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PYRIMIDINE CATABOLISM
IN Nocardia Corallina

A thesis presented in partial
fulfilment for the degree of
Master of Science in Biochemistry.

at

Massey University

Douglas Opie Mountfort
1971
I wish to thank Professor R.D. Batt for his supervision throughout the course of the investigation.

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SECTION 1

INTRODUCTION
The degradation of pyrimidines in living systems may be initiated by either a reductive step leading to the formation of a dihydropyrimidine intermediate, or an oxidative step yielding the corresponding barbituric acid. In the case of thymine however, the oxidative step can lead alternatively to the formation of 5-hydroxymethyluracil.

In the following discussion of the literature oxidative catabolism is treated separately from reductive catabolism.

1. Oxidative catabolism of pyrimidines.
   A. Barbituric acids as intermediates.

   Studies by Lara (1952a), based in the principle of sequential induction, indicated that uracil and barbituric acid could be intermediates in the catabolism of thymine in N. corallina.

   From the observation that organisms induced for thymine oxidation were simultaneously induced for uracil oxidation whereas organisms not induced for thymine catabolism did not attack uracil, Lara suggested that uracil was a possible intermediate in the breakdown of thymine. Further, since barbituric acid was oxidised more rapidly by thymine grown organisms, than those grown on yeast extract, it was proposed as the intermediate to follow uracil in the thymine catabolic sequence.

   Lara (1952b) showed that cell-free extracts derived from
organisms grown on either thymine, uracil or barbituric acid were active towards thymine and uracil in oxidising these compounds to the corresponding barbituric acids.

The finding of 5-methylbarbituric acid as the oxidative product of thymine did not support the conclusion reached in earlier studies (Lara, 1952a). The earlier proposal that uracil was degraded through barbituric acid was supported by studies with cell-free extracts. It was feasible that barbituric acid could have been formed in a reaction involving a demethylation of 5-methylbarbituric acid.

Some of the conclusions of Lara (1952b) were substantiated by the subsequent work of Wang and Lampen (1952) and Hayashi and Kornberg (1952).

Wang and Lampen (1952) prepared extracts from an unidentified bacterium (Strain U-1) by alumina grinding and found that such extracts catalysed the oxidation of uracil and thymine with the uptake of 1 atom of oxygen per mole of pyrimidine. These workers were successful in isolating barbituric acid from the uracil reaction mixture, but did not isolate the product formed from thymine under similar conditions.

Hayashi and Kornberg (1952) working with crude enzyme preparations of uracil-thymine oxidase from strains of Mycobacterium and Corynebacterium, isolated and identified both the oxidation products from thymine and uracil as 5-methylbarbituric acid and barbituric acid respectively.
The oxidation of pyrimidines to the corresponding barbituric acids is shown as part of an overall scheme for pyrimidine catabolism in Figure 1.

Batt and Woods (1961) questioned the possibility that in *N. corallina* the pathways for pyrimidine catabolism were likely to operate solely via the corresponding barbituric acids. 5-methylbarbituric acid was established as the only oxidative product of thymine catabolism by uracil grown organisms, but other oxidative pathways could have been operating in thymine grown organisms. Likewise, barbituric acid was established as the only oxidative product of uracil catabolism by thymine grown organisms, but again alternative pathways could be present in cells grown on uracil.

Manometric studies were carried out on thymine and uracil grown organisms oxidising thymine, uracil, barbituric acid and 5-methylbarbituric acid. For the oxidation of thymine and 5-methylbarbituric acid the difference in total oxygen uptake was not equivalent for both thymine and uracil grown organisms (Table 1). Further, the difference in total oxygen uptake was comparable with the theoretical value only in the case of uracil grown cells. The discrepancy in oxygen uptake together with the uncertainty of 5-methylbarbituric acid as an intermediate of thymine catabolism in *homologous cells led Batt and Woods to suggest that in such cells, a second pathway could exist in which 5-methylbarbituric acid was not an intermediate.

