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

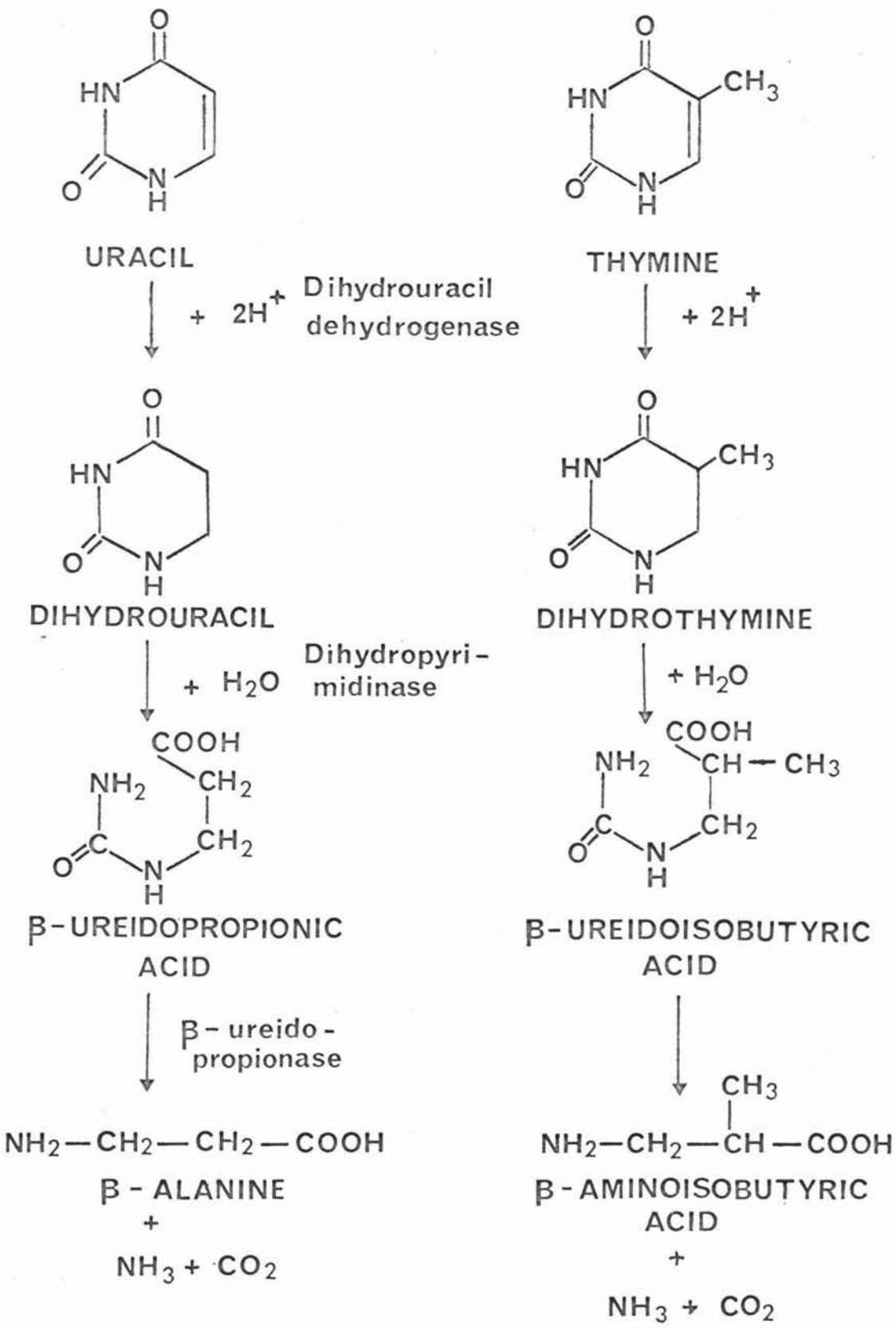


Fig.1-1 Pathway for the reductive catabolism of uracil and thymine.

