INDUCTION OF ENZYMES FOR PYRIMIDINE CATABOLISM IN NOCARDIA CORALLINA

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Induced enzymes for the oxidation of uracil and thymine have been described in several bacterial species (Hayaishi and Kornberg, 1952; Lara, 1952; Batt and Woods, 1951). Two enzymes have been named and studied in detail: (i) uracil-thymine oxidase which catalyzed the oxidation of either uracil or thymine to the corresponding barbituric acid and (ii) barbiturase, which hydrolyzed barbituric acid, but not 5-methyl-barbituric acid, yielding malonic acid and urea. Little is known concerning the factors which affect the induction of these enzymes. In 1952, Lara reported the results obtained when the principle of sequential induction (Stanier, 1947; Cohn et al., 1953) was used to determine intermediate steps on the pathways for pyrimidine catabolism in Nocardia corallina. Cells grown on thymine as the main source of carbon and nitrogen, rapidly oxidized thymine, uracil, and barbituric acid and the following scheme was suggested:

\[
\text{thymine} \rightarrow \text{uracil} \rightarrow \text{barbituric acid}
\]

The possibility that N. corallina formed enzymes of low specificity for pyrimidine catabolism was not discussed by Lara (1952). This communication considers (i) factors which influence the induction of the uracil-thymine oxidase in N. corallina strain S, and (ii) difficulties encountered when the principle of sequential induction is used to study a catabolic sequence in which the enzymes do not exhibit rigid specificity.

METHODS

Organism. A bacterial species, capable of growing on uracil as the main source of carbon and nitrogen, was isolated by aerobic selective culture from medium A (KH₂PO₄, 5 g; MgSO₄·7H₂O, 2 g; CaCl₂, 2 g; H₂O, 1 liter; pH 7.2) to which uracil (5 g/liter) was added. After successive single colony isolations on tryptic meat agar, the organism was identified by Professor H. L. Jensen as a strain of N. corallina, and has been referred to subsequently as strain S (Martin and Batt, 1957; Midwinter and Batt, 1960).

The master culture has been subcultured once every 4 to 6 weeks since the original isolation in 1950 and, by spontaneous mutation, now contains a relatively high proportion of rough variants of the organism (originally the smooth variant). The two variants, isolated from single colonies differed markedly in their behavior on centrifugation. The rough variant packed well and could be readily washed unlike the smooth variant which proved difficult to centrifuge (3,000 × g).

The two variants showed identical properties in all other respects. In experiments involving prior inoculation of cells with inducers and subsequent assay of the organism for enzymatic activity, efficient washing of cell suspensions became necessary. For this work, therefore, the rough variant has been used.

Growth of the organism. Cells of strain S, not induced for pyrimidine catabolism were grown in either a tryptic meat broth, medium B (KH₂PO₄, 5 g; MgSO₄·7H₂O, 2 g; (NH₄)₂SO₄, 0.56 g; Lab Lemco, 2.5 g; H₂O, 1 liter; pH 7.2), or medium C (sodium propionate, 2 g; (NH₄)₂SO₄, 1.5 g; thiamine, 1.0 mg; and inorganic salts as in medium A; H₂O, 1 liter; pH 7.2). Cells induced for pyrimidine catabolism were cultured in a medium containing inorganic salts (medium A), uracil or thymine (1 g/liter), and yeast extract (0.1 g/liter). In all cases, media were autoclaved (121 C for 20 min) and inoculated with an aqueous suspension of cells taken from a tryptic meat agar slope (24 hr at 30 C). Incubations were carried out aerobically either in incubator bottles or in conical flasks (500 ml), containing 200 ml media, and shaken slowly (60 oscillations/min; amplitude 7.5 cm) at 30 C. After 65 hr the cells were harvested by centrifugation (2,000 × g for 20 min), washed with 0.1 M phosphate buffer (pH 7.2), and resuspended in buffer at a cell concentration equivalent to 10 mg dry weight/ml.