* Homologous cells are those oxidising the pyrimidine on which they were grown.
Figure 1. Scheme for the oxidative catabolism of pyrimidines with barbituric acids as intermediates.

Thymine $\xrightarrow{1/2O_2}$ 5-Methylbarbituric acid $\xrightarrow{1/2O_2}$ Barbituric acid $\xrightarrow{2H_2O}$ Urea $\xrightarrow{2H_2O}$ Malonic acid

Established Reactions

Tentative Reactions
TABLE 1

Total oxygen consumption during oxidation of various pyrimidines.

(After Batt and Woods, 1961)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Oxygen Uptake</th>
<th>Theoretical Oxygen Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmole/μmole pyrimidine</td>
<td>μmole/μmole pyrimidine</td>
</tr>
<tr>
<td></td>
<td>for organisms grown on</td>
<td></td>
</tr>
<tr>
<td>Uracil</td>
<td>1.01</td>
<td>2.5</td>
</tr>
<tr>
<td>Thymine</td>
<td>1.48</td>
<td>2.0</td>
</tr>
<tr>
<td>Barbituric acid</td>
<td>0.87</td>
<td>2.0</td>
</tr>
<tr>
<td>Thymine</td>
<td>2.07</td>
<td>4.0</td>
</tr>
<tr>
<td>5-methylbarbituric</td>
<td>1.47</td>
<td>3.5</td>
</tr>
<tr>
<td>acid</td>
<td>0.88</td>
<td></td>
</tr>
</tbody>
</table>

The oxidation of uracil and barbituric acid by uracil and thymine grown organisms presented a similar case (Table 1). It was concluded accordingly, that for uracil catabolism by homologous cells a second pathway was likely in which barbituric acid was not an intermediate.

B. The fates of 5-methylbarbituric acid and barbituric acid.

Although in the microbial oxidation of thymine and uracil the corresponding barbituric acids have been established as intermediates, only the fate of barbituric acid has been elucidated in some detail.

Lara (1952) found that extracts obtained from N. corallina grown on thymine were active in hydrolysing barbituric acid with the formation
of 1 mole of CO\textsubscript{2}, two moles of NH\textsubscript{3}, and one mole of malonic acid/mole of substrate (Figure 1). Urea, which accounted for the total CO\textsubscript{2} and NH\textsubscript{3} production was established as a direct product of barbituric acid hydrolysis. Hayaishi and Kornberg (1952) working with extracts of uracil adapted organisms of a strain of Mycobacterium likewise established that barbituric acid was hydrolysed to malonic acid and urea. These workers partially purified the barbiturase enzyme using a technique of protamine sulphate fractionation, followed by cation exchange column chromatography.

Batt and Woods (1961) expressed doubt that free malonic acid was likely to be an intermediate in the breakdown of barbituric acid in intact cells. These workers showed that although barbituric acid could be converted anaerobically to malonic acid, CO\textsubscript{2} and NH\textsubscript{3} by enzyme preparations of \textit{N. corallina}, malonic acid could not be degraded by whole cells under the same conditions. Such results were also supported by those of Hayaishi and Kornberg (1952) in which it was shown that whole cells of a uracil adapted strain of \textit{Mycobacterium} could not attack barbituric acid under anaerobic conditions, as opposed to cell-free extracts which could.

Batt and Woods (1961) further showed that there was no oxidation of malonic acid by pyrimidine adapted cells of \textit{N. corallina} and similar results were obtained by Hayaishi and Kornberg (1952) working with uracil adapted cells of \textit{Mycobacterium}.

From their results Hayaishi and Kornberg (1952) proposed that the absorption of barbituric acid into the cell was energy requiring,
and could only be facilitated under aerobic conditions. The poor utilisation of malonic acid by uracil adapted organisms was attributed to a permeability barrier. Thus malonic acid was not excluded as a likely product of barbituric acid degradation.

