containing the starch was polymerized using ammonium persulfate.

A small volume (less than 0.1 ml) of sample, from the top layer of the 100,000 x g supernatant fluid was layered over the spacer gel. The current was turned on, to a maximum of 2mA per tube to avoid convection of the sample. After 10 min., the voltage was increased to 200 volts, and the electrophoresis was continued at constant voltage. Constant voltage was maintained by using a Heathkit regulated power supply (Model IP-32). When the marker dye had migrated 4 cm into the separation gel, current was turned off and the gels were rimmed out from the tubes.

The gels were placed in 0.01 M, \( \text{H}_2\text{O}_2 \) in 0.01 M, Tris-HCl buffer, pH 7.4 and shaken on a Yankee Rotater. After 10 min the gels were taken
out, rinsed with fresh buffer and placed in a freshly mixed staining solution of the following composition:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium iodide</td>
<td>0.12 M</td>
</tr>
<tr>
<td>HCl</td>
<td>0.10 N</td>
</tr>
<tr>
<td>Ammonium molybdate</td>
<td>0.20 mM</td>
</tr>
</tbody>
</table>

The gels were removed immediately after the dark color began to appear and they were washed well with 95% ethanol to remove the excess free iodine. The gels can be stored in 5% acetic acid with no visible change in color for at least 24 hrs.

\[
H_2O_2 + 2I^- + 2H^+ \rightarrow 2H_2O + I_2
\]

\[
I_2 + \text{starch} \rightarrow \text{color}
\]

Since catalase decomposes the \(H_2O_2\), iodide cannot be oxidized to iodine where there is catalase activity in the gel. This results in a colorless band in an otherwise dark-blue gel.

G. Induction of Enzyme:

When air was used as an inducer for catalase in anaerobically grown cells, an aliquot of the culture was transferred to a 18 x 150 mm tube and air was bubbled through. The cultures were incubated under saturating light intensity at 30°C.

When \(H_2O_2\) was used as an inducer, the O.D.680nm of the culture was adjusted to 1.00 using fresh MAD-medium. Then, 40 \(\mu\)m \(H_2O_2\) (in \(H_2O\)) was added. Nitrogen was bubbled through the tubes to drive off the oxygen liberated from \(H_2O_2\) by catalase and peroxidase activity. Since no difference was observed between nitrogen bubbled cultures and static cultures, nitrogen bubbling was deleted in the later experiments. The
cultures were incubated at 30°C under saturating light intensity.

2. Materials

All chemicals used were of reagent grade, meeting ACS specifications. Biochemicals were purchased either from Calbiochem or Nutritional Biochemicals Corporation.
RESULTS AND DISCUSSION

Table I gives the results of a comparative study on catalase induction in various organisms belonging to the family, Athiorhodaceae. All these organisms are grouped in the family, because of their ability to grow both aerobically and anaerobically on organic acids. Morphology and pigment characteristics place the locally-isolated strains in the genus, *Rhodopseudomonas*. TL-1, TL-4, Rps. D, AT-3 & AT-9 could be grouped as *R. spheroides*, due to their morphology and pigment characteristics. As the table indicates, only *R. spheroides* and related organisms had any inducible catalase. Although some organisms did produce considerable amounts of catalase during aerobic growth, eg. *R. palustris*, the inducibility of an anaerobic culture was very low. For these reasons, only *R. spheroides* and related organisms were selected for further studies.

When an anaerobically grown culture of *R. spheroides* was aerated in the presence of light, catalase synthesis began almost immediately. The synthesis followed a constant exponential rate. After 50 minutes the rate of synthesis began to decrease (Fig. 2). Similar results were obtained by Clayton (1960), with the same organism. Jones *et al.* (1964) also observed an increase in the non-heme catalase levels of *Streptococcus faecalis* by aeration. The increase in catalase was also enhanced by iron, manganese and zinc ions. None of these metal ions had any effect on the activity of the enzyme. Heady *et al.* (1964) observed with *Staphylococcus epidermidis*, that addition of hemin at a concentration of 0.5 μg/ml to an anaerobic culture led to catalase levels as high as that of aerated cultures.
<table>
<thead>
<tr>
<th>organism</th>
<th>Catalase level (volt/min/ml of cells of 1 O.D. 80)</th>
<th>Anaerobically grown cells</th>
<th>Induced with 40µM H₂O₂</th>
<th>Aerobically grown cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>uninduced</td>
<td>induced</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhodopseudomonas capsulata</td>
<td>0.021</td>
<td>0.054</td>
<td>0.080</td>
<td></td>
</tr>
<tr>
<td>Rh. gelatinosa</td>
<td>0.009</td>
<td>0.009</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>Rh. palustris</td>
<td>0.018</td>
<td>0.012</td>
<td>0.110</td>
<td></td>
</tr>
<tr>
<td>Rh. spheroides</td>
<td>0.0044</td>
<td>0.252</td>
<td>0.168</td>
<td></td>
</tr>
<tr>
<td>Rhodospirillum molischianum</td>
<td>0.009</td>
<td>0.015</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>R. rubrum</td>
<td>0.0025</td>
<td>0.015</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>Locally isolated strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TL-1</td>
<td>0.001</td>
<td>0.275</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TL-4</td>
<td>0.076</td>
<td>0.290</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rps. A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.009</td>
<td>0.009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.012</td>
<td>0.011</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.048</td>
<td>0.410</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At - 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.034</td>
<td>0.044</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.052</td>
<td>0.047</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.011</td>
<td>0.143</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.011</td>
<td>0.041</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.055</td>
<td>0.027</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.022</td>
<td>0.047</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.035</td>
<td>0.046</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.023</td>
<td>0.044</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Time course of catalase induction in *Rh. spheroides* with air as the inducer.
As in many other inducible systems, the substrate, $H_2O_2$, could act as an inducer of catalase in \textit{Rh. sphaeroides} (Clayton, 1960b). As shown in Fig. 3, no enzyme was formed in the first 6 min. after the addition of hydrogen peroxide (40 $\mu$M). After this initial lag, catalase synthesis began. Catalase content of the cells increased linearly. After 20 min. the rate of synthesis began to decrease and reached zero at about 60 min. Clayton (1960c), in his studies on the effect of concentration of $H_2O_2$ on induction, observed that increasing the inducer concentration did not affect the rate of formation of the enzyme. Instead the total quantity of enzyme formed varied with the concentration of $H_2O_2$. Similar results were obtained in this study as shown in Fig. 4. Clayton explained this in the following manner. The added inducer was destroyed by the small amounts of catalase and peroxidase present in the uninduced culture. As the concentration of cells decreased, the length of time that the inducer could stay in contact with the cells increased. This in turn gave the same effect as the addition of a higher concentration of inducer. In this study 40 $\mu$M of $H_2O_2$ was used in all the experiments.

