THYMINE CATABOLISM IN NOCARDIA CORALLINA

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ABSTRACT

The oxidation of thymine, 5-methylbarbituric acid, methylmalonate and succinate was studied in cells grown on thymine, uracil, 5-methylbarbituric acid, barbituric acid, methylmalonate and succinate. In agreement with the results of Batt and Woods (1961) it was shown that thymine-grown cells oxidise thymine to 5-methylbarbituric acid which is in turn rapidly metabolised. Uracil-grown cells were shown to oxidise thymine to 5-methylbarbituric acid which accumulates and is metabolised only after thymine is all used up. Methylmalonate and succinate were oxidised significantly only in cells grown on the same carbon source, probably reflecting a requirement for permease.

Metabolism of 5-methylbarbituric acid by cell-free extracts (but not by boiled cell-free extracts) was demonstrated, but the products remained unidentified. The use of [^{14}C] 5-methylbarbituric acid in experiments with cell-free extracts was complicated by the gradual auto-oxidation of 5-methylbarbituric acid before and after the incubation period.

The stability of 5-methylbarbituric acid under various experimental conditions was examined. Chromatographic separation of 5-methylbarbituric acid from growth medium resulted in up to 42% yield of 5-methylbarbituric acid. On storage, it was shown that [^{14}C] 5-methylbarbituric acid was converted to 5-hydroxy-5-methylbarbituric acid and two other products, the major one of which was probably methyl-tartronyl urea.

In long term incubations (1.5 to 6 hr.) of uracil-

grown cells with [methyl-^{14}C] thymine, most of the radioactivity incorporated in the ethanol soluble extract was in glutamate. Labelled methylmalonate was also produced, but in very low levels (this confirms the report of Mountfort, 1971). The long term incubation period and the presence of impurities in [^{14}C] thymine made interpretation of results difficult.

The remainder of the work was devoted to short term incubations by thymine-grown cells with high specific activity [^{14}C] thymine. The incorporation of ^{14}C into various compounds was followed by two-dimensional thin layer chromatography (in phenol : water and n-butanol : acetic acid : water solvents) and autoradiography; and co-chromatography of radioactive compounds in various solvents.

Kinetic studies with [2-^{14}C] thymine suggest the following labelling sequence of thymine breakdown products: Thymine ⟶ 5-methylbarbituric acid ⟶ urea ⟶ CO_2. At very early times, an additional, rapidly metabolised compound appeared, and it is suggested that this may be thymidine.

By a combination of results obtained by incubating cells with [2-^{14}C] and [methyl-^{14}C] thymine it could be shown that no 5-hydroxymethyluracil, uracil, barbituric acid, dihydrothymine, or β-ureidoisobutyrate were formed. This suggests that neither the reductive pathway nor the oxidative pathway via uracil operates in thymine-adapted Nocardia corallina under the experimental conditions used here.

Kinetic studies with [methyl-^{14}C] thymine suggests the following scheme of labelling of intermediates: Thymine ⟶ 5-methylbarbituric acid ⟶ methylmalonyl CoA

(activated methylmalonate) ⟶ succinate ⟶ aspartate and alanine and then glutamate. A large pool of aspartate and glutamate present in Nocardia corallina acts as a trap for ^{14}C.

Activated methylmalonate was identified by hydrolysis to methylmalonate and also by treatment with hydroxylamine to form methylmalonyl hydroxamate. Some activated succinate was also present since hydroxylamine treatment led to the formation of a hydroxamate, which on acid hydrolysis formed succinate.

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INTRODUCTION

I. Pyrimidine Catabolism

Thymine is catabolised in living organisms by three known routes, namely a reductive pathway via dihydrothymine, as illustrated in Figure 1.1, an oxidative pathway via 5-methylbarbituric acid as depicted in Figure 1.2 and an oxidative conversion to uracil shown in Figure 1.3.

A. Reductive Pathway

The reductive catabolism of the pyrimidines commences with an enzyme-catalysed conversion of the pyrimidines to dihydropyrimidines. This process requires NADH + H^+ or NADPH + H^+ depending on the source of the enzyme. The dihydropyrimidines then proceed through a series of hydrolytic steps to form β-amino acids, ammonia and carbon dioxide.

A variant reductive pathway, with dihydroorotic acid as an intermediate in cytosine catabolism, was proposed by Di Carlo (1952) for the yeast Torula utilis, but Batt et al., (1953, 1954) showed that this organism cannot degrade exogenous dihydroorotic acid. No further work with yeasts has been reported.

In animals, the reductive pathway was demonstrated by Fink et al. (1953, 1956), Canellakis (1956), Fritzson (1957) Fritzson and Pihl (1957); using ^{14}C-labelled uracil and thymine. Rat liver slices, intact rats and other animals were used.

The existence of the reductive pathway has also been demonstrated in a wide variety of bacteria. Pseudomonas