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

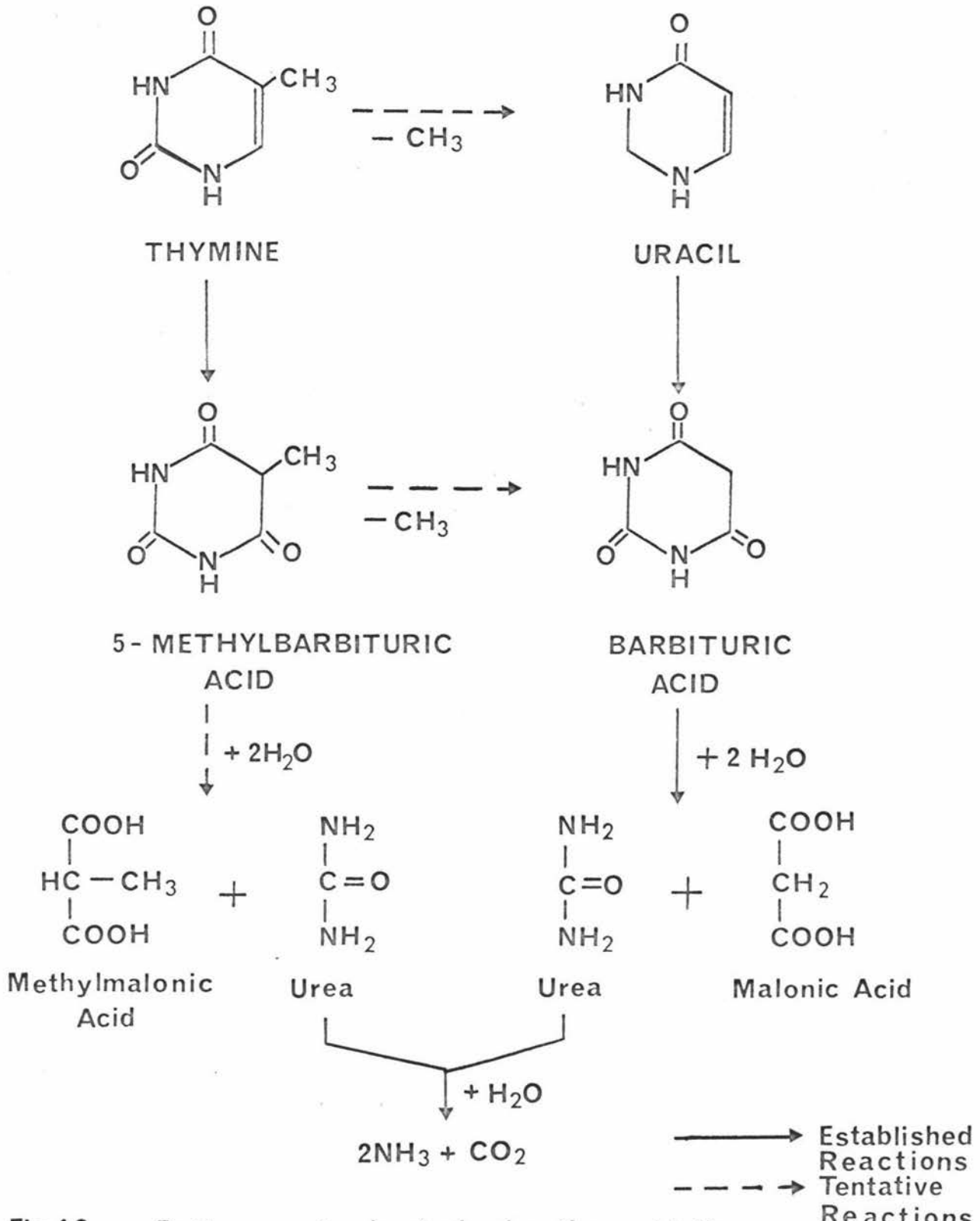


Fig.1·2 Pathways in bacteria for the oxidative catabolism of uracil and thymine through the barbituric acids.

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

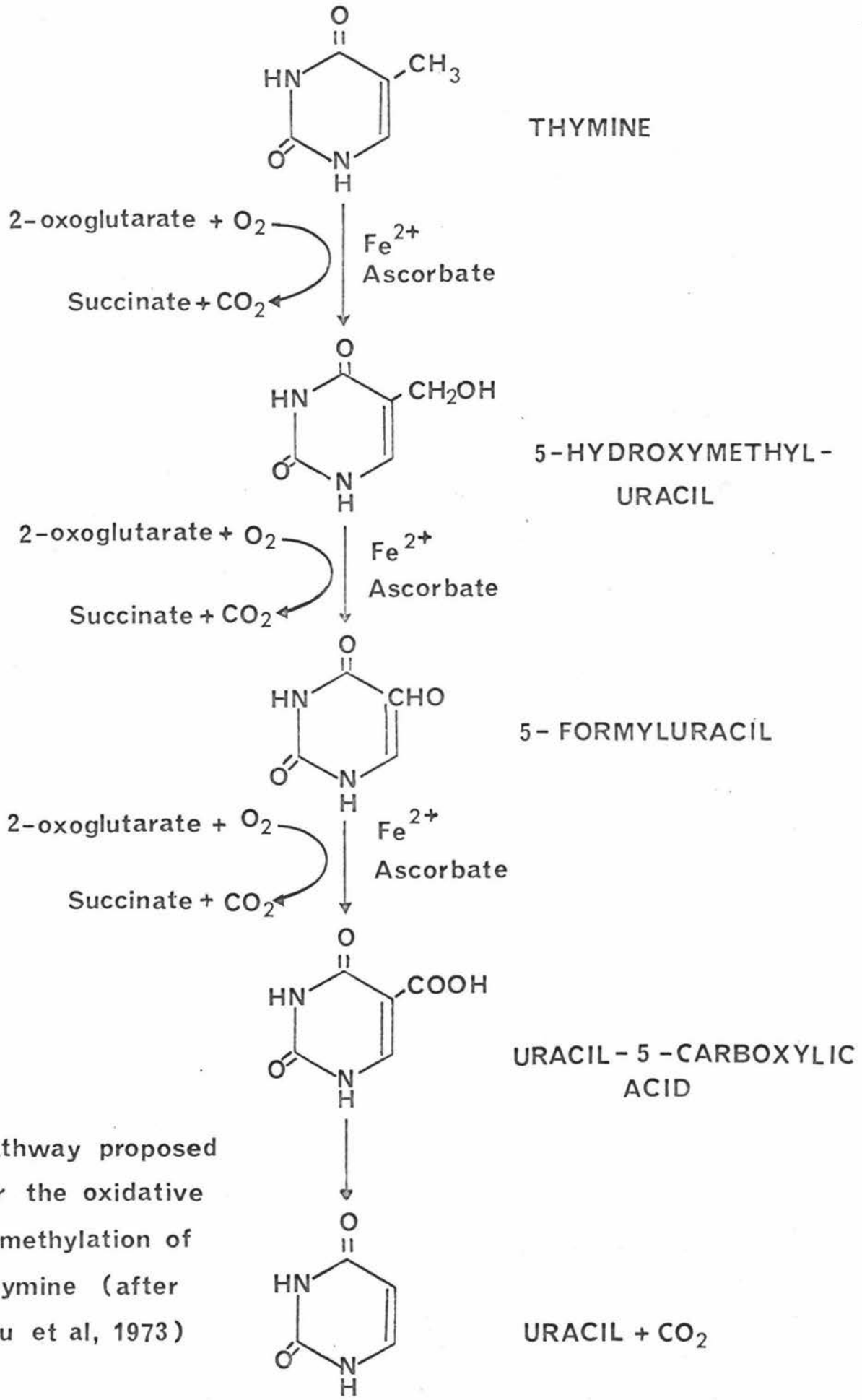
required by theory assuming methylmalonate was produced from 5-methylbarbituric acid. Their results suggest the hydrolysis of 5-methylbarbituric acid to urea and methylmalonate, a pathway analogous to the breakdown of barbituric acid. However, as in the case of barbituric acid metabolism, methylmalonyl 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.