Batt and Woods (1961) showed that malonic acid although not oxidised itself, could inhibit succinate oxidation by cells of N. corallina. Presumably, therefore, the malonic acid could permeate the cells to the site of succinate oxidation. They suggested from their results that a derivative of malonic acid is formed from barbituric acid and this derivative is further metabolised in vivo without the intermediate formation of free malonic acid.

Less is known about the degradation of 5-methylbarbituric acid. Attempts to obtain bacterial homogenates or cell-free extracts active upon 5-methylbarbituric acid have all been unsuccessful. Hayashi and Kornberg (1952) used both sonication and alumina grinding to obtain homogenates of a Mycobacterium strain. It was found that homogenates prepared by either method were uniformly inactive towards 5-methylbarbituric acid. Similarly, Lera (1952) prepared cell-free extracts from a pyrimidine adapted strain of N. corallina, and found that such extracts were active towards thymine, uracil and barbituric acid, but not 5-methylbarbituric acid. Similar results were obtained by Batt and Woods (1961) and more recently Biggs and Doumas (1963) reported that cell-free extracts of a Corynebacterium strain were active towards barbituric acid but not 5-methylbarbituric acid.

Because bacterial cell-free extracts were found to be inactive
towards 5-methylbarbituric acid, there was some doubt as to whether an enzyme existed which acted on the compound. Batt and Woods (1961) showed that with adequate aeration, 5-methylbarbituric acid was rapidly oxidised in phosphate buffer at neutral pH, to 5-hydroxy - 5-methylbarbituric acid. This result was substantiated by the findings of Biggs and Doumas (1962), who further showed that 5-hydroxy - 5-methylbarbituric acid was slowly fragmented to give methyltartronylurea. Such findings presented the possibility that 5-methylbarbituric acid might be spontaneously oxidised within the cell. However, studies by Batt and Woods (1961) showed that when 5-methylbarbituric acid was incubated with a cell suspension of *N. corallina* no 5-hydroxy - 5-methylbarbituric acid was formed under their experimental conditions. It was considered that the presence of an actively metabolising suspension of organisms, suppressed, in some way, the formation of 5-hydroxy-5-methylbarbituric acid. Further, 5-hydroxy - 5-methylbarbituric acid was not oxidised by whole cells of *N. corallina*, and was considered unlikely to be an intermediate of 5-methylbarbituric acid catabolism.

Biggs and Doumas (1962) assessed the possible roles of 5-hydroxy-5-methylbarbituric acid and methyltartronylurea as intermediates of 5-methylbarbituric acid catabolism in *Corynebacterium*. Neither compound supported growth of the organism. Furthermore, urea, which is normally an end product of thymine catabolism, was not detected in the media. From such results it was considered unlikely that 5-hydroxy - 5-methylbarbituric acid or methyltartronylurea were intermediates of 5-methylbarbituric acid catabolism. It did not necessarily follow however, that these compounds could not be enzymatically formed from 5-methylbarbituric acid.
In 1963 Biggs and Doumas working with Corynebacterium provided evidence for 5-methylbarbituric acid being hydrolysed to methylmalonic acid and urea. When 5-methylbarbituric acid - 2 - C\textsuperscript{14} was used as the substrate, the isolated urea was not only radioactive, but also had the same specific activity as the starting material. The activity of the isolated methylmalonic acid was only 2\% of that of 5-methylbarbituric acid - 2 - C\textsuperscript{14}. However it was found by chromatography that the methylmalonic acid was contaminated with other labelled compounds, and because of the small amount of acid isolated, a further purification was not attempted.

When 5-methylbarbituric acid - 5 - C\textsuperscript{14} was used as the substrate, the methylmalonic acid isolated had a specific activity which was 96\% of the theoretical maximum value. The amount of accumulated acid was small (only 1.66mg. compared with the 500 mg. of 5-methylbarbituric acid - 5 - C\textsuperscript{14} originally used). The specific activity of urea in this experiment was 1000 times lower than that of the original substrate.