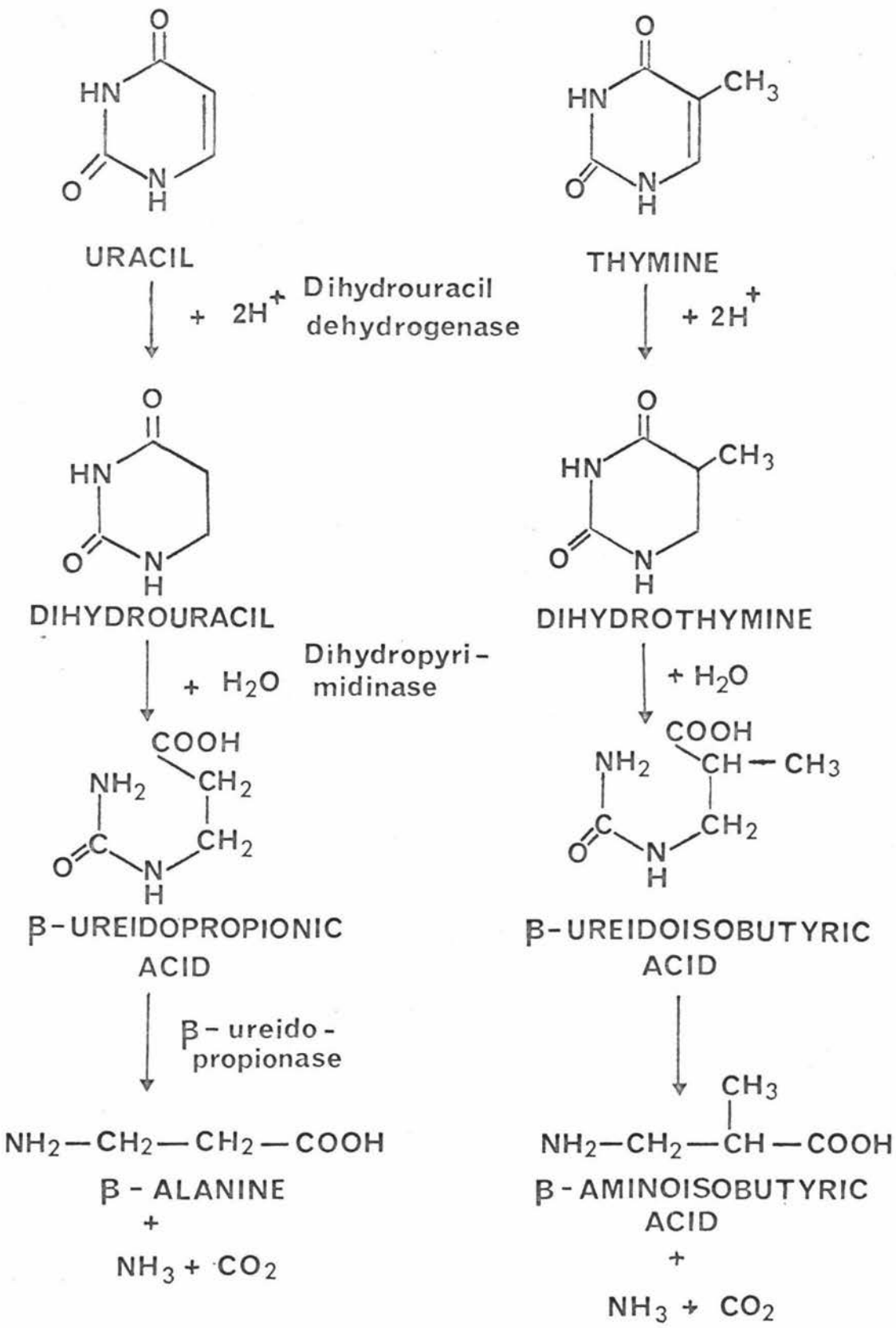


Fig.1-1 Pathway for the reductive catabolism of uracil and thymine.

aeruginosa (Fink et al., 1954) and Clostridium uracilicum (Campbell, 1957) were shown to convert uracil and thymine to dihydropyrimidines and (ultimately to) β-amino acids, ammonia and CO_2. With C. uracilicum, β-ureidopropionic acid was demonstrated as a product of uracil catabolism. Analogous intermediates were found in the catabolism of orotic acid by Zymobacterium oroticum and by Corynebacterium species (Liebermann and Kornberg, 1953, 1954, 1955; Reynolds et al., 1955). Kraemer and Kaltwasser (1969), demonstrated the pathway in intact cells and in cell-free extracts of Hydrogenomonas facilis. Dihydrouracil, β-ureidopropionic acid and β-alanine were each able to serve as sole source of carbon and nitrogen for this organism, whereas barbituric acid and malonate, products of the oxidative catabolism of uracil, could not support growth.

The reductive pathway has also been demonstrated in plants (Evans and Axelrod, 1961; Ross 1965) and in Chlorella fusca (Knutsen, 1972).

B. Oxidative Pathway via 5-Methylbarbituric acid

This oxidative pathway begins by the oxidative catabolism of thymine and uracil to 5-methylbarbituric acid and barbituric acid respectively; catalysed by the enzyme 'thymine-uracil oxidase'. Early work suggested that barbituric acid is further hydrolysed to malonate and urea. By analogy, the breakdown of 5-methylbarbituric acid to methylmalonate and urea was suggested (Biggs and Doumas, 1963). The end products of this pathway are the malonic acids, ammonia and carbon dioxide.

Proposals for the oxidative pathway of thymine and

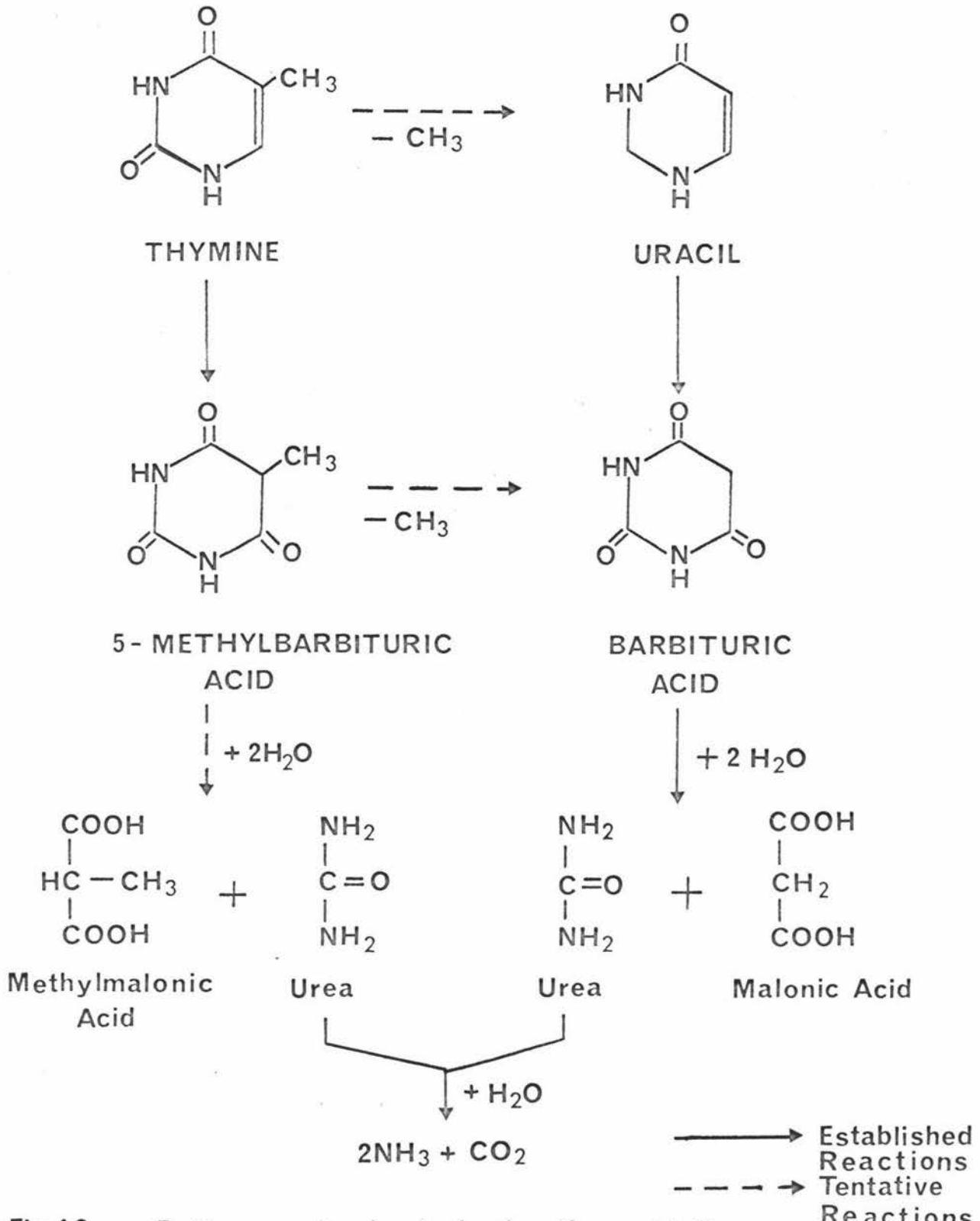


Fig.1·2 Pathways in bacteria for the oxidative catabolism of uracil and thymine through the barbituric acids.

uracil breakdown in microorganisms were first put forward in 1951 (Batt and Woods, Hayaishi and Kornberg, and Wang and Lampen). Cell-free extracts were prepared from Corynebacterium and Mycobacterium (Hayaishi and Kornberg, 1951) which oxidised uracil to barbituric acid with the consumption of one mole oxygen per mole substrate. Thymine was similarly oxidised to 5-methylbarbituric acid. Hayaishi and Kornberg (1952) and Wang and Lampen (1952) were able to partially purify the enzyme involved in the above oxidation and gave it the name 'thymine-uracil oxidase'.