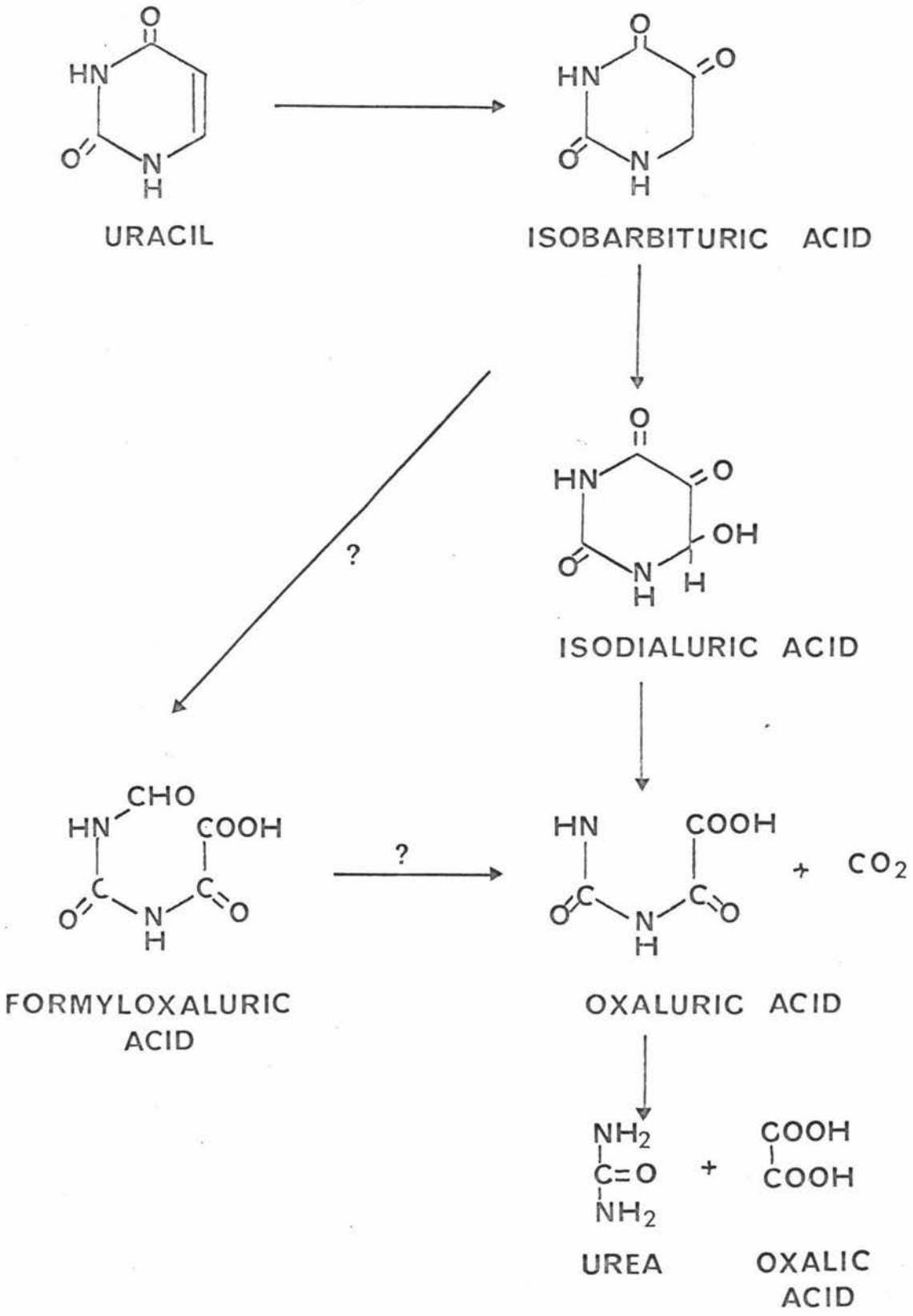
Each of the oxidation steps shown in Figure 1.3 was coupled to the decarboxylation of 2-oxoglutarate with molecular oxygen being incorporated into both the oxidation product and succinate. The oxidation product, succinate and carbon dioxide were all produced in equimolar amounts.

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)

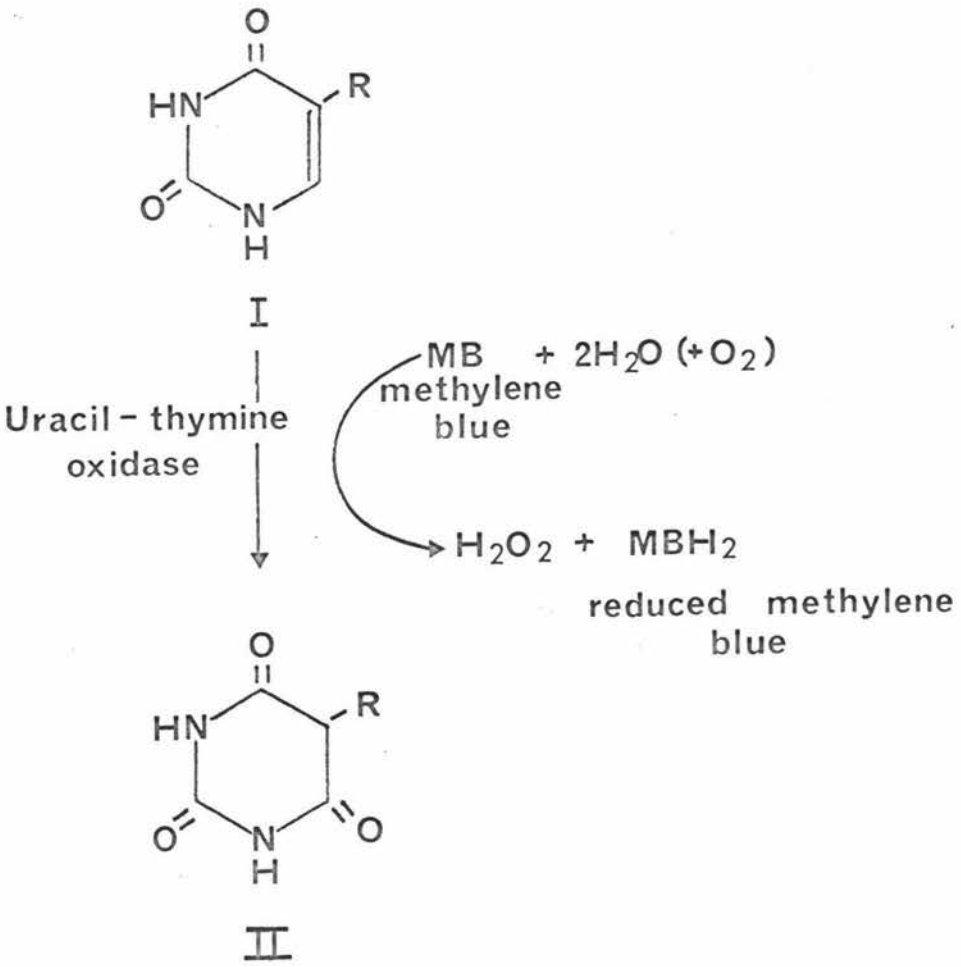
and thymine breakdown would result in the formation of acetol, carbon dioxide and urea.