Induction of enzymes in washed cells. Washed
cells (equivalent to 100 mg dry weight) which had been grown in either a tryptic meat broth, medium B, or medium C were incubated aerobically in phosphate buffer (0.02 M; 12.5 ml) with the inducer (50 μmoles) at 30 C for 16 hr on a mechanical shaker (60 oscillations/min; amplitude, 7.5 cm). Control cells were similarly incubated with chloramphenicol (4.3 mg in 12.5 ml) and the inducer. At the end of the induction period the systems were centrifuged and the cells washed twice with 0.1 M phosphate buffer, pH 7.2.

Assay of cells for oxidase activity. Oxidase activity was assayed by following the ability of cells to oxidise uracil or thymine to the corresponding barbituric acids. However, incubation of cells with either of these pyrimidines constituted an induction system for strain S and accordingly all assays were carried out in the presence of chloramphenicol (final concentration 0.3 mg/ml). Systems for determining oxidase activity contained cells (10 mg dry weight), phosphate buffer (0.1 M; pH 7.2; 1.0 ml) and either uracil or thymine (10 μmoles); total volume, 3.0 ml. After incubating aerobically at 30 C for 90 min with shaking (100 oscillations/min; amplitude, 5 cm), the pyrimidine concentrations in the supernatants were determined spectrophotometrically, using a Hilger "Uvispec" spectrophotometer. The pyrimidines present in the supernatants were separated and identified by paper chromatography using the method of Markham and Smith (1949) and a solvent composed of isopropanol (100 volumes) and water (30 volumes).

Manometry. Conventional methods (Umbreit, Burris, and Stauffer, 1949) were followed for the manometric experiments. The manometer cups contained (unless otherwise stated) phosphate buffer (1.0 ml; 0.2 M; pH 7.2), cell suspension (0.5 ml; 10 mg dry weight) and distilled water (0.5 ml) in the main compartment and the substrate solution (0.5 ml; 0.02 M) or distilled water (for the controls) in the side bulb. The experiments were carried out at 30 C. For the experiments which lasted for more than 6 hr, the manometer cups and stoppers were previously heated in an air oven for 12 hr at 110 C, all solutions were autoclaved, and all transfers, made while setting up the experiment, were carried out under sterile conditions.

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tbody>
<tr>
<td><strong>Effect of yeast extract and glucose on growth of strain S on uracil</strong>*</td>
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<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td><strong>Growth Medium Containing</strong></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td><strong>Uracil removed</strong></td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>47</td>
</tr>
<tr>
<td>78</td>
</tr>
</tbody>
</table>

All media contained uracil, 2 g/liter, and inorganic salts as in medium A; pH 7.2.

Additions: glucose, 10 g/liter; yeast extract, 0.1 g/liter.

The inoculum was equivalent to 0.5 mg dry weight cells/100 ml media and the incubations were carried out in sloped Roux bottles at 30 C. Samples were removed at intervals for turbidity and pyrimidine estimations. Turbidity was measured in an EEL (Evans Electroselenium, Ltd.) colorimeter using a neutral density filter.

**RESULTS AND DISCUSSION**

Cell suspensions of *N. corallina* strain S grown on either uracil or thymine, as the main source of carbon and nitrogen, rapidly oxidized uracil, thymine, barbituric acid, or 5-methylbarbituric acid. Cells grown in media to which no pyrimidine had been added (e.g., tryptic meat broth, medium B, or medium C) did not oxidize uracil, thymine, or the barbituric acids rapidly within a period of at least 4 hr and it was concluded that the catabolism of pyrimidines in strain S was dependent on the presence of induced enzymes.

**Inhibition of pyrimidine induction.** The effect of glucose on the removal of uracil by strain S from growth media is shown in table 1. The availability of glucose markedly reduced the amount of uracil degraded by the organism. The cells from the three different media were centrifuged after 78 hr growth, washed, and tested, as suspensions, for uracil oxidase activity. Uracil was oxidized rapidly and without a lag by cells grown on uracil, or on uracil plus yeast extract. However cells grown in the uracil-glucose-yeast extract medium did not initially attack uracil. No oxidase activity was detected in cells grown in the presence, but
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washed free, of glucose, and presumably the carbohydrate prevented the induction of the oxidase (Cohn and Horibata, 1959). The effects of chloramphenicol and sodium azide were compared with the action of glucose on enzyme induction; the three substances all greatly reduced the ability of cells to form enzymes for the oxidation of thymine to 5-methylbarbituric acid in response to the inducer, thymine (table 2).