Lara (1952), on the basis of experiments on simultaneous adaptation, suggested the decomposition of thymine in N. corallina by way of uracil and barbituric acid. However, he could detect uracil in only one of his incubations of cell-free extracts of thymine-grown N. corallina with thymine and was forced to give up the idea, pending further evidence. Batt (1960) showed that 'thymine-uracil oxidase' was a non-specific enzyme which catalysed the oxidation of uracil, thymine, 2-thiouracil or 2-thiothymine to the corresponding barbituric acids; therefore, Lara's methods could not be directly applied to studies on pathways of pyrimidine catabolism.

Barbituric acid was converted to malonate, ammonia and carbon dioxide by various cell-free extracts either in aerobic or anaerobic conditions (Hayaishi and Kornberg, 1952; Lara, 1952). These workers showed the conversion of barbituric acid to urea and malonate catalysed by the enzyme 'barbiturase' and the further decomposition of urea to ammonia and carbon dioxide by cell-free extracts. Hayaishi and Kornberg (1952)

were able to partially purify 'barbiturase' from Corynebacterium and free it of urease. Work by Batt and Woods (1961) and by Pearce (1974) (see section II) has led to the suggestion that 'barbiturase' is an artifact of cell extraction and that the true products from barbituric acid are malonyl CoA and urea.

The oxidation of 5-methylbarbituric acid was observed only in intact cells; with the end products of ammonia, carbon dioxide and water (Hayaishi and Kornberg, 1952). The detailed mechanism was not known.

Following their finding that 5-methylbarbituric acid was autooxidised to 5-hydroxy-5-methylbarbituric acid and methyltartronylurea, Doumas and Biggs (1962) tested these compounds as intermediates of 5-methylbarbituric acid catabolism in Corynebacterium. But 5-hydroxy-5-methylbarbituric acid and methyltartronylurea were not utilised by the organism, hence they are unlikely to be intermediates. However, Biggs and Doumas (1963) were able to extract urea and methylmalonate from the supernatant solution when 5-methylbarbituric acid or thymine were incubated with intact Corynebacterium cells. The yield of methylmalonate was less than 0.5%, whereas urea was isolated in 35% yield. Using ^{14}C-labelled 5-methylbarbituric acid, they showed that methylmalonate and urea were derived directly from this substrate. With [2-^{14}C] 5-methylbarbituric acid as substrate, the urea produced had the same specific activity as the starting material, whereas the methylmalonate produced had an activity only 2% that of the labelled 5-methylbarbituric acid. With [5-^{14}C] 5-methylbarbituric acid, the activity recovered in methylmalonate was 96% of that

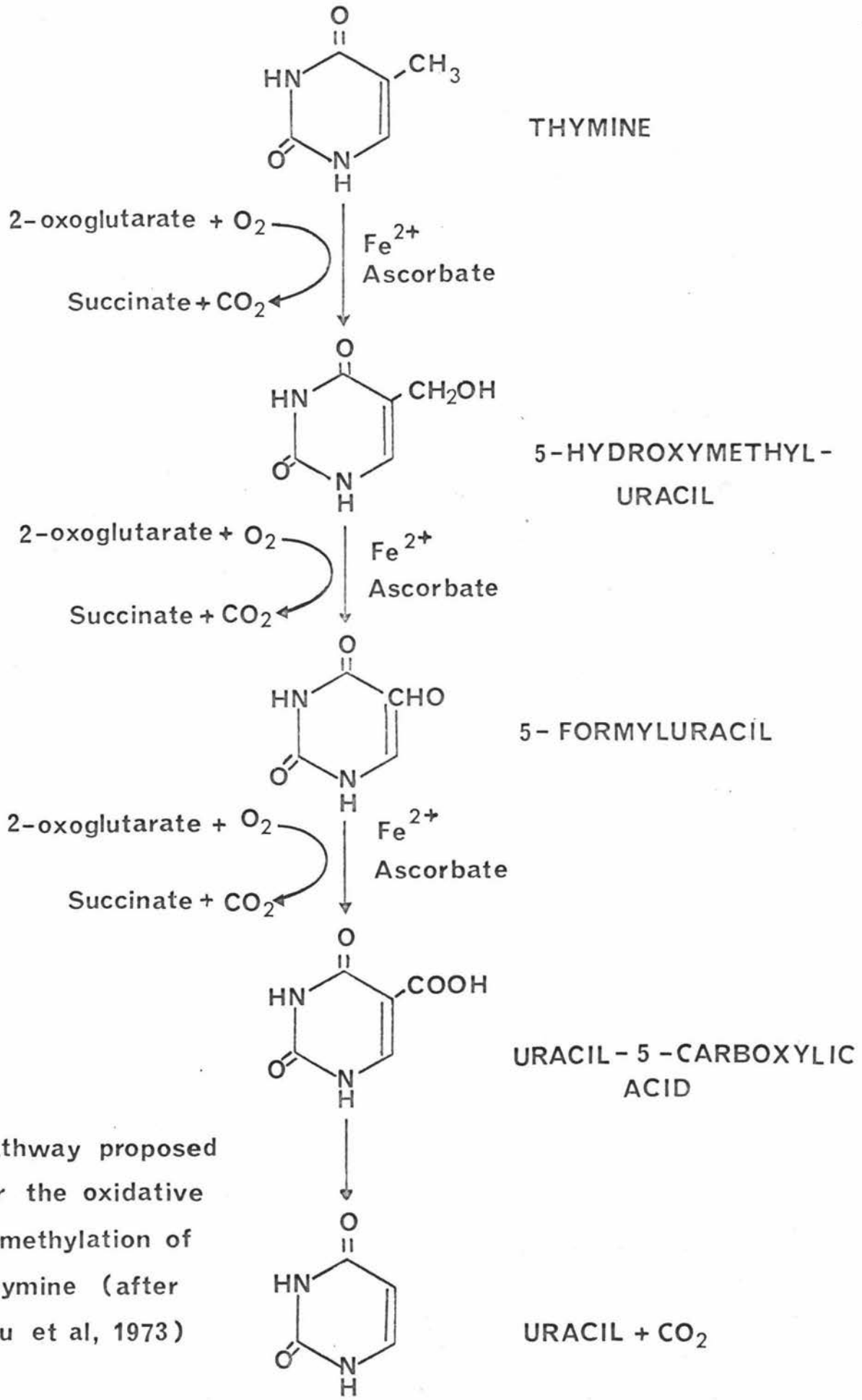
required by theory assuming methylmalonate was produced from 5-methylbarbituric acid. Their results suggest the hydrolysis of 5-methylbarbituric acid to urea and methylmalonate, a pathway analogous to the breakdown of barbituric acid. However, as in the case of barbituric acid metabolism, methylmalonyl CoA might be the immediate *in vivo* product from 5-methylbarbituric acid; the methylmalonate would result from hydrolysis of methylmalonyl CoA. (If 5-methylbarbituric acid were hydrolysed to methylmalonate and urea, one might expect that the enzyme, being a hydrolase would be relatively stable *in vitro*.)