Inhibitors of protein synthesis inhibited the net synthesis of catalase as shown in Table II, indicating a 'de novo' protein synthesis. Using radio-active isotopes, Clayton (1960c) obtained more direct evidence for this. Acriflavine, which is 3,6-diamino, N-methyl, acridine is known to bind with DNA and thus inhibit the transcription of messenger RNA (Blake, 1968; Waring, 1968). The results of this study are consistent with these observations. Fig. 5 shows, that after addition of 20 $\mu$M acriflavine in the first 5 min. after the initiation of induction,
Figure 3. Time course of catalase induction in *Rh. spheroides* with 40 μM H₂O₂ as the inducer.
Figure 4. Effect of concentration of cells on catalase induction in Rh. spheroides with H$_2$O$_2$ (40 μM) as the inducer.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (μg/ml)</th>
<th>Amount of catalase induced (v/min/ml)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>--</td>
<td>0.097</td>
<td>--</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>5 0.000</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 0.000</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>5 0.015</td>
<td>74.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 0.008</td>
<td>91.75</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5. Effect of the time of addition of acriflavine (20 μM) on catalase induction. 40 μM H₂O₂ was added at zero-time.
completely inhibited the formation of new enzyme. After the first 5 min.,
the inhibitory effect of acriflavine decreased with time, and reached a
value of zero at 30 min. As shown in Fig. 3, the synthesis of catalase
proceeded under these conditions up to 60 min., although the rate of
synthesis decreased after the first 20 min. This data, combined with the
results of Fig. 5 indicates that m-RNA was formed during the first 20 min.
at a constant rate. Synthesis of m-RNA rapidly decreased in the next 10
min. After 30 min., new synthesis of m-RNA did not occur and the exist­
ing m-RNA continued to be translated for another 30 min.

Clayton (1960a,d) in his studies with Rh. spheroides, observed that
the catalase inducibility of the cells, increased when the culture reached
stationary phase. He observed that the inability of the log phase cells
to be induced to high levels could be abolished by removal of acetate and
glutamate from an acetate-glutamate-malate medium. Clayton explained
this in the following way. During log phase, many organic intermediates
were formed from acetate and glutamate. One of these compounds may have
acted as a repressor of catalase induction. As the culture reached sta­
tionary phase, both acetate and glutamate were exhausted from the medium.

McCarthy and Hinshelwood (1959) observed with Aerobacter aerogenes,
that the catalase content of the cells increased when the culture reached
stationary phase. During growth, the pH of the culture also fell down to
4.5. When the pH of the culture was maintained at 7.0, no catalase
synthesis occurred during the stationary phase. This data indicates a
relationship between the pH of the medium and catalase autoinduction.
These authors also observed an increase in catalase content of the cells,
on aeration. White (1962) also observed an increase in catalase content
of *Haemophilus parainfluenzae* by aeration. While studying the amount of catalase present during the growth cycle under conditions of poor aeration, a maximum was observed in the stationary phase. Increasing the rate of aeration brought the maximum to the log phase. The growth rate also was much higher under rapid aeration. In the stationary phase, a decrease in the cytochrome-c level was also observed.

Pinsky and Stokes (1952) observed a stationary phase induction of formic hydrogenlyase and nitrate reductase in *Escherichia coli*. They obtained similar results with a benzoate oxidation system in *Pseudomonas fluorescens*.

As shown in Fig. 6a, no stimulation of catalase induction in the log phase was observed under these conditions in *Rh. spheroides*. The medium had malate as the sole source of carbon and reducing power. The increase of catalase inducibility in the stationary phase alone cannot be explained by the hypothesis of Clayton. Although it is possible to have an internal repressor for catalase synthesis in *Rh. spheroides* it is hard to visualize how an organic intermediate of metabolism can act as a repressor for an enzyme, like catalase.

*Rh. spheroides* has been known to excrete a 405nm-absorbing material into the medium. This was observed by van Niel (1944). Lascelles (1956) characterised it as coproporphyrin-III. As shown in Fig. 6a, the rate of excretion was low during the growth period. When the culture reached stationary phase, it increased and continued at a higher rate until about 60 hrs. Porphyrins are the prosthetic groups of chlorophylls and some catalases. Bchl has magnesium as the metal cation while catalase has iron. It has been shown in *Rh. spheroides* (Jones, 1967) that the synthesis
Fig. 5 GROWTH AND CATALASE INDUCTION IN
A NITROSPENIOMA NITROGENIFICA
B TIL-1
C TIL-4
D TIL-8

Legend:
- O, D, 650 nm
- BChl.
- Carotenoids
- A 405 nm
- CATALASE INDUCTION
of both magnesium and iron branches of the tetrapyrrole synthesis share the common pool up to the protoporphyrin level. Coproporphyrinogen, a precursor of the stable coproporphyrin occurs before protoporphyrin. This indicates that both branches share a common pool of coproporphyrinogen. Porphyrin excretion has not been found in other species of the family. This excretion is believed to be an impaired control mechanism in the magnesium branch. Lascelles (1966, '68) observed that an albino mutant, which cannot form Bchl, also did not excrete porphyrin under conditions in which the wild type did. This indicated that the excreted porphyrin was under the control of Bchl synthesis, rather than the heme or B12 branch (Cauthen et al., 1967; Lascelles & Altshuler, 1967). During growth, the synthesized porphyrins were completely used up in Bchl and heme synthesis. When the culture reached stationary phase, Bchl synthesis ceased. This presumably resulted in an increased accumulation of coproporphyrinogen in the intracellular pool, leading to excretion as the stable form of coproporphyrin. When H2O2 was added at this stage, a greater amount of catalase was synthesized due to a high availability of porphyrin. In other words, the amount of protoporphyrin which can enter the iron branch is controlled by Bchl synthesis. This may explain the greater inducibility of catalase in the stationary phase. Clayton (1959a) observed a competition between Rhodospirillum heme protein (RHP) and catalase synthesis in Rh. spheroides. When the RHP level was lower, the catalase level increased and vice versa.

This hypothesis relating the porphyrin synthesis with catalase induction in the stationary phase of the Rh. spheroides culture, can be
tested in 2 ways. The first test would be to study the effect of inhibition of the excretion of porphyrin on catalase induction. In this case, no induction maximum would be observed in the stationary phase. The second test would be to inhibit Bchl synthesis during log phase. This should lead to a stimulation of catalase induction.

Some of the locally isolated Rhodopseudomonads did not excrete any porphyrins. TL-1, TL-4 and Rps. D were such bacteria and they also had an inducible catalase. In these three organisms inducibility of catalase reached a maximum during the mid-log phase (Fig. 6b,c,d). Catalase inducibility decreased to a low value as the culture reached stationary phase (during which no synthesis of porphyrins occurred). In both TL-1 and Rps. D, catalase inducibility was maximum when the Bchl content was maximum. As the rate of Bchl synthesis decreased, the inducibility also decreased. Bchl synthesis could be used in these three organisms as an indication of the rate of porphyrin synthesis. Since, there was no porphyrin excretion, the rate of synthesis of catalase also fell down to a very low value, when the culture reached stationary phase.