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)

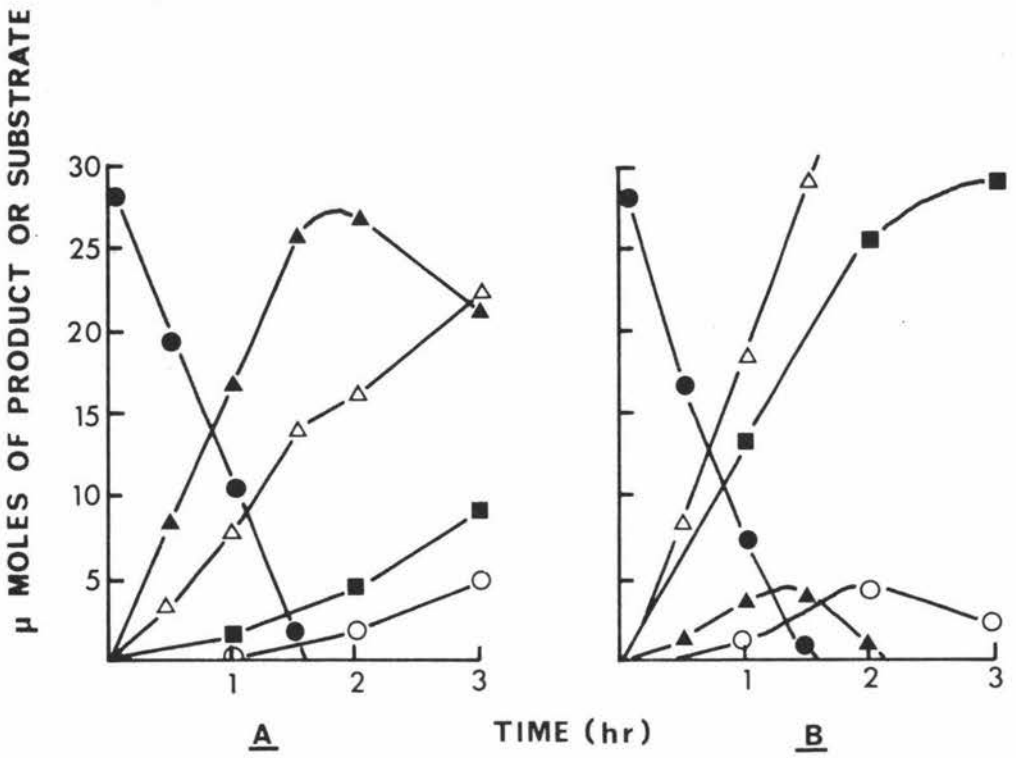


Figure 1.6. A. Metabolism of thymine (28 μmoles) by organisms grown on uracil. ● Thymine, ▲ 5-methyl-barbituric acid, △ O_2 uptake, ■ NH_3 and ○ urea. All values corrected for control values without substrate.

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

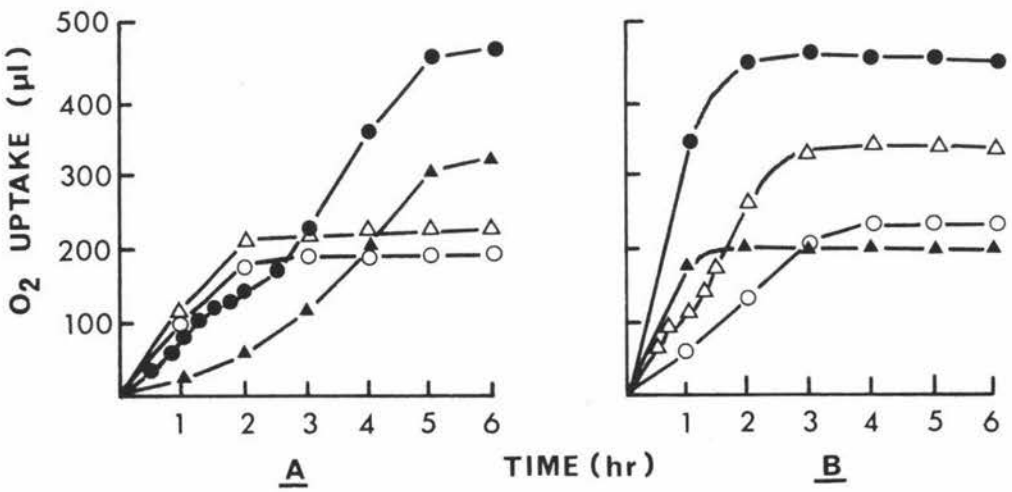


Figure 1.7. A. Uptake of oxygen by uracil-grown organisms acting on ● thymine, ▲ 5-methylbarbituric acid, △ uracil and ○ barbituric acid. 10 μmoles of substrate was used. All values corrected for endogenous O_2 uptake.