Although methylmalonic acid was proposed as an intermediate in the catabolism of 5-methylbarbituric acid, on the basis of the above results, Biggs and Doumas (1963) did not consider the possibility that it could be produced from a minor reaction sequence.

C. The oxidation of thymine to 5-hydroxymethyluracil.

5-hydroxymethyluracil has been established as a product of thymine oxidation in rat liver systems. (Fink, Cline, Henderson and Fink, 1956).
In microbial systems the only reported cases of thymine conversions to 5-hydroxymethyluracil have been shown to occur in *Neurospora*.

Abbott, Kadner and Fink (1964) studied the activity of extracts from ground *Neurospora* mycelia, towards thymine. Initial experiments showed that the extracts were inactive towards the compound in the absence of co-factors. However in the presence of glutathione, reduced nicotinamide adenine dinucleotide phosphate (NADPH + H⁺) and oxygen, extracts were shown to convert thymine to 5-hydroxymethyluracil. Thymine-7-hydroxylase was the name proposed for the enzyme which catalysed this reaction.

Holme, Lindstedt, Tofft and Lindstedt (1970) working with an enzyme preparation obtained from a strain of *Neurospora* demonstrated that the hydroxylation of thymine was dependent on 2-oxoglutarate. Furthermore, a stoichiometric relationship was found between the hydroxylation of thymine, and the decarboxylation of 2-oxoglutarate. Carbon dioxide and succinate were found to be the products of 2-oxoglutarate degradation.

II. The reductive catabolism of pyrimidines

The first significant studies on the reductive catabolism of pyrimidines in micro-organisms, were carried out by DiCarlo, Schultz and Kent in 1952.

These studies were a continuation of earlier work on the nature of cytosine breakdown in yeasts. (Hahn and Haarmann, 1926; Chargaff and Kream, 1948; DiCarlo, Schultz and McManus, 1951.)
Hahn and Haarmann (1926) and Chargaff and Kream (1948) demonstrated with cell-free extracts of yeast, the conversion of cytosine to uracil. The enzyme catalysing the conversion was referred to as a cytosine deaminase. DiCarlo, Schultz and McManus showed that both *Saccharomyces cerevisiae* and *Torula utilis* could utilise cytosine as a source of nitrogen for growth. However, only *T. utilis* could utilise uracil. The results indicated that although both organisms were likely to possess the necessary deaminase for cytosine, only *T. utilis* possessed the enzymes necessary for the further degradation of uracil.

DiCarlo, Schultz and Kent (1952) further showed that neither *S. cerevisiae* nor *T. utilis* could utilise barbituric, isobarbituric or isodialuric acids for growth. This ruled out the possibility that the initial step in the catabolism of uracil was oxidative.

The possibility that the initial step involved carboxylation seemed unlikely, since the yeasts failed to grow on uracil - 5 - carboxylic acid or orotic acid.

When dihydrouracil was tested, it was found to be completely utilised by *T. utilis*, but supported no growth of *S. cerevisiae*.

*β*-Aminopropionamide, *β*-ureidopropionic acid, hydro-orotic acid, and hydrouracil - 5 - carboxylic acid, were tested as possible degradation products of hydrouracil. Hydrouracil-5-carboxylic acid did not support the growth of either yeast and so was eliminated as a catabolic product of hydrouracil. *β*-ureidopropionic acid and *β*-Aminopropionamide served as excellent nitrogen sources for *T. utilis*, but per-
mitted no growth of *S. cerevisiae*. It was suggested that both compounds were converted to P-alanine which could also be utilised by *T. utilis*. Dihydro-orotic acid was shown to be utilised by both yeasts, and this led DiCarlo, Schultz and Kent to suggest it was possibly produced from dihydrouracil.

It was further suggested from the ability of a variety of other compounds to serve as nitrogen sources for growth, that urea was likely to be produced from dihydro-orotic acid. The scheme for the degradation of cytosine via uracil to urea and possibly succinate acid as proposed by DiCarlo et al. on the basis of growth studies and structural considerations, is shown in Figure 2.