Mountfort (1971) using whole cells of *N.corallina* was able to extract methylmalonate from thymine incubation medium which contained diethyl malonate as inhibitor (see section II).

C. Oxidative Conversion to Uracil

Fink *et al.* (1956) found that 5-hydroxymethyluracil and uracil-5-carboxylic acid accumulated in rat liver slices incubated with labelled thymine.

Abbot *et al.* (1964, 1967, 1968) showed that cell-free extracts from *Neurospora crassa* mycelia convert thymine to 5-hydroxymethyluracil and then to 5-formyluracil, only in the presence of oxygen, Fe^{2+}, ascorbate and 2-oxoglutarate. They proposed the name 'thymine-7-hydrooxylase' for this enzyme. Watanabe *et al.* (1970) showed that 5-formyluracil was converted to uracil-5-carboxylic acid by cell-free extracts, and this conversion required the same cofactors as above. But in 1970, Palmatier partially purified an enzyme which catalysed the decarboxylation of uracil-5-carboxylic acid without a



Fig·1·3 Pathway proposed for the oxidative demethylation of thymine (after Liu et al, 1973)

requirement for cofactor.

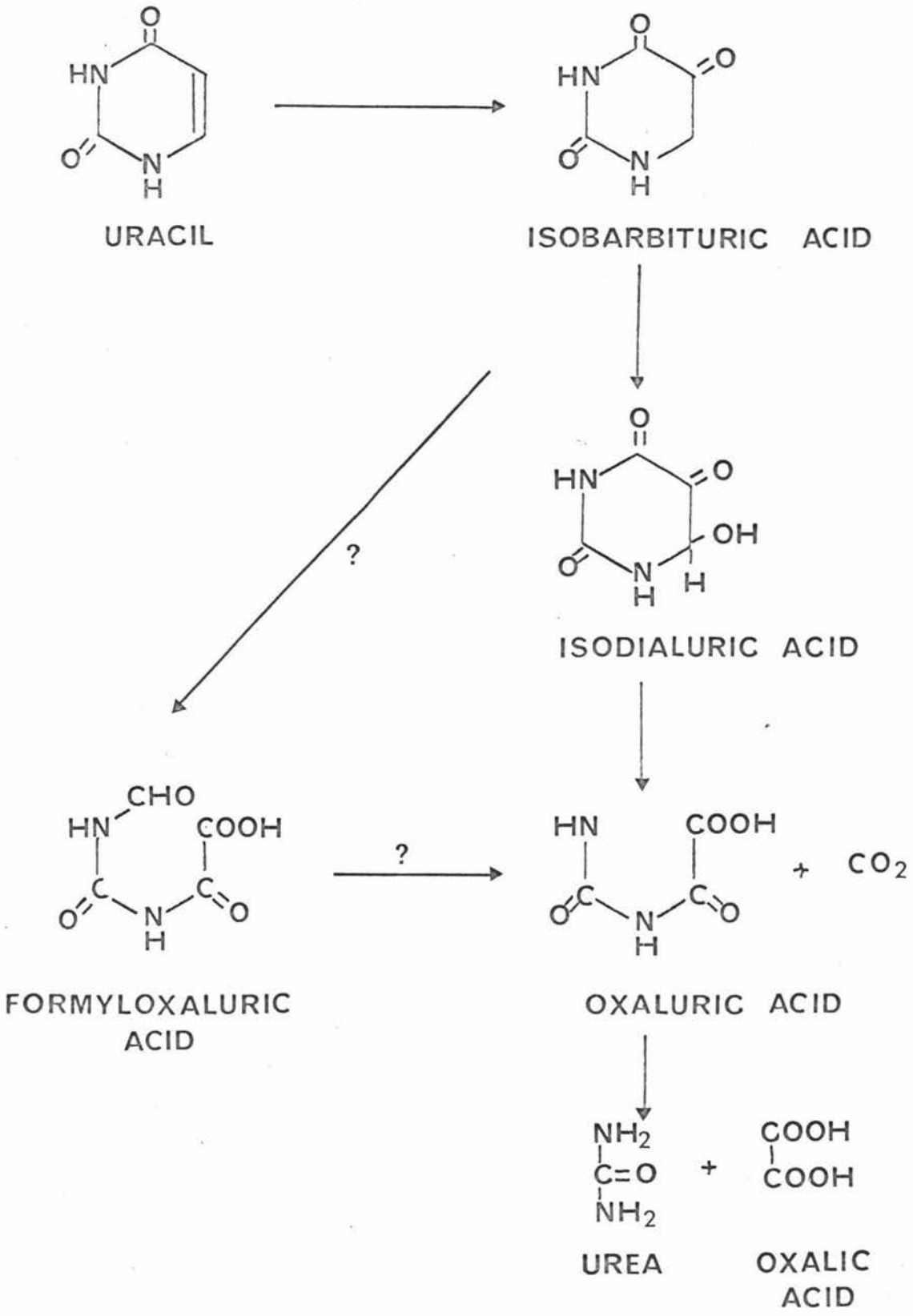
Each of the oxidation steps shown in Figure 1.3 was coupled to the decarboxylation of 2-oxoglutarate with molecular oxygen being incorporated into both the oxidation product and succinate. The oxidation product, succinate and carbon dioxide were all produced in equimolar amounts.

Similar pathways may exist in micro-organisms. Vilks et al. (1972) reported that Rhodotorula glutinis utilised thymine as a sole nitrogen source and a pathway of thymine to uracil was suggested. 5-hydroxymethyluracil and uracil-5-carboxylic acid were shown to accumulate when Rhodotorula was grown on thymine as the sole carbon source and that cell suspensions were capable of converting uracil-5-carboxylic acid to uracil (Vilks, 1973).

Zvyagintseva and Mamulina (1969) showed that a mixed culture of Pseudomonas species and Nocardia ruber utilised 6-methyluracil as sole nitrogen and carbon source, converting it oxidatively to uracil and then barbituric acid and urea. The pathway from 6-methyluracil to uracil was not reported in the paper.