A known chelater of metal ions, 8-hydroxy quinoline (8-OH Q), was found to inhibit Bacteriochlorophyll synthesis (Jones, 1963b, 1967). Jones also observed that 8-hydroxy quinoline actually catalyzed the incorporation of copper in the place of magnesium into protoporphyrin, thus inhibiting Bacteriochlorophyll synthesis. As Figure 7 shows, 8-hydroxy quinoline had no effect on catalase induction up to a concentration of $2 \times 10^{-5}$M. Higher concentrations did inhibit enzyme formation but concentrations as high as $2 \times 10^{-4}$M
Figure 7. Effect of the concentration of 8-hydroxy quinoline on catalase induction with 40 μM H₂O₂ as the inducer.
had no effect on the activity of preformed enzyme. 8-OH Q was added to a growing culture of *Rh. spheroides*, during the mid-log phase at a final concentration of $4 \times 10^{-5}$ M (Fig. 8b). Bchl synthesis, though slightly slowed down was not affected. The important observation was that the rate of synthesis of carotenoids was decreased. The total amount of carotenoids produced was also less. The cell growth, measured as O.D. 680nm was not affected. Excretion of porphyrins was completely arrested. High inducibility during the stationary phase was also abolished. When the same concentration of 8-OH Q was added to the culture just before the beginning of stationary phase, porphyrin excretion was arrested 3.5 hrs. later. The excretion resumed at a lower rate after 8 hrs. Under these conditions also, high catalase induction was abolished.

Since, 8-OH Q did not inhibit Bchl synthesis at $4 \times 10^{-5}$ M, its concentration was increased to $2 \times 10^{-4}$ M. This concentration did inhibit catalase induction (Fig. 7). The cells were therefore resuspended in fresh MAD-medium containing no 8-OH Q before addition of inducer. There was an initial inhibition of Bchl synthesis from which the cells recovered after 4 hrs. Porphyrin excretion showed the opposite effect. A low catalase inducibility during the stationary phase could be observed (Fig. 8d,e). Similar experiments done with TL-1 had slightly different results (Fig. 9). Carotenoid synthesis was not affected by 8-OH Q but catalase synthesis fell to a very low value. If the culture was resuspended in fresh medium inducibility increased but it did not reach the control levels. High concentration of 8-OH Q had little effect.

8-Hydroxy quinoline incorporated copper into the protoporphyrin at a rate equal to that of iron incorporation. Burnham and Lascelles (1963)
Figure 8. Effect of 8-hydroxy quinoline on growth and catalase induction in Rh. spheroides.

A & D  control
B & C  $4 \times 10^{-4}$ M 8-hydroxy quinoline
E      $2 \times 10^{-4}$ M 8-hydroxy quinoline

8-hydroxy quinoline was added at the times indicated by the arrows.

Scales and legend as in Fig. 6.
Figure 9. Effect of 8-hydroxy quinoline on the growth and catalase induction in TL-1.

A  control
B  $4 \times 10^{-5}$ M 8-hydroxy quinoline
C  $2 \times 10^{-4}$ M 8-hydroxy quinoline

8-hydroxy quinoline was added at the times indicated by the arrows.

Scales and legend as in Fig. 6.
observed that cu-protoporphyrin inhibited the δ-amino levulinic acid synthetase. Jones (1967) also observed that in the presence of iron and 8-OH Q, the excretion of porphyrin was inhibited. In the absence of iron (added as ferric citrate), porphyrin excretion was noted. This porphyrin was shown to be Mg-protoporphyrin mono methyl ester. Jones (1963a) identified this compound as an intermediate in the normal Bchl synthetic reactions. Addition of oxygen to the system inhibited only the incorporation of iron but not copper indicating an uncontrolled mechanism for copper incorporation.

Vernon and Garcia (1967) observed that treatment of R. rubrum chromatophores with Pancreatin (4 mg/ml) and Triton X-100 (0.5%) yielded 3 fractions on sucrose density gradient (a top green band, a middle brown band and a third blue band). When the chromatophores were hydrolysed aerobically, only the top two bands were observed. The middle brown band was found to contain carotenoids and protein while the other two had only Bchl and proteins. From the kinetics of formation of these bands, the authors concluded that the green band was derived from the blue band which was an aggregate, formed preferentially under anaerobic digestion conditions. Irrespective of the conditions of hydrolysis, a carotenoid-protein complex could be separated from a protein-Bchl complex. Rh. spheroides, R. rubrum and Chromatium were found to have similar membrane structures in the cytoplasm, carrying out photosynthesis. Gibson (1965) observed spherical particles, about 570 Å in diameter, in the cytoplasm of Rh. spheroides. Holt and Marr (1965) observed similar particles in R. rubrum. Similar protein-pigment complexes may be present in Rh. spheroides chromatophores. If 8-OH Q interferes with the aggre-
gation of the Bchl-protein and/or carotenoid-protein complexes, then its addition may also interfere with the formation of the complexes.

Carotenogenesis in the *Rh. spheroides* was decreased and porphyrin excretion was inhibited by 8-OH Q. The loss of the catalase inducibility could be the result of any one of the two or both. The results of these experiments do not distinguish between the two possibilities. Further study of Fig. 6, also indicates that a correlation exists between porphyrin synthesis and catalase inducibility. An increase in catalase inducibility during the growth period was observed in Fig. 6a. This increase stopped when the Bchl synthesis stopped. Similar results were obtained with the other three organisms. The effect is more pronounced with Rps. D (Fig. 6d). The fall in catalase inducibility correlated well with the cessation of Bchl synthesis.

In TL-1, the O.D. \(_{680\text{nm}}\) increased for another 6 hrs. even after the Bchl synthesis stopped. As the photomicrograph (Plate-I) shows TL-1 began to accumulate intracellular granular material in the late log phase. This was reflected by an increase in O.D. Poly-β-hydroxybutyrate is found in many photosynthetic bacteria (Stanier *et al.*, 1959). In *R. rubrum* (Merrick & Doudoroff, 1964), this accumulated material was used up as the carbon source became exhausted. The authors also obtained evidence for an enzyme system which can depolymerize the poly-β-hydroxybutyrate granules. In TL-1, the granular material remained throughout the stationary phase, indicating the inability of the organism to depolymerize it. No granular inclusions were seen in Rps. D. A good correlation between the amount of Bchl present and the catalase induced at the peak of inducibility was observed in these two organisms (Table III).
Plate I. Phase contrast photomicrographs of