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

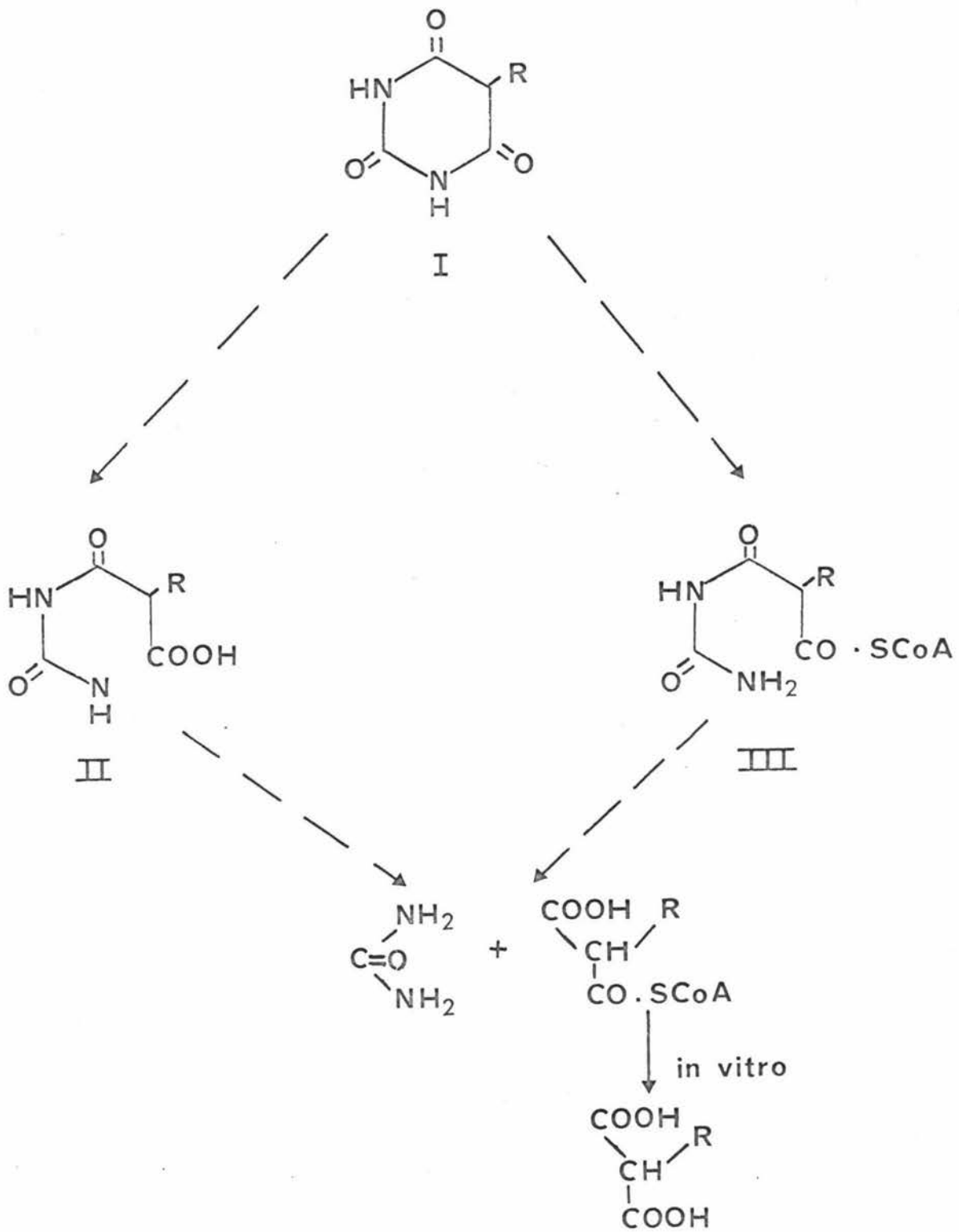
as a free intermediate in vivo. Rather, an activated form of malonate may be formed, which is rapidly hydrolysed in cell-free extracts.

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.

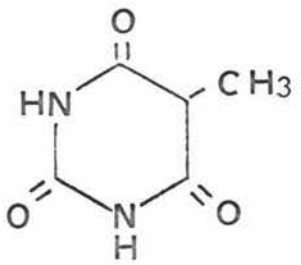
Mountfort tentatively identified 5-methylbarbituric acid, methylmalonate, succinate and malonate as compounds which accumulated when N. corallina cells were incubated with unlabelled thymine in the presence of diethylmalonate. The malonate would presumably be derived from diethylmalonate.

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.

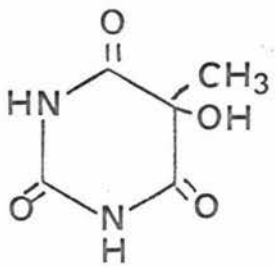
III. Stability of 5-Methylbarbituric Acid

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.

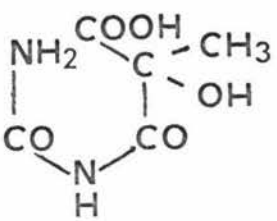
Batt and Woods (1961) reported that it may be completely oxidised to 5-hydroxy-5-methylbarbituric acid in 30 minutes when shaken in phosphate buffer in air, although it appears to be stabilised by the presence of a suspension of N. corallina cells. Doumas and Biggs (1962) showed that 5-



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.

CHAPTER 2

MATERIALS AND METHODS

I. MATERIALS

A. Reagents and Chemicals

The suppliers of some of the chemicals and materials used were:

1. [Methyl-^{14}C]-Thymine was obtained from Schwarz Mann, Division of Becton, Dickinson and Co., Orangeburg, New York 10962. Specific activities were 54 and 59 mCi/mmole.
2. [2-^{14}C]-Thymine was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. Specific activity was 59 mCi/mmole.
3. Thymine, Dihydrothymine and Hydroxymethyluracil were obtained from the Sigma Chemical Co., St. Louis Mo. 63178, U.S.A.
4. Uracil and Barbituric acid were obtained from BDH Chemicals Ltd., Poole, England.
5. Methylmalonic acid and Diethylmethyl malonate were obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis. 53233, U.S.A.
6. Yeast extract was obtained from Cockeysville, Maryland 21030, U.S.A.
7. Davis agar was obtained from Davis gelatine N.Z. Ltd., Christchurch.
8. Cellulose powder MN 300 was obtained from Machery, Nagel and Co., D.516 Düren.
9. PPO(2-5-Diphenyloxazole) was obtained from the Sigma Chemical Co.