**Rate of induction.** The rate at which strain S responded to the presence of inducers was followed manometrically by observing the oxygen uptakes when noninduced cells (grown in a tryptic meat broth) were incubated with uracil, thymine, barbituric acid, and 5-methylbarbituric acid (figure 1). The maximal rates of oxygen uptake were reached at the following times for the different substrates: thymine, 6 hr; 5-methylbarbituric acid, 8 hr; uracil, 12 hr; barbituric acid, 14 hr.

**Specificity of the induction response.** At the times indicated by arrows on the oxygen uptake curves in figure 1, the cells from duplicate sets of manometers were centrifuged, resuspended in buffer, and tested with four pyrimidines for catabolic activity (figure 2). The cells from the control manometers (i.e., cells with no added TABLE 2

Inhibition of induction of thymine oxidase in strain S

<table>
<thead>
<tr>
<th>Additions to Incubation System</th>
<th>Final Incubation</th>
<th>Pyrimidines Present in Systems after 5 hr Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>Thymine</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.016</td>
<td>8.7</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0.001</td>
<td>8.0</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.01</td>
<td>7.4</td>
</tr>
</tbody>
</table>

The incubation system (3.0 ml) contained cells (grown in medium C; equivalent to 10 mg dry weight), phosphate buffer (1.0 ml; 0.1 M; pH 7.2) and thymine (10 μmoles). After 5 hr incubation, the samples were centrifuged and the pyrimidine concentrations determined.

*Figure 1.* Oxygen uptake curves obtained during the induction of enzymes in strain S by uracil (U), thymine (T), barbituric acid (BA), and 5-methylbarbituric acid (MBA). The arrows indicate the times at which cells were removed from duplicate manometers and tested for their ability to degrade the same four pyrimidines (figure 2). Each manometer cup contained 10 μmoles of substrate.
substrate) were similarly tested after 17 hr incubation (figure 2). Each of the cell suspensions attacked all four pyrimidines more rapidly than the control cell suspension.

The ability of the cells to oxidize both uracil and thymine, irrespective of whether the inducing substrate was uracil, thymine, barbituric acid, or 5-methylbarbituric acid, indicated the formation in each type of cell suspension of a single enzyme which activates both uracil and thymine. In addition, it appeared that as inducers of enzymes for pyrimidine catabolism thymine was equivalent to 5-methylbarbituric acid and uracil was equivalent to barbituric acid.

The induction of the uracil oxidase by barbituric acid was also demonstrated by incubating a cell suspension of strain S (grown in medium C) aerobically, (shaking at 60 oscillations/min) at 30 C with barbituric acid for 16 hr. The resultant cells had a high oxidase activity when compared with control cells (similarly incubated in the absence of barbituric acid) and did not remove any barbituric acid during the assay period (90 min). The uracil oxidase has not been shown to catalyze a reversible reaction and it was concluded that barbituric acid had induced an enzyme for a reaction in which it was a product but presumably not a substrate.

**Induction of uracil oxidase by 6-methyluracil.** From tests with a series of compounds to determine the specificity of inducers for uracil oxidase, it was found that noninduced cell suspensions of strain S, after shaking slowly for 16 hr at 30 C with 6-methyluracil, showed high uracil oxidase activity. Strain S did not attack 6-methyluracil and this substance did not support the growth of the organism. 6-Methyluracil was therefore an inducer of an enzymatic reaction for which it was neither substrate nor product.

**Specificity of enzyme for uracil and thymine oxidation.** In 1952 Hayaishi and Kornberg described the properties of an induced enzyme (uracil-thymine oxidase) in a strain of Mycobacterium which oxidized uracil to barbituric acid or

![Diagram](https://example.com/diagram.png)
TABLE 3
Rate of pyrimidine removal by noninduced cells of strain S

<table>
<thead>
<tr>
<th>Media in Which Cells Were Grown</th>
<th>17 hr Uracil Removed</th>
<th>65 hr Thymine Removed</th>
<th>17 hr Uracil Removed</th>
<th>65 hr Thymine Removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract (1%) +</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>sodium acetate (1%)</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Casein hydrolyzate*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Casein hydrolyzate +</td>
<td>0</td>
<td>79</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>yeast extract (0.01%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Casein hydrolyzate +</td>
<td>0</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>thiamine (18 μg/ml)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

The incubation systems (10 ml in 50-ml conical flasks) contained cells (20 mg dry weight), phosphate buffer (4.0 ml; 0.1 M; pH 7.2) and either uracil or thymine (80 amoles). The flasks were shaken aerobically at 30 C and samples removed at 17 hr and 65 hr for pyrimidine analysis.