A  Rh. spheroides
B  Rps. D
C  TL-1
D  TL-4  18 hours old
E & F TL-4  35 hours old

s-spheroplasts
<table>
<thead>
<tr>
<th>Organism</th>
<th>Bchl (mg/100ml culture)</th>
<th>Catalase induced (v/min/ml)</th>
<th>Catalase Bchl</th>
<th>Rate of increase in inducibility Rate of synthesis of Bchl</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Rh. spheroides</td>
<td>0.214</td>
<td>0.120</td>
<td>0.56</td>
<td>0.28</td>
</tr>
<tr>
<td>**Rh. spheroides</td>
<td>0.260</td>
<td>0.280</td>
<td>1.08</td>
<td>--</td>
</tr>
<tr>
<td>TL-1</td>
<td>0.298</td>
<td>0.330</td>
<td>1.11</td>
<td>1.06</td>
</tr>
<tr>
<td>Rps. D</td>
<td>0.725</td>
<td>1.000</td>
<td>1.38</td>
<td>1.34</td>
</tr>
<tr>
<td>TL-4</td>
<td>0.305</td>
<td>0.792</td>
<td>2.60</td>
<td>2.65</td>
</tr>
<tr>
<td>+TL-4</td>
<td>0.50</td>
<td>0.51</td>
<td>1.00</td>
<td>0.80</td>
</tr>
</tbody>
</table>

* Log Phase Values
** Stationary Phase Values
+ Grown in the pH-stat at pH 7.3 (log phase values)
Appleman (1952) found a relationship between catalase and chlorophyll synthesis in barley. In etiolated seedlings, the level of catalase was much higher. As the chlorophyll synthesis began, the catalase level decreased. Inhibition of chlorophyll synthesis by dark led to a higher level of catalase. Shukla (1968) also observed a possible correlation between catalase levels and chlorophylls in algae.

The amount of catalase induced per unit of Bchl is much higher in TL-4. During the late-log phase, spheroplasts could be seen in TL-4 culture (Plate I). Berger (unpublished data) observed pleomorphic cells in Rps. D culture in a lactate-yeast extract medium. He also observed that the pleomorphism was pH-dependent. At pH 8.0, large, involuted, pleomorphic forms were formed; at pH 7.0 few were observed. When chloramphenicol (10 μg/ml) was added to the culture at pH 8.0, fewer irregular forms were observed. Addition of phenethyl alcohol (PEA), which inhibits initiation of DNA-replication also had the same effect. The pleomorphism was explained as being due to an unbalance between the rate of synthesis of cellular materials and cell wall synthesis. Formation of autolytic enzymes have been found in various bacteria. Strominger and Ghysen (1967) reviewed the literature on autolytic systems and also the various enzyme systems responsible for them. Kawata et al. (1968) obtained an autolysin from the cell wall fractions of Clostridium botulinum type A. This attacked the newly synthesized wall preferentially. Levin & Vaughn (1968) while studying the autolytic formation of spheroplasts in Desulfovibrio aestuarii found that the spheroplasts were non-viable and osmotically stable. They also found that the formation of spheroplasts was temperature dependent occurring at a maximum at 37-40°C.
Under the conditions of the experiment (Plate I) more and more of the irregular structures were observed as the TL-4 culture reached stationary phase. Lysis was noted. As the medium became viscous, spheroplasts were observed. The decrease in catalase inducibility could be explained by the lysis. The pH of the culture was usually 8.8.

Maintaining the pH of the culture at neutral values might obviate the formation of spheroplasts. This notion led to studies of catalase induction in cultures grown in the pH-stat.

The only morphological change observed among the various pH-cultures in the pH-stat experiments was a slight increase in size (Plate II) with increasing pH values. A maximum in the log phase catalase inducibility was seen around pH 7.3 (Fig. 10). At both pH 6.8 and 7.3, the inducibility decreased to a lower value as the culture reached stationary phase. But no such marked decrease was observed with the cultures at pH 7.8 and 8.3. In both cultures at the lower pH-values, the carbon source was completely exhausted 3 to 4 hours after the culture reached stationary phase. This is deduced from the stoichiometry of the addition of acid in the pH-stat. Because of the exhaustion of malate, no new synthesis could occur and the organism lost inducibility. Presence of malate in the induction medium did not reverse this. At pH 7.8 and 8.3, small amounts of malate were present throughout the experiment leading to a low rate of metabolism. This was deduced from the continuous slow rise in the pH of the culture. This may be the reason for a slightly greater inducibility of these cultures.

At all 4 pH-values, a drop in inducibility was observed as the Bchl synthesis ceased. This can be seen from the Bchl curves. Presumably due to the metabolic repression of porphyrin synthesis, no new synthesis of
Plate II. Composite of phase contrast photomicrographs of TL-4 grown in the pH-stat.
Figure 10. Effect of growing TL-4 at constant pH on catalase induction.

A pH 6.8
B pH 7.3
C pH 7.8
D pH 8.3

Scales and legend as in Fig. 6.
porphyrin occurred as the culture reached stationary phase. This led to the formation of lower amounts of enzyme. As the culture goes from log to stationary phase, there seems to be a change in metabolism. Small granular material (presumably poly-β-hydroxy butyrate granules), which were seen in all 4 cultures in the late-log phase began to disappear. Utilization of the poly-β-hydroxy butyrate would provide additional carbon and reducing power. The increase in inducibility at this time may not be trivial. The increase during the stationary phase was as high as that of the log phase (Fig. 6c). As shown in Table IV, the peak at the early stationary phase of the pH-stat cultures was comparable to that found in cultures whose pH was not controlled during growth.

In summary, one may conclude that the stationary phase inducibility is not affected by growing the cultures at constant pH. The inducibility of cells in the log phase was lower at each of 4 pH-values tested than in the culture whose pH was not controlled. The inducibility also had a maximum at pH 7.3. It is possible that some phenomenon related to chromatophore organization or energetics may depend on pH and plays a role in the pH-effect of catalase inducibility. Mitchell (1966) proposed a chemiosmotic mechanism for energy production in biological systems. Jagendorf (1967) observed a pH-dependent phosphorylation with spinach chloroplasts. von Stedingk (1967) also observed a light-dependent reversible pH-change with *R. rubrum*. Further, he observed an optimum pH of 7.4 for adenosine triphosphate production. It is possible that the maximum inducibility at pH 7.3 may be related to energy production.

If growing cells under constant pH had no effect on the stationary phase induction of catalase, then no change should be observed with *Rh.*
### TABLE IV

Amount of Catalase Induced During the Stationary Phase in TL-4 in the pH-stat.

<table>
<thead>
<tr>
<th>pH of the culture</th>
<th>Maximum Catalase induced (volt/min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.8</td>
<td>0.43</td>
</tr>
<tr>
<td>7.3</td>
<td>0.52</td>
</tr>
<tr>
<td>7.8</td>
<td>0.53</td>
</tr>
<tr>
<td>8.3</td>
<td>0.42</td>
</tr>
<tr>
<td>uncontrolled pH</td>
<td>0.62</td>
</tr>
</tbody>
</table>
**spheroides.** No change in morphology of the cells was observed (Plate III). Figure 11 shows catalase induction at all 4 pH-values. Although the cells reached the same O.D. 680nm, pigment content of the cells decreased with increasing pH. Porphyrin excretion reached a maximum at pH 7.8. Inducibility had a maximum at pH 7.3. This maximum value in inducibility was observed also in TL-4. The relationship between this pH and inducibility cannot be explained. The catalase assay in all cases was carried out at pH 7.4 as given under Materials and Methods. Chance (1952) observed no change in the catalase activity between the pH-values 5 and 9. Thus the higher activity of induced cells cannot be explained as a change in activity with pH. It is somehow related to the inducibility of the cells.