10. POPOP [1,4-Di(2-5-phenyloxazolyl)benzene] was obtained from Hopkin and Williams, Chadwell Heath, Essex, England.
11. Kodak film (Rapid Processing Medical X-Ray film), X-Ray Developer type 2 and X-Ray fixer were obtained from Kodak Australasia Pty. Ltd., Australia.

Other chemicals used were the reagent grade or higher purity from the following suppliers -

May and Baker Ltd., Dagenham, England.
Sigma Chemical Co.
BDH Chemicals Ltd.
Hopkin and Williams, and
J.T. Baker Chemical Co., Phillipsburg, N.J.

Phosphate buffer and Tris/HCl buffer were prepared according to the method of Gomori (1968).

B. Purification of [Methyl-^{14}C] Thymine

The [methyl-^{14}C] thymine, as purchased, showed several radioactive impurities when chromatographed in n-butanol : acetic acid : water (2 : 1 : 1) and autoradiographed (Figure 2.1A). To obtain pure [methyl-^{14}C] thymine for some of the experiments, 20μCi was chromatographed in the above solvent alongside an authentic thymine marker. The chromatograph was autoradiographed and the band corresponding to the position of pure thymine was scraped off the plate, and the thymine extracted with water. Figure 2.1B shows an autoradiograph of the thymine after this purification.

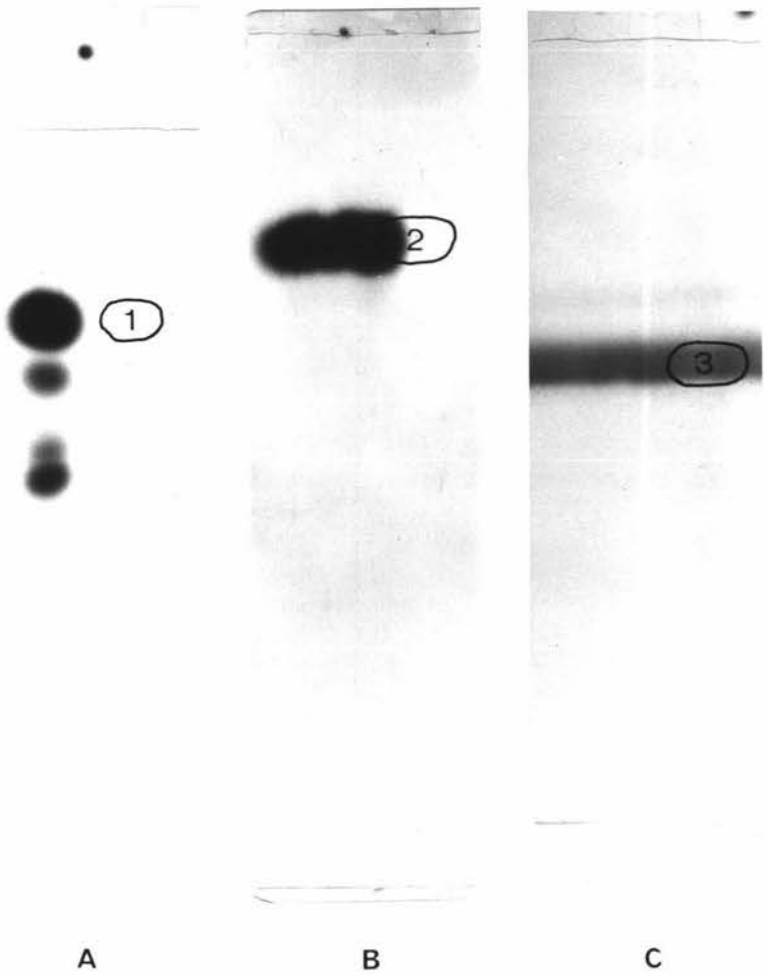


Figure 2.1. A is an autoradiograph of [methyl-^{14}C] thymine chromatographed in solvent 1 before purification.

B shows purified [methyl-^{14}C] thymine in the same solvent.

C is an autoradiograph of prepared [methyl-^{14}C] 5-methylbarbituric acid.

1 and 2 are thymine markers and 3 is 5-methylbarbituric acid marker.

C. Preparation of [Methyl-^{14}C] 5-Methylbarbituric acid

Some [methyl-^{14}C] 5-methylbarbituric acid was prepared from [methyl-^{14}C] thymine, by incubating with a suspension of uracil-grown cells. When all the thymine had disappeared and the absorbance at 268 nm had reached its maximum, the incubation medium was collected and centrifuged. The supernatant containing [methyl-^{14}C] 5-methylbarbituric acid (checked spectrophotometrically and chromatographically) was evaporated to dryness and redissolved in 0.1 M NaOH. This was then chromatographed in n-butanol : acetic acid : water solvent (2 : 1 :1v/v) and the band corresponding to 5-methylbarbituric acid detected by autoradiography (Figure 2.1C). This radioactive band was eluted with 60% ethanol and used directly for the experiments reported in the results section (see also Methods section 3.1.ii).

D. Chemical Synthesis of 5-Methylbarbituric acid

5-methylbarbituric acid was chemically prepared from urea and diethylmethylmalonate following the method of Holmberg (1945), as described by Mountfort (1971).

Sodium (2.5g) was dissolved in absolute ethanol (40 cm^3) to which was added urea (6.6g). The mixture was warmed until a clear solution was obtained. Diethyl methylmalonate (17.4g) was added and a thick paste formed which was heated under reflux for 4 hours at 115°C to 120°C. Absolute alcohol (50 cm^3) was added and the mixture was boiled for a few minutes and the sodium salt filtered from the mixture. The sodium salt was recrystallised twice from boiling water and dried at 110°C. This is a monosodium salt of 5-methylbarbituric acid which was used for most of the experimental work.

The free acid was obtained by dissolving the sodium salt in a minimum volume of boiling water acidified to pH 2 with concentrated HCl, and recrystallising from water until the filtrate was chloride free. The final product obtained after washing with absolute ethanol and drying over $CaCl_2$ in a dessicator, had a melting point of 195°C (literature value 203°C).