D. Pathway Proposed by Cerecedo

Another pathway of pyrimidine catabolism in dogs was proposed by Cerecedo (1927, 1930, 1931), Figure 1.4. He measured the changes in the urinary excretion of urea after feeding pyrimidines and related substances to dogs. His results led him to propose an initial oxidation at carbon 5, which in the case of uracil would yield isobarbituric acid and with thymine would result in thymine glycol. Oxalic acid, formic acid and urea would be ultimately obtained from uracil



Fig·1·4 Pathway for the oxidative catabolism of uracil proposed by Cerecedo (1931)

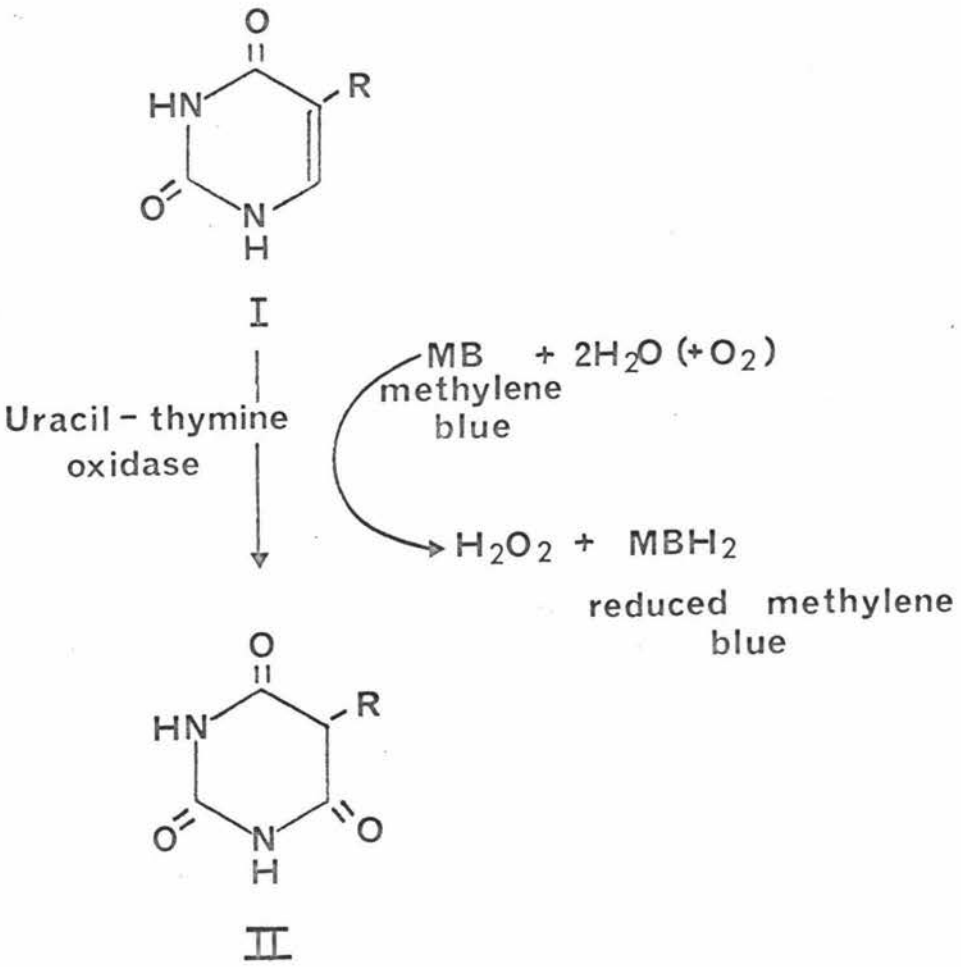
and thymine breakdown would result in the formation of acetol, carbon dioxide and urea.

Di Carlo (1952) showed that while T. utilis assimilated all of the nitrogen of uracil for growth, it was unable to grow on isobarbituric acid or isodialuric acid implying that the yeast did not catabolise uracil by the pathway suggested for dogs. Lara (1952) found evidence that this pathway was not followed in Corynebacterium. Further work on mammalian systems by Fink et al. (1956) rendered Cerecedo's pathway unlikely.

II. Pyrimidine Catabolism in Nocardia corallina

In Nocardia corallina the major pathway of pyrimidine catabolism is the oxidative pathway via the corresponding barbituric acids (Figure 1.2). Batt and Woods (1961), showed that growth on either uracil or thymine induced the 'uracil-thymine oxidase' necessary for the initial reaction in this pathway. This enzyme, first demonstrated by Hayaishi and Kornberg (1952) in Mycobacterium and Corynebacterium strains catalyses the oxidation of either uracil or thymine to the corresponding barbituric acids. Payakachat (pers. comm.) has purified the enzyme from N. corallina and shown it to be a metalloflavoprotein catalysing the dehydrogenation of thymine (or uracil) at carbon 6 in the presence of an artificial electron carrier such as methylene blue. This is shown in Figure 1.5.

Batt and Woods (1961) showed that when uracil-grown N. corallina cells were allowed to oxidise thymine, 5-methylbarbituric accumulated in up to 90% yield (Figure 1.6). Further metabolism of 5-methylbarbituric acid was initially slow and



R = H, I = Uracil, II = Barbituric Acid

R = CH_3, I = Thymine, II = 5-Methylbarbituric Acid

Fig.1·5 Proposed reaction of the oxidation of Uracil or Thymine, in the presence of Methylene Blue to Barbituric Acid or 5-Methylbarbituric Acid, respectively. (after Payakachat, personal communication)

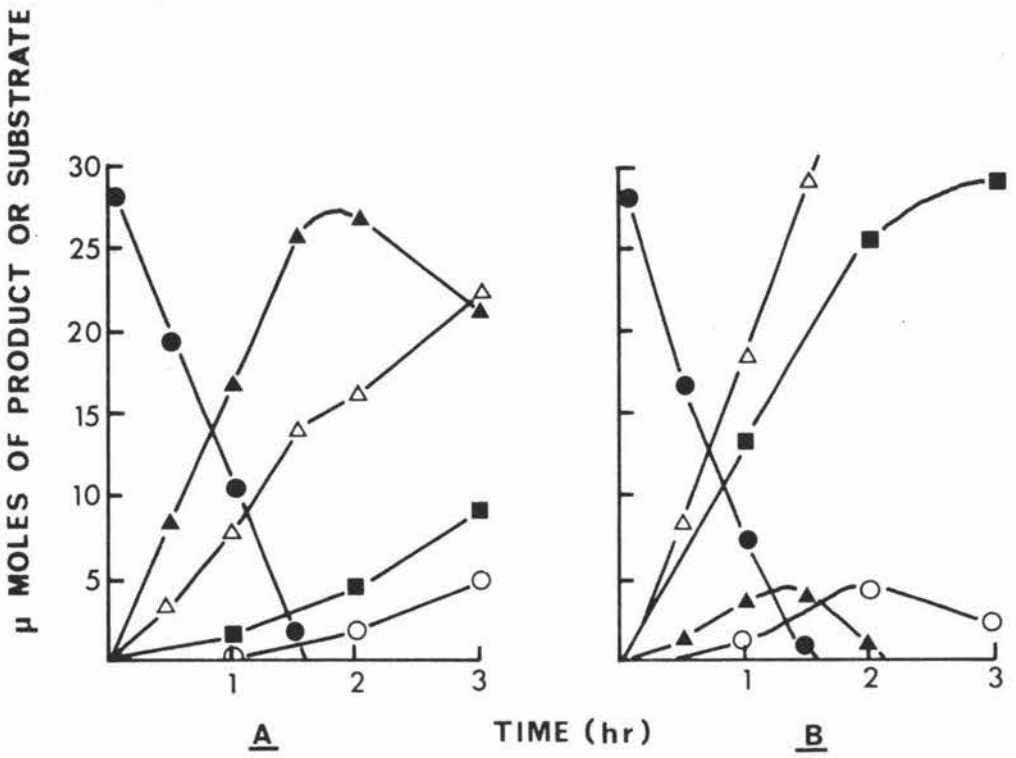


Figure 1.6. A. Metabolism of thymine (28 μmoles) by organisms grown on uracil. ● Thymine, ▲ 5-methyl-barbituric acid, △ O_2 uptake, ■ NH_3 and ○ urea. All values corrected for control values without substrate.