The initial control in porphyrin synthesis can be seen with respect to the A405nm curves (Fig. 11). As the Bchl synthesis reached the maximum, an initial plateau in A405nm was observed which increased thereafter. This plateau value could be correlated with low synthesis of catalase. Immediately after, the synthesis began to increase to a maximum at all 4 pH-values. This indicates that the mechanism of induction in Rh. spheroides is not altered by growing the culture at constant pH.

**Mechanism of Catalase Induction:**

Clayton (1960b) while studying the mechanism of catalase induction showed evidence for the formation of an internal inducer (II). This II decayed at a constant rate when incubated in the dark at 25°C. Light accelerated the rate of decay. Incubating the induced cells at 4°C in the dark preserved the inducer, indicating the decay is a biochemical reaction. The amount of inducer present can be quantitatively assayed as the amount
Plate III. Composite of phase contrast photomicrographs of *Rh. spheroides* grown in the pH-stat.
Figure 11. Effect of growing Rh. spheroides at constant pH on catalase induction.

A  pH 6.8
B  pH 7.3
C  pH 7.8
D  pH 8.3

Scales and legend as in Fig. 6.
of catalase which could be induced under appropriate conditions. This II was synthesized within the first 7 minutes which was also the lag period in induction.

The current dogma of molecular biology dictates that the information flows from DNA to RNA to protein. In systems like β-Galactosidase in Escherichia coli (Kaempfer and Magasanik, 1967), the m-RNA is synthesized immediately after the addition of the inducer. The synthesis of m-RNA is arrested soon after the inducer is removed. But in the catalase system of Rh. spheroides, the external inducer, H₂O₂, decomposes within the first 5 min. Using the β-Galactosidase system for a model, the immediate question which arose was, whether the II described by Clayton (1960b) was m-RNA. Although acriflavine is known to inhibit catalase induction it did not answer the question. A high level of m-RNA, if induced could also be observed chemically. Gray et al. (1964) were unable to detect a specific m-RNA fraction.

That the II observed by Clayton was not m-RNA may be deduced from Fig. 5. During the first 5 min. no synthesis of m-RNA could have taken place. The synthesis continued however up to 30 minutes once it began.

Van Niel (1944,'47) observed that introduction of air into an anaerobic culture of Rh. spheroides led to the formation of red carotenoids. He further found that yellow carotenoids were quantitatively and irreversibly converted to red ones. Further evidence of this was obtained by Cohen-Bazire et al. (1957). The chemical reactions involved in this conversion were characterized by Jensen (1962, 1965) (Fig. 12). A keto group is introduced in the C₂ position of spheroidene to give rise to spheroidenone. This reaction occurs in Rh. spheroides. The oxygen atom which is introduced
Fig. 12  OXYGEN DEPENDENT CAROTENOID CONVERSION IN RHODOPSEUDOMONAS SPHEROIDES
(FROM JENSEN, 1965)
comes from molecular oxygen and not from $H_2O$ (Shneour, 1962). Although some other species of Rhodopseudomonas can produce these pigments, the major form which most produce is of the spirilloxanthin type. As Table I shows, only those organisms which can synthesize spheroidene as the major pigment have an inducible catalase. This suggested correlation between catalase induction and carotenoid conversion.

Some of the carotenoidless mutants isolated by Sistrom et al. (1956) were unable to survive when air was introduced into an anaerobic culture. These mutants, which were green, were capable of growing aerobically in the dark or photosynthetically. It was explained that these mutants were killed by peroxides, formed in the presence of both light and air. Carotenoids may play a major role in preventing the formation of peroxides from excited Bchl molecules. Clayton (1962) with a high catalase, blue-green mutant of Rh. spheroides observed that catalase protected the cell from low but not high concentration of oxygen. No studies on the formation of catalase have been done with these mutants. Mitchell and Anderson (1965) observed that catalase was photoinactivated in carotenoidless organisms (Zea mays, Sarcina lutea). Photoinactivation of crystalline catalase followed first order kinetics. Thus, any study made on the formation of catalase in blue-green mutants would be in doubt.

Di-phenylamine inhibits the formation of carotenoids in a wide variety of organisms. Jensen (1962,65) reviewed the literature on these effects in the photosynthetic bacteria. Cohen-Bazire and Stanier (1958) observed in Rh. rubrum and Rh. palustris that diphenylamine at a concentration of $7 \times 10^{-5}M$ inhibited only carotenogenesis. It had no effect on growth. The di-phenylamine-inhibited cultures of Rh. rubrum were sensitive to
oxygen in the presence of light. Nugent and Fuller (1967) observed that 4-phenoxy-2,6-diamino pyridine, which inhibits pteridine utilization also inhibited carotenogenesis much like di-phenylamine. Fuller and Anderson (1958) obtained chromatophores from carotenoid-depleted Chromatium cultures and found them to photosynthesize normally. This concentration of di-phenylamine inhibited the growth of Rh. spheroides only. Goodwin et al. (1953) made the specific suggestion that di-phenylamine inhibited the transfer of high-energy phosphate to an acceptor in the electron transport chain. Rilling (1965) observed that di-phenylamine and various acridine dyes inhibited the carotenogenesis in Mycobacterium. He suggested a molecular mechanism implicating a binding of these dyes to the carotenoids which thus inhibit the dehydrogenation of saturated carotenoids. Future use of these inhibitors may reveal the role of carotenoids in catalase induction, if any.

As shown in Fig. 13, the addition of di-phenylamine at both 0 and 15 minutes after the addition of H₂O₂, inhibited further catalase induction almost completely. When 3 x 10⁻⁴ M, di-phenylamine was added after 30 minutes, the inhibition was much lower and the induction pattern followed the control. At very low concentrations of di-phenylamine, a stimulation of catalase induction was noted (Fig. 14a). Increasing the concentration of di-phenylamine inhibited the induction. At a concentration of 3 x 10⁻⁴ M di-phenylamine inhibited as much as 95% of induction. Conversion of yellow to red carotenoid was also inhibited progressively. A compound absorbing at 615 nm was observed in the supernatants of the di-phenylamine inhibited cultures. Its production was found to require whole cells and light. Cell-free extracts or culture supernatant did not produce
Figure 13. Effect of the addition of di-phenylamine at various time periods after the initiation of induction.

(40 μM H$_2$O$_2$ was added at 0-time)

Arrows indicate the time of addition of $3 \times 10^{-4}$ M di-phenylamine.

A  control
B  0-time
C  15 minutes
D  30 minutes
Figure 14a. Effect of concentration of di-phenylamine on the induction of catalase and carotenoid interconversion in *Rh. spheroides*.