E. Preparation of 5-Hydroxy-5-methylbarbituric acid and Methyltartronylurea (Based on the method of Doumas and Biggs, 1962)

1. 5-Hydroxy-5-methylbarbituric acid was prepared by shaking 5-methylbarbituric acid (0.5g) in 30 cm^3 of 3% H_2O_2 until the absorption at 268 nm had disappeared. The solution was then evaporated to dryness at 60° under reduced pressure, and the residue was dissolved in water and again taken to dryness under reduced pressure. After being dried over $CaCl_2$, the compound had a melting point of 215°C (literature value 226°C).

2. Methyltartronylurea was prepared by adding 5N KOH to a solution of 5-hydroxy-5-methylbarbituric acid (0.2g in 100 cm^3 water) until the pH reached 7.15. The solution was incubated overnight at 30°C, with occasional addition of 5N KOH to maintain the pH at 7.15. The solution was then acidified to pH 1.7 with concentrated HCl, freeze dried and the dry residue extracted with acetone. The acetone solution was again taken to dryness and the residue obtained taken up in 2 cm^3 methanol. 25 cm^3 of toluene was then added and the solution left overnight at -10°C. The resulting voluminous precipitate was filtered and washed with ether. After being dried, the

compound had a melting point of 149°C (literature value 148-149°C).

F. Preparation of Methylmalonyl Hydroxamate (kindly performed by Dr. I.G. Andrew)

1. Methylmalonyl thiophenyl ester was prepared according to the method of Trams and Brady (1960). Hydroxamate assay by the hydroxamate method (Stadtman, 1957) showed a yield of 20%.
2. Methylmalonyl hydroxamate was prepared by incubating the thiophenyl ester with an excess of freshly prepared neutral hydroxylamine in methanol.

G. Preparation of β-ureidoisobutyric acid (kindly performed by Dr. I.G. Andrew)

β-ureidoisobutyric acid was prepared by incubating 6 mg of 5-6-dihydrothymine in 1 cm^3 0.1M NaOH, at room temperature until the absorbance at 230 nm had disappeared (after Batt et al., 1954).

II. METHODS

A. Bacteriological Methods and Analytical Techniques

1. Organism used

The organism used was a strain of *Nocardia corallina* isolated by Batt and Woods (1951). It is aerobic, gram positive, rod shaped and neither motile nor spore forming. A fuller description of the organism is given by Robertson (1968).

2. Maintenance of Stock Culture

Nocardia corallina was maintained on glucose agar slopes and subcultured at monthly intervals. After inoculation the glucose agar medium was incubated at 30°C for 30 hours and then stored at 2° to 4°C.

Glucose agar medium for slope cultures was made up of the following, dissolved in 450 cm^3 distilled water.

KH_2PO_4	1.70g
$(NH_4)_2SO_4$	1.50g
Oxoid yeast extract	0.25g
Thiamine HCl (Vitamin B1)	0.025g
Glucose	3.75g
$MgSO_4.7H_2O$	0.05g

The pH was adjusted to pH 7.2 using 5M NaOH and the volume was made up to 500 cm^3. 10g of Davis Agar was added, the medium heated to dissolve the agar, and prior to autoclaving, approximately 10 cm^3 was poured into each McCartney bottle for slope.

3. Growth in Liquid Culture

a) Growth media

Cells less than 2 days old from the agar maintenance medium were cultured onto glucose growth medium using sterile inoculation loops. The glucose growth medium was made up of the following constituents dissolved in 750 cm^3 water.

$(NH_4)_2 SO_4$	3.0g
$MgSO_4.7H_2O$	0.1g
KH_2PO_4	13.6g
Vitamin B_1	0.025g

The pH was adjusted to pH 7.0 using 5M NaOH and the volume made up to 800 cm^3. The medium was autoclaved in 1 litre flasks containing 200 cm^3 medium each, at 121°C for 15 minutes.

7.5g of glucose was dissolved in 200 cm^3 water and autoclaved in 50 cm^3 lots. This glucose was then added to the above medium prior to using.

Apart from growth studies, cells to be used were first grown in glucose medium and then used as the inoculum for other media. An inoculum volume of 10 cm^3 was routinely used.

The pyrimidine culture medium consisted of the following

KH_2PO_4	13.6g
$MgSO_4.7H_2O$	0.1g
Vitamin B_1	0.025g
Pyrimidines (0.2%)	
Thymine	2g
or Uracil	2g
or 5-Methylbarbituric acid	2g
or Barbituric acid	2g

The above were dissolved in 900 cm^3 water and the pH adjusted to pH 7.0 using 5M NaOH and the volume made up to 1 litre. 250 cm^3 of medium were autoclaved in 1 litre flasks.

Dicarboxylic acid medium consisted of the following

$(NH_4)_2SO_4$	3.0g
KH_2PO_4	13.6g
$MgSO_4.7H_2O$	0.1g
Vitamin B_1	0.025g
Dicarboxylic Acids (0.2%)	
Methylmalonate	2g
or Succinate	2g
or Malonate	2g

made up to 1 litre after the pH was adjusted to pH 7.0 using 5M NaOH and autoclaved in 1 litre flasks each containing 250 cm^3.

b) Growth conditions

Growth medium containing 10 cm^3 inoculum was incubated at 30°C with shaking on a new Brunswick gyro-rotatory shaker until the culture had reached the appropriate stage of growth. Absorbance and pH measurements were made on samples at intervals during growth.

4. Measurement of Growth

a) Absorbance: at appropriate time intervals, 5 cm^3 of culture was removed using a sterile pipette. The absorbance was read against a distilled water blank in a Bausch and Lomb 'Spectronic 20' spectrophotometer at 490 or 660 nm. Towards the end of the log phase, when the absorbance of the undiluted culture exceeded 0.6 on the absorbance scale, the cells were appropriately

diluted before determining the absorbance.

b) Determination of Dry and Wet Weight of Cells: The dry weight of cells was determined by two methods

i) 1 cm^3 of cell suspension on weighed clean milk bottle caps was dried in an oven overnight at 110°C. Before being weighed the caps containing the dried cells were cooled in a dessicator.

ii) The relationship between absorbance at 660 nm and dry weight determined by Robertson (1968) was used. The dry weight of cells (mg/cm^3 of solution in the Bausch and Lomb tube) = 0.43 x (O.D. at 660 nm). The relationship was linear for O.D. < 0.40 units.