B. Metabolism of thymine (28 μmoles) by organisms grown on thymine. Symbols as for A. (After Batt and Woods, 1961).

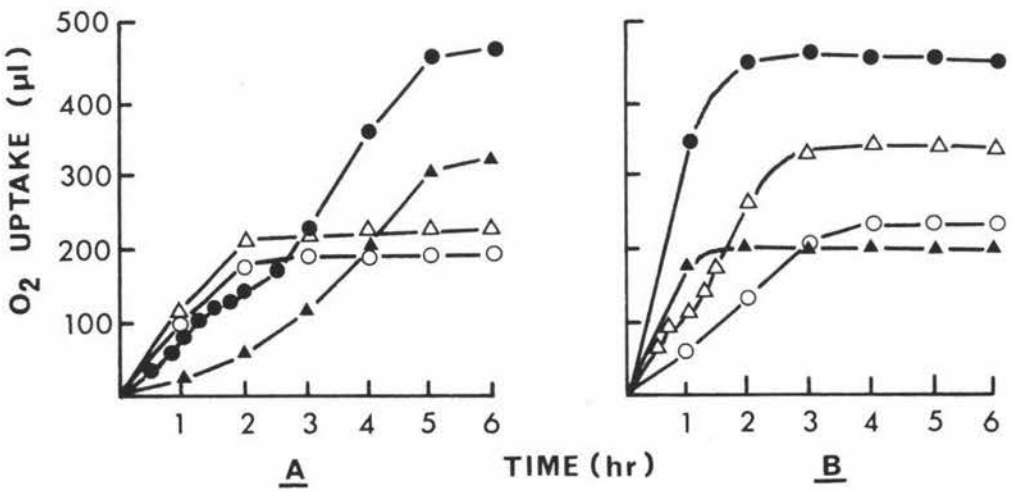


Figure 1.7. A. Uptake of oxygen by uracil-grown organisms acting on ● thymine, ▲ 5-methylbarbituric acid, △ uracil and ○ barbituric acid. 10 μmoles of substrate was used. All values corrected for endogenous O_2 uptake.

B. Uptake of oxygen by thymine-grown organisms acting on ● thymine, ▲ 5-methylbarbituric acid, △ uracil and ○ barbituric acid. Conditions were as for A. (After Batt and Woods, 1961).

evidently required the induction or activation of further enzymes either not present in the uracil-grown cells or present at reduced levels. Similarly, when thymine-grown cells were incubated with uracil, barbituric acid initially accumulated in almost quantitative yield.

However, when thymine-grown cells were incubated with thymine or uracil-grown cells with uracil, the 5-methylbarbituric acid or barbituric acid accumulated at only very low levels, evidently because it was rapidly metabolised further (Figure 1.6).

Batt and Woods (1961) suggested an alternative explanation (see below) for the low yield of 5-methylbarbituric acid from the action of thymine on thymine-grown cells. They incubated cells with pyrimidine substrate for 6 hours by which time oxygen-uptake had levelled off in all cases (Figure 1.7). In every case, they found that the actual oxygen uptake was far short of the theoretical uptake for complete oxidation of the substrate and accordingly suggested that extensive assimilation of the substrate into cell material was taking place. This latter conclusion was supported by experiments where sodium azide was present.

For example, thymine oxidation would involve a theoretical oxygen uptake of 4.0 μmole/μmole thymine:

$$C_5H_6N_2O_2 + 4\ O_2 \longrightarrow 5\ CO_2 + 2NH_3$$

The observed O_2 uptake on thymine oxidation by uracil and thymine-grown cells was, however, 2.07 and 2.05 μmole/μmole thymine, respectively.

The oxidation of thymine to 5-methylbarbituric acid requires only 0.5 μmole O_2/μmole thymine. Thus the oxidation

of 5-methylbarbituric acid should require 0.5 μmole O_2 less than the oxidation of thymine.

With uracil-grown cells, oxidation of 5-methylbarbituric acid required 1.47 μmole O_2/μmole compared with 2.07 μmole O_2/μmole for thymine oxidation. This is consistent with the other evidence that thymine is initially converted almost quantitatively to 5-methylbarbituric acid, which is then further metabolised.

With thymine-grown cells, however, incubation with 5-methylbarbituric acid resulted in consumption of only 0.88 μmole O_2 per μmole 5-methylbarbituric acid compared with 2.05 μmole O_2 per μmole for thymine oxidation. The difference, 1.17 μmole O_2 per μmole pyrimidine oxidised is much greater than the value expected, 0.5 μmole per μmole if all the thymine is first oxidised to 5-methylbarbituric acid. This observation led Batt and Woods (1961) to suggest that an additional pathway of thymine catabolism may operate in thymine-grown cells, and that this may, in part, account for the low yield of 5-methylbarbituric acid observed from such cells.

If there is an alternative pathway of thymine catabolism in thymine-grown N. corallina, by-passing the formation of 5-methylbarbituric acid, it might be expected that intermediates of this hypothetical pathway would be substrates for growth of N.corallina. Batt and Woods (1961) investigated this, and showed that, while 5,6-dihydrothymine and 5,6-dihydrouracil did not support growth, 5-hydroxymethyluracil was a suitable growth substrate. Hence, thymine catabolism was considered unlikely to involve the reductive pathway shown in Figure 1.1, but could involve the oxidation of thymine to uracil and carbon dioxide via the pathway in

Figure 1.3.

If a portion of the thymine in thymine-grown cells is converted to uracil, e.g. by the 5-hydroxymethyluracil pathway, we would expect the enzymes of uracil catabolism to be induced in such cells. Barbituric acid is indeed oxidised in thymine-grown cells, with no lag, although the rate of its oxidation is faster in uracil-grown cells (Batt and Woods, 1961). Brennan (1970) found ten times higher 'barbiturase' activity in uracil-grown cells than in thymine-grown cells.

Intermediates of Cerecedo's pathway, isobarbituric acid, isodialuric acid and oxaluric acid, were not able to support growth.

Mountfort (1971) attempted to determine whether an alternative pathway for thymine catabolism existed in thymine-grown cells. Under his conditions, he failed to demonstrate accumulation of 5-methylbarbituric acid when thymine-grown cells were incubated with thymine in the absence of inhibitors. But if diethylmalonate was present, 5-methylbarbituric acid accumulation was observed. (Diethylmalonate is probably converted to malonate by esterases inside the cell; the free malonate, by blocking succinate dehydrogenase, might lead to a general inhibition of cell metabolism.) The amount of accumulated labelled 5-methylbarbituric acid increased with time. In the sample taken at 30 minutes, it comprised 18% of the total radioactivity; and at 60 minutes, the relative radioactivity in 5-methylbarbituric acid and methylmalonate accounted for 70% of the total radioactivity scanned. This contrasted with results obtained from the incubation of thymine-grown cells with thymine in the absence of diethylmalonate. The band corresponding to

labelled 5-methylbarbituric acid was clearly discernible under the UV light. This band when eluted showed spectral characteristics identical to 5-methylbarbituric acid with λ max. values in 0.1 M HCl and 0.1 M NaOH at 268 nm and 269 nm respectively.