- O------O catalase induced
- □-----□ yellow carotenoid converted
- △-----△ red carotenoid formed
- •-----• $A_{615 \text{nm}}$

b. Effect of concentration of acridine on the induction of catalase in *Rh. spheroides*. 
while studying the photochemical conversion of N-methyl-di-phenylamine observed a 610 nm-absorbing material as an intermediate in the formation of N-methyl carbazole.

Linschity and Grellmann (1964) postulated a triplet state intermediate of di-phenylamine before the 610 nm-absorbing material. They also observed that the 610 nm-absorbing material was quite stable in polar solvents. Bowen and Eland (1963) also observed similar reactions with di-phenylamine. They were unable to observe any triplet-state intermediate. The conversion was also independent of concentration, ranging from 5 x 10^{-5} M to 7 x 10^{-3} M. The formation of carbazole was inhibited by oxygen but did occur at temperatures as low as -190°C with the evolution of H2. These reports indicate that di-phenylamine could be converted to carbazole through a stable intermediate. The 615 nm-absorbing compound obtained in this study might be a form of the 610 nm-absorbing material in polar solvents. The appearance of this compound in the supernatant was correlated with the beginning of inhibition of catalase synthesis.

Acridine also had the same effect on catalase induction. The effect was not as pronounced as with di-phenylamine (Fig. 14b). The effect of acridine could not be the binding with DNA. The acridines in order to bind to the nucleic acids should have an ionizable group yielding a cation
(Albert, 1966). At pH 7.3, only 1% of acridine is ionized as cation, while almost 100% of acriflavine is ionized under similar conditions. Ionization giving rise to anions (Acridine-9-carboxylic acid) or zwitterions (9-amino acridine-4-carboxylic acid) had no effect on the growth of various organisms tested. The ionized acridines (cations) were found to bind with DNA (Blake, 1968; Waring, 1968), and to inhibit transcription. As Fig. 5 shows, the complete inhibition of induction by acriflavine occurred at 20\mu M. Acridine as high as 100\mu M, actually had a stimulatory effect. It is hard therefore to visualize a mechanism for the interaction of acridine with nucleic acids, similar to that shown by acriflavine.

Based on Rilling's model, it is possible that both acridine and di-phenylamine inhibit the catalase induction by the same mechanism. The effect of di-phenylamine can be explained in the following way:

a. An interaction with the carotenoids.

b. An interaction with the electron transport system.

Intermediates may exist at low concentrations between the yellow and red carotenoids. These may be termed X and Y. As oxygen was introduced into an anaerobic culture, the electron transport in the chromatophores was also inhibited (Vernon, 1968). This would lead to an accumulation of excited Bchl molecules. Clayton (1967) observed with photosynthetic bacteria, an increase in Bchl fluorescence as the light harvesting Bchl (P_{870}) was bleached. When the P_{870} was completely bleached, it was unable to trap the absorbed energy and the light harvesting Bchl returned to the ground state by fluorescent emission. About 20 Bchl molecules were found per each P_{870}. Zankel et al. (1968) observed in Rh. spheroides
that reducing the electron acceptors from P_{870} increased the fluorescence while phenazine methosulfate (PMS) addition altered the kinetics of both absorption and kinetics of P_{870}. Arnold and Clayton (1960) observed that illumination of dried chromatophores with infrared light led to temperature independent reversible absorption changes in the carotenoid region of the spectrum. This indicated that carotenoids and Bchl interacted in radiation transfer. Fork and Amesz (1967) observed similar light induced carotenoid absorption changes in both system 1 and system 2 of photosynthesis in red and brown algae. The data has been interpreted in the following way:

"... that the 515 nm change in green algae may be due not to an oxidation-reduction reaction, but rather to a change in the environment of a compound, probably chlorophyll b, which gives rise to a relatively small change in its absorption spectrum. The change in the environment would then be caused in some way by electron transport in system 1 or 2. The same might be true for the carotenoid shift in red algae..." If carotenoids do absorb energy from chlorophyll molecules, epoxy carotenoids may be formed; these could be converted to keto forms. Heath and Packer (1968a,b) observed a cyclic photoperoxidation of triunsaturated fatty acids in spinach chloroplasts. It is possible that one of these intermediates of carotenoid interconversion may act as the internal inducer for catalase.

If at low concentrations, di-phenylamine inhibits the conversion of X to Y, then the intermediate X would accumulate giving rise to a higher concentration of the inducer, and a greater catalase inducibility. Similar mechanism may be operative with acridine. At higher concentrations
both may also inhibit the formation of $X$. As the concentration of di-phenylamine is increased (during the inhibition process), it may also get oxidized by the excited Bchl molecules. This is possible because of its binding with carotenoids. The oxidized di-phenylamine intermediate may be excreted into the medium as it is formed. This may be the 615 nm-absorbing material. When the concentration of di-phenylamine was further increased, an effective redox couple may be formed between this compound and its oxidation product, carbazole. The proper redox potential may actually help bypass the block introduced by oxygen in the electron transport. This would restore normal conditions, as far as electron flow is concerned blocking further flow to carotenoids. In other words, restoration of electron transport would inhibit formation of the internal inducer and thus inhibit catalase induction.

Heath and Packer (1968a,b,c) found that the addition of $\text{H}_2\text{O}_2$ also acts as an inhibitor of electron flow which leads to the formation of fatty-acid epoxides. They also observed that addition of various co-factors like ferricyanide, PMS, 2,6-dichlorophenol-indophenol (DCPIP) had inhibitory effects on epoxide formation. From this, they concluded that overloading chlorophyll light collecting systems resulted in the photoperoxidation. They also obtained evidence for some fluorescent quenching by the above mentioned co-factors. Similar results were obtained by Arnon and his co-workers (1965). Fluorescence of spinach chloroplasts was better quenched by the co-factors of the cyclic photophosphorylation system (menadione, PMS) than by the non-cyclic ones. Murata et al. (1966) observed that mainly chlorophyll a fluoresced and that DCPIP quenched it.

The model proposed, here, for the formation of the internal inducer
Fig. 15  A SUGGESTED MODEL FOR THE MECHANISM OF CATALASE INDUCTION IN RHODOPSEUDOMONAS SPHEROIDES
(Fig. 15) predicts that only those organisms which can produce that particular carotenoid intermediate can be inducible for catalase. A look at Table I shows, inducible catalase is found only in *Rh. spheroides* and its related organisms. As discussed earlier, only these organisms can effect the conversion of carotenoids.

This model also predicts that, if there is a higher concentration of pigments, the induction of catalase would also be higher. This is borne out by the experiments with TL-1 and *Rps. D* (Table III). The higher the pigment content the greater is the maximum inducibility. The ratio of catalase to Bchl remains constant.

The effect of pH also can be explained by this hypothesis. The internal integration might vary depending on the pH in which the organism is growing. At higher pH-values less interaction between the various components may occur.