* The casein hydrolyzate (10 per cent) was a vitamin-free product supplied by Difco and used at a level, in the growth medium, of 1 ml/100 ml.

thymine to 5-methylbarbituric acid. The similar enzyme in strain S, however, activates a wider range of substances; in addition to uracil and thymine, it could be oxidized to the corresponding barbituric acids, 2-thiouracil and 2-thiouracil, which were oxidized to 2-thiobarbituric acid and 2-thio-5-methylbarbituric acid, respectively, by the strain S oxidase.

Further information concerning the specificity of the enzyme in vivo was obtained by varying the composition of the growth medium to give cells in which the oxidase could be induced by thymine but not by uracil (table 3). Cells grown on a casein hydrolyzate were washed thoroughly and added to a buffered medium containing either uracil or thymine. The suspension completely removed both uracil and thymine in 17 hr when incubated aerobically at 30 C with shaking. Approximately 30 per cent of the uracil which had disappeared could be accounted for as barbituric acid which had accumulated during the experiment. The results had shown, therefore, that cells, preincubated with thymine, attacked both uracil and thymine, although uracil (in the preincubation system) was not an inducer, and these findings were consistent with the proposition that (in strain S) a uracil-thymine oxidase was induced which could activate more than one substrate.

Application of the principle of sequential induction. In 1952, Lara used the principle of sequential induction to determine the intermediate steps in pyrimidine catabolic pathways for a strain of N. corallina. From the observation that cells induced for thymine oxidation were simultaneously induced for uracil oxidation, whereas cells not induced for thymine catabolism did not attack uracil lead Lara to suggest that uracil was a possible intermediate in the thymine catabolic sequence. With the demonstration, by Hayashi and Kornberg (1952), and from the present study, that pyrimidine oxidation in bacteria may be initiated by an oxidase which was active with several substrates, the validity of the conclusions given by Lara (1952) became questionable. Stanier (1951) stated that "the validity of the biochemical inferences drawn from such experiments rests on two assumptions which may not always be correct: rigid specificity of adaptive response and free permeability of the cell to all compounds tested.

Cell suspensions of strain S grown on thymine, oxidized rapidly thymine, uracil, 2-thiouracil, 5-methylbarbituric acid, and 5-hydroxymethyluracil. If uracil was an intermediate for thymine breakdown (on sequential induction principles) a single catabolic pathway could not include both 5-methylbarbituric acid and 5-hydroxymethyluracil. Although thymine may be degraded to uracil by some, as yet uncharacterized, bacterial system, such a conversion cannot be deduced from these induction studies.

Differential induction. Although the induction of a nonspecific enzyme invalidates conclusions based on the principle of sequential induction, the nonspecific enzyme system may be used to demonstrate enzymic reactions in vivo.

Cells of strain S grown on uracil have been shown to convert thymine to 5-methylbarbituric acid; the 5-methylbarbituric acid accumulated quantitatively from the thymine oxidized because the cells, although induced for thymine oxidation were not induced for the oxidation of 5-methylbarbituric acid. Subsequently the 5-methylbarbituric acid was degraded by the cells as the enzymes for its catabolism were induced by the acid itself.
SUMMARY

A uracil-thymine oxidase has been induced in Nocardia corallina strain S by uracil, thymine, barbituric acid, 5-methylbarbituric acid, 5-hydroxymethyluracil, and 6-methyluracil. Induction of the oxidase was inhibited by glucose, chloramphenicol, and sodium azide. The oxidase was shown to be a nonspecific enzyme which activated uracil, thymine, 2-thiouracil, or 2-thiothymine for oxidation to the corresponding barbituric acids. The principle of sequential induction could not be directly applied to studies on pathways for pyrimidine catabolism.

REFERENCES