The catabolism of barbituric acid and 5-methylbarbituric acid in bacteria appear to follow parallel pathways (Figure 1.2) but involving different enzymes.

Malonic acid and urea have been identified as products of barbituric acid catabolism in cell-free extracts of N. corallina (Lara, 1952), and of uracil-adapted organisms of a strain of Mycobacterium (Hayaishi and Kornberg, 1952). Urea, but not malonic acid (Batt and Woods, 1961) was observed as a product in vivo.

Urea is readily hydrolysed to carbon dioxide and ammonia by urease, and this presumably accounts for the disappearance of urea with concomitant increase in ammonia observed with N. corallina cell suspensions.

Malonic acid would be expected to be metabolised via malonyl-CoA. Thus, in Pseudomonas fluorescens, Hayaishi (1955) demonstrated that malonate is degraded to acetate with the intermediate formation of the corresponding CoA derivatives. However, Batt and Woods (1961) showed that malonate was not metabolised by whole cells (N. corallina). Also, they were unable to observe activation of malonate to malonyl hydroxamate with cell-free extracts of N. corallina, although these same cell-free extracts readily activated acetate and propionate to their hydroxamate detivatives. For this reason, they postulated that malonate may not be formed

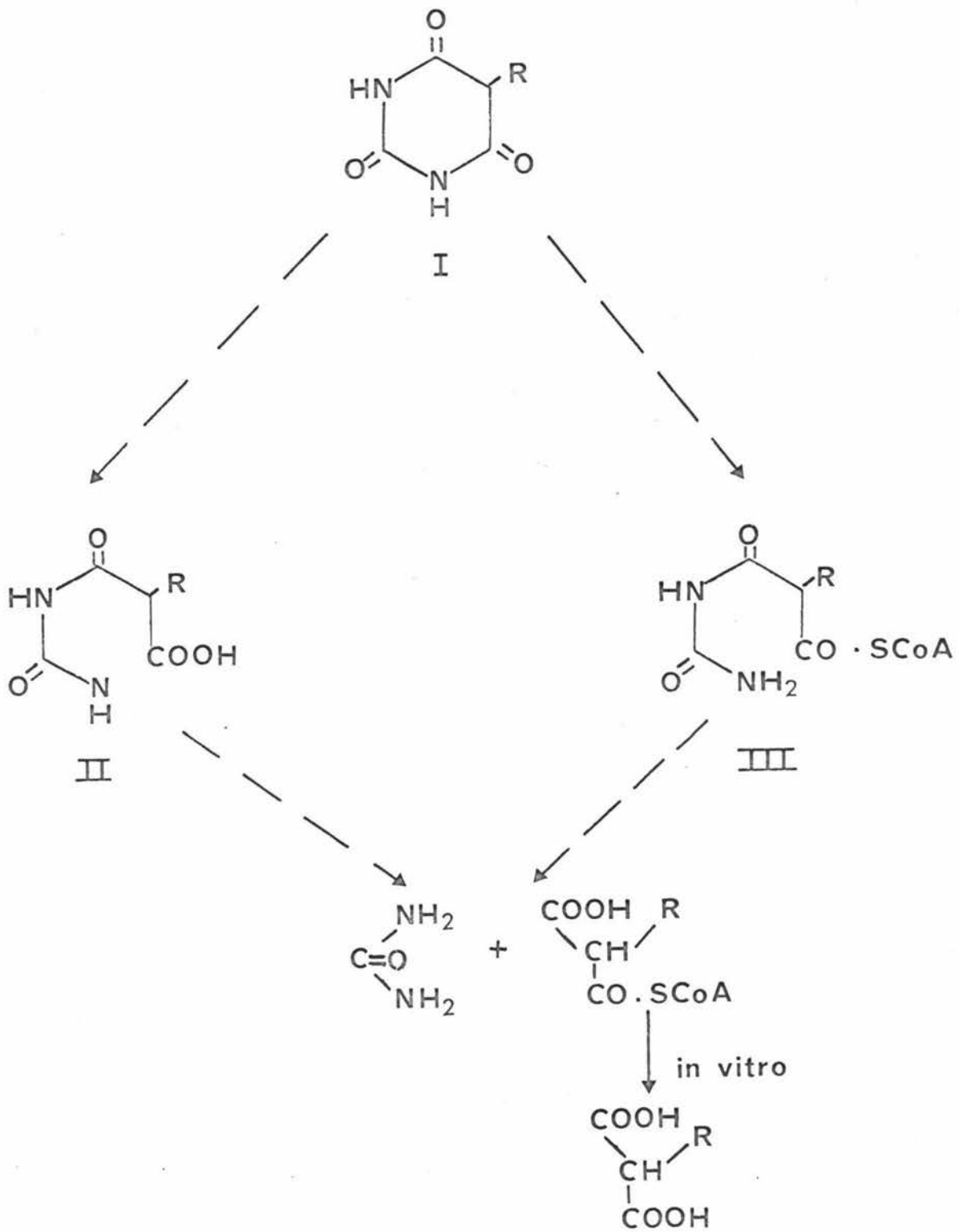
as a free intermediate in vivo. Rather, an activated form of malonate may be formed, which is rapidly hydrolysed in cell-free extracts.

Pearce (1974) incubated [5-^{14}C] barbituric acid with cell-free extracts of N. corallina and demonstrated the initial formation of a ^{14}C product which was chromatographically indistinguishable from malonyl-CoA, and which, on treatment with hydroxylamine gave a product chromatographically indistinguishable from malonyl monohydroxamate. The formation of 'malonyl-CoA' (identified by the above criteria) was dependent on the addition of ATP, CoA and Mg^{++} to the incubation mixture.

Pearce further showed that [2-^{14}C] malonate did not give rise to malonyl-CoA when incubated under identical conditions. The malonyl-CoA observed was therefore likely to have been formed directly from barbituric acid, without the intermediate formation of free malonate. [5-^{14}C] barbituric acid did give rise to free malonate in this experiment, but from the time course data, it did not appear to be a precursor of the malonyl-CoA. Rather, malonyl-CoA appeared to be the earlier formed product.

The name 'barbiturase' has been given to the enzyme involved in the conversion of barbituric acid to malonate and urea, although it would appear that it is not a simple hydrolase, but a mixture of perhaps several enzyme activities with no in vivo function. It is possible that 'barbiturase' is produced as an artifact of isolation.

A possible pathway for the formation of malonate and urea from barbituric acid is given in Figure 1.8.



a) R= H : Barbiturase , I= Barbituric Acid, II= Malonyl Urea

b) R= CH_3 , I= 5-Methylbarbituric Acid, II= Methylmalonyl Urea

Fig. 1·8 A possible pathway for the formation of malonic acid and urea from barbituric acid, and of methylmalonic acid and urea from 5-Methyl-barbituric acid·

Pearce (1974) attempted to synthesise malonylurea in order to test it as a possible intermediate in barbituric acid degradation, but it is evidently unstable, being readily decarboxylated to acetylurea.