In conclusion, catalase induction in Athiorhodaceae occurs only in those organisms which can produce spheroidene and spheroidenone as major pigments. Some form of the carotenoid plays the role of internal inducer. The external inducer, oxygen or hydrogen peroxide acts only indirectly in the formation of internal inducer. The synthesis of catalase is dependent on the rate of porphyrin synthesis, which is the prosthetic group of the enzyme.
SUMMARY

Catalase induction was investigated in various organisms belonging to the family Athiorhodaceae. Catalase induction could be demonstrated only in *Rhodopseudomonas spheroides* and related locally-isolated organisms. Both RNA and protein synthesis were found to be necessary for the induced synthesis. Although $H_2O_2$, the added inducer, was decomposed in the first 5 min., m-RNA synthesis continued up to 30 min. In *Rh. spheroides*, maximum induction occurred during the early stationary phase, while in TL-1, TL-4 and Rps. D, the induction was during the logarithmic phase of growth. *Rh. spheroides* alone excreted porphyrins into the medium during the stationary phase. Inhibition of porphyrin synthesis by 8-hydroxy quinoline ($4 \times 10^{-5}$ M) also inhibited catalase induction. The induced synthesis of catalase has been interpreted to require porphyrin synthesis. Growing TL-4 at constant pH decreased the log phase level of induction. Inducibility under these conditions was maximum at pH 7.3. Growing either *Rh. spheroides* or TL-4 at constant pH had no effect on the stationary phase level of induction.

Induction of catalase was observed only in those organisms which can effect an oxygen dependent conversion of the carotenoid spheroidene to spheroidenone. Inhibition of this carotenoid conversion by inhibitors like diphenylamine ($3 \times 10^{-4}$ M) or acridine ($5 \times 10^{-4}$ M) also inhibited the catalase induction. Lower concentration (0.5 to 1.0 $\times 10^{-4}$ M) of the inhibitors had a stimulatory effect. The data is discussed in terms of the involvement of carotenoids in the induction of catalase.
APPENDIX A

Media used in this study:

1. Stock culture agar:

   MgSO$_4$·7H$_2$O  0.01%
   (NH$_4$)$_2$SO$_4$  0.05%
   K$_2$HPO$_4$  0.10%
   Lactic acid  0.20%
   Yeast extract  0.05%
   Agar  0.5%

   pH 7.0 before autoclaving.

2. Medium for *Rhodospirillum*

   A synthetic Malate-Ammonia-Defined (MAD) medium, developed by Ormerod *et al.* (1961) was used for the growth of *Rhodospirillum*.

STOCK SOLUTIONS

1. Basal Salts Solution

   water  800.0 ml
   (NH$_4$)$_2$SO$_4$  10.0 gm
   MgSO$_4$·7w  2.0 g
   CaCl$_2$·2w  0.75 g
   FeSO$_4$·7w  118.0 mg
   EDTA  200.0 mg

   dissolve each substance separately; adjust to pH 6.8 with NaOH

   trace element soln  10.0 ml

   water qs to  1 Liter
2. Trace elements

\[
\begin{align*}
&MnSO_4 \cdot 4H_2O & 2.1 \text{ g} \\
&H_3BO_3 & 2.8 \text{ g} \\
&Cu(NO_3)_2 \cdot 3H_2O & 40.0 \text{ mg} \\
&ZnSO_4 \cdot 7H_2O & 240.0 \text{ mg} \\
&Na_2MoO_4 \cdot 2H_2O & 750.0 \text{ mg} \\
\text{water qs to} & 1 \text{ Liter}
\end{align*}
\]

3. Phosphate solution

\[
\begin{align*}
&KH_2PO_4 & 40.0 \text{ g} \\
&K_2HPO_4 & 60.0 \text{ g} \\
\text{water qs to} & 1 \text{ Liter}
\end{align*}
\]

4. 20% DL-malic acid; pH 6.8 with NaOH

Store in deep freeze

5. Biotin

10 mg per 100 ml of 50% ethanol

DILUTE 1:20 before use

Store in deep freeze

FINAL MEDIUM

\[
\begin{align*}
\text{Water} & 800.0 \text{ ml} \\
\text{basal salts} & 100.0 \text{ ml} \\
\text{biotin (diluted)} & 3.0 \text{ ml} \\
\text{DL-malate} & 4.0 \text{ g} \\
\text{phosphate soln} & 15.0 \text{ ml}
\end{align*}
\]

pH before autoclaving is 6.8. Sterilize 121°C, 15 mins

Medium will be cloudy when hot but clears on cooling.
3. Medium for Rhodopseudomonas

The following vitamins were supplemented to the MAD-medium for the growth of Rhodopseudomonas.

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinic acid</td>
<td>1.0 mgm</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>0.5 mgm</td>
</tr>
<tr>
<td>p-Amino Benzoic Acid</td>
<td>0.2 mgm</td>
</tr>
<tr>
<td>MAD-medium</td>
<td>1.0 Liter</td>
</tr>
</tbody>
</table>

0.01% vitamin-free casaminoacids (Difco) was added to the Rhodopseudomonas medium for the cultivation of Rh. palustris.
APPENDIX B

GROWTH OF THE ORGANISM AT CONSTANT pH.

Growth of the organism was maintained at constant pH by using the 'pH-stat' apparatus described by Matsumoto (1966) with slight modifications (see Fig. A-1). pH of the culture was measured by a Beckman (Zeromatic) pH-meter and recorded in a potentiometric recorder (Varian Assoc.). The recorder was so adjusted that a raise in pH (due to the utilization of malate) discharged a condenser through a relay which activated a solenoid valve. As the valve opened, a constant volume (0.07 ml) of 0.5N H₂SO₄ was added to the culture. This series of events could repeat automatically every 15 seconds until the pH returned to the set point.

100 ml of the culture was grown in a specially modified 500 ml erlenmeyer flask. Either argon or helium was bubbled through the stirred culture. Samples were periodically taken with a syringe by puncturing the serum stopper.
Fig. A-1  pH-STAT APPARATUS

KEY
A EVENT MARKER
B CONTROL SWITCH
C MOTOR-DRIVEN SWITCH
20SEC CYCLE; 53SEC ON
D SOLENOID VALVE
E RELAY; 10K CCIL
F ACID-BASE SWITCH
G SILICON DIODE OR
SELENIUM RECTIFIER

1 CULTURE VESSEL
2 SAMPLING PORT
3 pH ELECTRODE
4 30°C WATER BATH
5 AIR FILTERS
6 HEAT FILTER
7 LAMP
APPENDIX C

Catalase Assay

Gui1dbau1t (1966a) developed an electrochemical system for enzyme assays, in which either the product or substrate was electroactive. The catalase assay described by this author (1966b) has been used with few modifications in this study. The apparatus used is a slight modification of the one described by Kramer et al. (1962) (Fig. A-2 & 3).