In 1953 Batt, Martin and Ploesser claimed that dihydro-orotic acid was not utilised by *T. utilis*; the compound was shown to be chemically unstable under the experimental test conditions which had been used in the above studies. This cast doubt on the proposal by DiCarlo, Schultz and Kent, that dihydro-orotic acid was likely to be an intermediate in the reductive catabolism of uracil.

Campbell (1957b) working with a strain of *Clostridium uracilicum* identified dihydrouracil, and P-ureidopropionic acid as intermediates in the conversion of uracil to P-alanine, CO₂, and NH₃. The scheme for the degradation of uracil according to Campbell is shown in Figure 3. Such findings would also offer support to the possibility that in *T. utilis*, P-ureidopropionic acid could serve as an intermediate in the reductive catabolism of uracil instead of dihydro-orotic acid.
Figure 2. Suggested scheme for the breakdown of cytosine in yeasts. (after DiCarlo, Schultz and Kent, 1952.)

\[
\begin{align*}
\text{Cytosine} & \xrightarrow{+H_2O} \text{Uracil} \\
\text{Uracil} & \xrightarrow{+2H} \text{Hydouracil} \\
\text{Hydouracil} & \xrightarrow{+CO_2} \text{Hydro-orotic acid} \\
\text{Hydro-orotic acid} & \xrightarrow{} \text{Succinic acid + Urea}
\end{align*}
\]
Figure 3. Scheme for the reductive catabolism of uracil.
(After Campbell, 1957; Kraemer and Kaltwasser, 1970.)

\[
\begin{align*}
\text{Uracil} \\
\text{+ 2H} & \xrightarrow{\text{Dihydrouracil Dehydrogenase}} \\
\text{Dihydrouracil} \\
\text{+ H}_2\text{O} & \xrightarrow{\text{Dihydrouracil Hydrase}} \\
\beta\text{-ureidopropionic acid} \\
\text{+ 2H}_2\text{O} & \xrightarrow{\beta\text{-ureidopropionase}} \\
\text{CO}_2 + \text{NH}_3 + \text{NH}_2\text{-CH}_2\text{-CH}_2\text{-COOH} & \\
\beta\text{-alanine}
\end{align*}
\]
Campbell (1957c) isolated and partially purified the dihydro­uracil dehydrogenase which catalysed the reductive step in uracil catabolism. The results of Campbell (1957) were further substantiated by the findings of Kraemer and Kaltwasser (1970a). These workers demonstrated with cell-free extracts of cytosine grown <i>Hydrogenomonas facilis</i>, that cytosine was converted to uracil, which in turn was converted to β-alanine, CO₂ and NH₃ via dihydrouracil and β-ureidopropionic acid. Barbituric acid and urea were not detected. Cytosine deaminase, dihydrouracil dehydrogenase, dihydrouracil hydrase, and 3-ureidopropionase but not uracil oxidase, were demonstrated in cell-free extracts.

Kraemer and Kaltwasser (1970b) carried out further studies on the metabolism of cytosine and uracil in wild type and mutant strains of <i>H. facilis</i>. Mutant strains unable to utilise uracil as a nitrogen source were derived by treatment of organisms with l-methyl-3-nitro-l-nitroso guanidine and incubation at various concentrations of penicillin.

One group (A) of these mutants lacked dihydrouracil dehydrogenase and did not utilise thymine, orotic acid, or uracil, while a second group (B) lost the ability to form dihydrouracil hydrase and was unable to utilise dihydrouracil and dihydrothymine, as well as the compounds not utilised by group (A). Group (A) excreted uracil and group (B) dihydrouracil during incubation with cytosine. Wild type organisms did not possess dihydro-orotic dehydrogenase or dihydro-orotase as demonstrated with cell-free extracts.

The results from these studies indicated that uracil and thymine
were utilised by a non-specific dehydrogenase and that both dihydro-
uracil and dihydrothymine were acted upon by a non-specific hydrase.