The degradation of 5-methylbarbituric acid may follow a pathway similar to that of barbituric acid, as in Figure 1.8. Urea was demonstrated as a product in vivo by Lara (1952) and by Batt and Woods (1961) with N. corallina; and by Hayaishi and Kornberg (1952) with Mycobacterium and Corynebacterium.

Biggs and Doumas (1963) were able to demonstrate the accumulation of urea and methylmalonate when intact cells of Corynebacterium species were incubated with either thymine or 5-methylbarbituric acid. /5-^{14}C/ 5-methylbarbituric acid gave rise to ^{14}C methylmalonate of the same specific activity and /2-^{14}C/ 5-methylbarbituric acid gave rise to ^{14}C urea of the same specific activity, thus confirming the catabolic pathway.

Mountfort (1971) was able to demonstrate the accumulation of ^{14}C methylmalonate in N. corallina cells when these were incubated with ^{14}C thymine, but only if diethylmalonate was also present. As suggested above, diethylmalonate would presumably be converted to malonate in the cells; and thus cause inhibition of terminal oxidation and hence of all energy dependent processes.

If methylmalonate were a normal product of 5-methylbarbituric acid metabolism in N. corallina it might be formed from methylmalonyl CoA as in Figure 1.8. In normal metabolism, methylmalonyl CoA might be converted to succinyl CoA via

methylmalonyl CoA isomerase and thence to succinate. Further metabolism of succinate would be blocked in the presence of diethylmalonate.

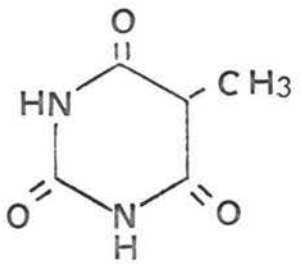
Mountfort tentatively identified 5-methylbarbituric acid, methylmalonate, succinate and malonate as compounds which accumulated when N. corallina cells were incubated with unlabelled thymine in the presence of diethylmalonate. The malonate would presumably be derived from diethylmalonate.

Hitherto, the catabolism of 5-methylbarbituric acid by cell-free extracts has not been observed. Unsuccessful attempts at utilisation of 5-methylbarbituric acid by cell-free extracts have been reported by Hayaishi and Kornberg (1952) with mycobacterium; Biggs and Doumas (1963) with Corynebacterium; and Lara (1952) and Batt and Woods (1961) with N. corallina. Thus, no enzyme similar to 'barbiturase' has been detected despite intensive efforts by several investigators to detect such a comparable system for 5-methylbarbituric acid.

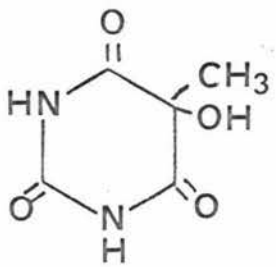
III. Stability of 5-Methylbarbituric Acid

A major aim of this thesis is the investigation of 5-methylbarbituric acid catabolism by Nocardia corallina. Experimental difficulties with 5-methylbarbituric acid have resulted from its instability. Hence some effort was put into attempts to stabilise 5-methylbarbituric acid in solution.

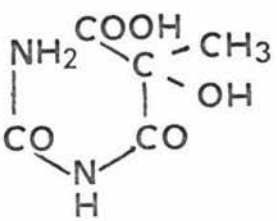
Batt and Woods (1961) reported that it may be completely oxidised to 5-hydroxy-5-methylbarbituric acid in 30 minutes when shaken in phosphate buffer in air, although it appears to be stabilised by the presence of a suspension of N. corallina cells. Doumas and Biggs (1962) showed that 5-



5 - Methylbarbituric Acid



5 - Hydroxy - 5 - methylbarbituric Acid



Methyl - tartronyl - urea

Fig.1·9 The spontaneous oxidation of 5- Methylbarbituric Acid.

methylbarbituric acid is readily oxidised to 5-hydroxy-5-methylbarbituric acid and this in turn is hydrolysed at neutral pH to methyltartronylurea (see Figure 1.9). Hayaishi and Kornberg (1952) found that at neutral and acidic pH it was readily oxidised by air to 5-hydroxy-5-methylbarbituric acid. However, at pH 9.7 and low temperatures it was quite stable.

Doumas and Biggs (1962) observed that EDTA or zinc ions are capable of inhibiting the oxidation by air of 5-methylbarbituric acid for long periods of time and that both are effective at very low concentrations.

IV. Aim of the Investigation

The oxidative catabolism of thymine is thought to proceed via a pathway analogous to that of uracil catabolism, namely via 5-methylbarbituric acid and methylmalonate. Although Biggs and Doumas (1963) were able to show the presence of a very small amount of methylmalonate in the incubation medium of *Corynebacterium* cells with /2-^{14}C/ and /5-^{14}C/ 5-methylbarbituric acid; in incubations of *Nocardia corallina*, methylmalonate was shown to be a product only in the presence of diethylmalonate as inhibitor (Mountfort, 1971). The problems concerning the pathway of oxidative catabolism of thymine in *N. corallina* during normal growth still remained.

This investigation is aimed at confirming the pathway of the oxidative catabolism of thymine in *N. corallina* during normal growth and thus the results of Biggs and Doumas, and Mountfort (above). The objectives are -

1. To determine whether the oxidative catabolism of thymine in pyrimidine-adapted N. corallina operates solely via the 5-methylbarbituric acid pathway. This could be tested by feeding [2-^{14}C] thymine to the cells. If thymine is converted to uracil or barbituric acid, then the 2-^{14}C label would appear in these compounds (Figure 1.2). If barbituric acid were an intermediate, addition of excess unlabelled barbituric acid should cause the label to accumulate in it.
2. To determine whether methylmalonate or methylmalonyl CoA is an essential intermediate of 5-methylbarbituric acid metabolism. This could be tested by incubating pyrimidine-adapted cells (whole cells or cell-fractions) with [methyl-^{14}C] 5-methylbarbituric acid. Failing this (bearing in mind the instability of 5-methylbarbituric acid), [methyl-^{14}C] thymine could be used as substrate provided that thymine had been shown to be metabolised solely via 5-methylbarbituric acid. If normal catabolism of 5-methylbarbituric acid proceeds through methylmalonate or methylmalonyl CoA, then these two compounds should be amongst the early labelled products of thymine or 5-methylbarbituric acid catabolism.

By establishing the sequence in which label is incorporated into various compounds from the methyl-^{14}C-labelled substrates, a tentative pathway of 5-methylbarbituric acid metabolism might be put forward. Radioactivity should accumulate in early intermediates if an excess of unlabelled intermediate is included in the incubation mixture. This approach might work better in a cell-free system where there

is no permeability barrier for the postulated intermediate.

The first part of the experimental work described in this thesis is concerned with testing the growth of N. corallina on possible intermediates, the right conditions for the accumulation and extraction of 5-methylbarbituric acid from the growth media and the stability of 5-methylbarbituric acid. The remainder is devoted to a study of the other questions already listed.