The reaction mixture in a specially constructed vessel contained in 4 ml, 10 mM of Tris-Maleate buffer, pH 7.4, 2 mM of H$_2$O$_2$ and the appropriate amount of enzyme. Before addition of the enzyme the two platinum electrodes were immersed into this solution. Tip of a saturated calomel electrode (SCE) (Beckman) was immersed into the same solution through the sidearm. The solution was stirred with a 3 x 10 mm magnetic stirrer bar. A constant current of 13 μA was passed across the two platinum electrodes from either one of the two regulated power supplies. The potential difference between the anode of the Pt electrodes and the SCE was fed to an electrometer (Keithley Instruments Inc. Model 610A). In an alternate system the output from the reaction vessel was fed through a voltage divider to the input of a Corning pH-meter (Model 7), operated in ±mv range (full scale 0 to 1.4 v). In both cases, the output voltage was recorded by a strip-chart recorders. When the output voltage reached a constant value, appropriate amount of enzyme preparation was added. Increase in the voltage was automatically recorded. Fig. A-4 is a tracing of an actual recording.

Theory:

When H$_2$O$_2$ is added to the buffer, the peroxide undergoes electrolysis.
Fig. A-2  POWER SUPPLY AND CATALASE ASSAY SYSTEM

1. REACTION VESSEL
2. Pt ELECTRODES (24G)
3. SATURATED CALOMEL ELECTRODE
4. MAGNETIC STIRRER
Fig. A-3 POWER SUPPLY AND CATALASE ASSAY SYSTEM

1. REACTION VESSEL
2. Pt ELECTRODES (24G)
3. SATURATED CALOMEL ELECTRODE
4. MAGNETIC STIRRER
Figure A-4. Voltage - time curve for the decomposition of \( \text{H}_2\text{O}_2 \) by 1 ml of a 19 hours old catalase-induced \textit{Rh. sphaeroides} culture.
Since the current applied between the two Pt electrodes is small, the amount of electrolysis is also trivial

\[ \text{2H}_2\text{O}_2 \rightarrow \text{2H}_2\text{O} + \text{O}_2 \quad \text{- 1} \]

According to Hickling and Wilson (1951), the anodic decomposition of H\textsubscript{2}O\textsubscript{2} occurs in 3 steps in neutral solutions.

\[ \text{2OH}^- \rightarrow \text{2OH} + 2\text{e}^- \quad \text{- 2} \]

\[ \text{OH} + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{HO}_2^- \]

\[ \text{OH} + \text{HO}_2^- \rightarrow \text{H}_2\text{O} + \text{O}_2 \]

Since H\textsubscript{2}O\textsubscript{2} is easily decomposed, compared to the other components in the reaction mixture, the Pt electrodes become depolarized with the addition of H\textsubscript{2}O\textsubscript{2}. As a result, a low potential is registered between the anode and the SCE. This whole electrolytic process obeys the Nernst equation (Guildbault et al., 1963).

\[ E = E_o + \frac{RT}{nF} \ln K \]

Where

- \( E \) = observed potential
- \( E_o \) = standard potential
- \( R \) = gas constant
- \( T \) = absolute temperature
- \( n \) = number of electrons transported
- \( f \) = Faraday constant
- \( K \) = equilibrium constant

From eq. 2

\[ E = E_o + \frac{RT}{nF} \ln \frac{(\text{H}_2\text{O})^2(\text{O}_2)}{(\text{H}_2\text{O}_2)(\text{OH})^2} \]
Since oxygen has unit activity and the reaction occurs in an aqueous phase, the terms in the numerator reduce to unity. The medium is well buffered preventing any change in OH-ions. Hydrogen peroxide remains as the only variable.

\[
E = E_0 + \frac{RT}{n_f} \ln \left( \frac{1}{(H_2O_2)} \right)
\]

\[
= E_0 - \frac{RT}{n_f} \ln (H_2O_2)
\]

Differentiating the above equation with respect to time, \( t \), gives

\[
\frac{dE}{dt} = -\frac{RT}{n_f} \cdot \frac{1}{(H_2O_2)} \cdot \frac{d(H_2O_2)}{dt}
\]

The equation predicts that, the drop in the electrode potential becomes proportional to the concentration of \( H_2O_2 \) in the reaction mixture.

Addition of catalase to this reaction mixture decreases the concentration of \( H_2O_2 \). The voltage begins to increase proportionately until it reaches a maximum value corresponding to a complete decomposition of \( H_2O_2 \). Since the rate of decomposition is proportional to the concentration of the enzyme, a measure of the rate of increase in voltage with time gives the amount of enzyme present.

Fig. A-5 is a standard curve run with Beef liver catalase. This preparation was assayed volumetrically according to the method of Herbert (1955) and found to be 1.945% pure. The figure indicates that the reaction rate increases linearly with increasing concentration of the enzyme.
Figure A-5. Effect of beef liver catalase concentration on reaction velocity.

Reaction mixture contained in a final volume of 4.0 ml, 10 mM of Tris-Maleate buffer, pH 7.4, 2 mM of $H_2O_2$ and the appropriate amount of the enzyme.
BIBLIOGRAPHY


Heath, R.L. and L. Packer 1968b. Photoperoxidation in Isolated
Chloroplasts II. Role of Electron Transfer. ibid., 125: 850-857

chloroplasts and Electron Flow. ibid., 125: 1019-1021

Herbert, D. 1955. Catalase from Bacteria (Micrococcus lysodeikticus)
In Methods in Enzymol. Bol. II. Ed. by S.P. Colowick and N.O. Kaplan


of Inhibitors of Nucleic Acid and Protein Synthesis on the Induced
Syntheses of Bacteriochlorophyll and 6-Amino levulinic acid Synthetase

Intracytoplasmic Membranes of Rhodospirillum rubrum. J. Bact., 89:
1413-1420

Jagendorf, A.T. 1967. Acid-base transitions and Phosphorylation by

Jensen, S.L. 1962. The Constitution of some Bacterial Carotenoids and
Their Bearing on Biosynthetic Problems. KGL. NORSKE VID. SELSK. SKR.,
RN.8 (Trondheim, Norway)

Jensen, S.R. 1965. Biosynthesis and Function of Carotenoid Pigments in

Johnston, M.A. and E.A. Delwiche 1965. Isolation and Characterisation of
the Cyanide-Resistant and Azide-Resistant Catalase of Lactobacillus
plantarum. J. Bact. 90(2): 352-356

Jones, R., R.H. Deibel and C.F. Niven Jr. 1964. Catalase Activity of
two Streptococcus faecalis strains and its Enhancement by Aerobiosis
and added cations. J. Bact., 88: 602-610

Jones, O.T.G. 1963a. The Production of Magnesium Protoporphyrin
Monomethyl ester by Rhodopseudomonas spheroides. Biochem. J., 86:
429-432

Jones, O.T.G. 1963b. The inhibition of Bacteriochlorophyll Biosynthesis
in Rhodopseudomonas spheroides by 8-OH Quinoline. Biochem. J., 88:
335-343

Jones, O.T.G. 1967. Intermediates in Chlorophyll Biosynthesis in
Rhodopseudomonas spheroides: Effects of Substrates and Inhibitors.
Phytochem., 6: 1355-1362


Waring, M.J. 1968. Drugs which affect the Structure and Function of DNA. Nature, **219**: 1320-1325