The wet weight of cells was determined directly by weighing the washed cell pellet. 1.0 g wet weight was equivalent to 0.13g dry weight.

5. Preparation of Cell Suspension

Nocardia cells which had reached the later part of the log phase (approximately three quarters of the log phase) were centrifuged at 3000g for 20 minutes in screw capped polypropylene cups (250 cm^3 Nalgene) in a Sorval Centrifuge (Model RC2-B) at 4°C. The supernatant was discarded and the cells washed in 100 cm^3 0.067 M phosphate buffer, pH 7.2 and again centrifuged (3000g, 20 min.). The pellet was then suspended in the required volume of 0.067 M phosphate buffer, pH 7.2.

As a normal procedure the cell suspension was used immediately.

6. Preparation of Cell-Free Extract

The pellet of packed cells was resuspended in 0.067 M phosphate buffer, pH 7.2 (25% cell suspension wet weight) and disrupted by passage through a French pressure cell (Aminco, Silver Springs, Maryland) at 7000 lb/sq in., twice. This gives almost complete cell disintegration (Mountfort, 1971). The broken cells were centrifuged at 35,000g for 5 minutes in the Sorval RC2-B centrifuge at 4°C. The cell-free extract was carefully decanted and used immediately.

In some experiments, the cell debris was used or the disrupted cells were used without separating the debris.

In one experiment, cells suspended in buffer as above were shaken in 1% toluene (aqueous) to render them permeable to small molecules.

7. Determination of Pyrimidines

The pyrimidines were determined by their characteristic UV absorbance. In neutral solution all four pyrimidines have an absorbance maximum in the region of 255-270 nm: thymine (264 nm), uracil (258 nm), 5-methylbarbituric acid (268 nm), and barbituric acid (257 nm). In 0.1 M NaOH the absorbance maximum of thymine is shifted to 290 nm and that of uracil to 285 nm although there is still appreciable absorbance at 268 nm and 258 nm respectively.

The calibration curves for uracil, thymine, 5-methyl-barbituric acid and barbituric acid were prepared by diluting 0.015 M solutions in 0.1 M NaOH to concentrations giving absorbance values within the range 0 to 1.0 on the absorbance scale. 0.1 M NaOH was used as a blank. The absorbance of uracil, thymine, 5-methylbarbituric acid and barbituric acid were measured at 285 and 258 nm, 264 and 290 nm, 268 nm, and 257

nm respectively in a Hitachi spectrophotometer model 101, and the concentrations estimated from the standard curves.

In the presence of thymine, the absorbance of 5-methylbarbituric acid was determined in the following manner. The absorbance of thymine at 290 nm was read, and the corresponding value of its absorbance at 268 nm determined from the calibration curve. This value was then the absorbance contributed by thymine, and had to be subtracted from the apparent absorbance of 5-methylbarbituric acid at 268 nm, to get the real value for the absorbance of 5-methylbarbituric acid.

The amount of pyrimidines remaining in the growth medium during cell growth was determined after removal of the cells by centrifugation. The absorbance of the supernatant was measured in appropriately diluted samples to give an absorbance between 0 and 1.0 absorbance units on the spectrophotometer scale. The concentration of the pyrimidines were then read off the concentration versus absorbance, standard curves. One drop of chloroform was added to the supernatant to kill any remaining cells, if the samples had to be stored for any length of time.

8. Measurement of Oxygen Uptake

a) Oxygen Electrode

The oxygen electrode used was a Yellow Springs YSI oxygen electrode, Model 5301, connected to a YSI model 53 oxygen monitor and a Servoscribe Potentiometric recorder.

b) Assay of Oxidation of Substrates

The following incubation mixture was set up in the electrode chamber

2 cm^3 substrate (0.02 M) in 0.067 M phosphate buffer, pH 7.2.

2 cm^3 cell suspension (3 mg dry weight).

4 cm^3 0.067 M phosphate buffer, pH 7.2.

All solutions used had been equilibrated at 30°C prior to being used. After a few seconds stirring, a 2 cm^3 aliquot was removed from the above incubation mixture for the determination of substrate concentration, according to the method described in section II.A.7 above (for substrates that were pyrimidines).

The remaining 6 cm^3 incubation mixture in the electrode chamber was allowed to equilibrate for three minutes, then the plunger was inserted and the oxygen uptake measured on the chart for three to five minutes. The plunger was removed and the solution stirred for a further 30 minutes before a duplicate oxygen reading was taken on the same sample. After this, another 2 cm^3 aliquot was immediately removed for substrate determination (as above).

c) Calibration and Calculation of Oxygen Uptake

The scale expansion of the recorder was adjusted with air saturated distilled water at 30°C in the electrode vessel such that a full-scale deflection (100 chart recorder units) was equivalent to 0.235 x µmoles oxygen. $\overline{x}$ was the volume of the incubation mixture used in cm^3, and 1 cm^3 of air saturated water contained 0.235 µmoles oxygen (Umbreit et al., 1964).

The following is a typical calculation for a cell suspension oxidising 5-methylbarbituric acid. A determination of the initial 5-methylbarbituric acid content

gave a value of 34 µmoles; at the end of a 28 min. period, 24.8 µmoles remained.

The volume of incubation mixture = 6 cm^3

The rate of oxygen uptake = 7.5 recorder units per minute.

Therefore the total oxygen uptake in 28 minutes

$= \frac{7.5 \times 0.235 \times 6 \times 28}{100}$ µmoles oxygen

= 2.96 µmoles oxygen.

Over this same period, 9.2 µmoles of 5-methylbarbituric acid was oxidised.

Therefore for each mole of 5-methylbarbituric acid consumed, $\frac{2.96}{9.2}$ = 0.32 moles oxygen were used up.

d) Assay of Substrate Uptake (kindly performed by Dr. I.G. Andrew)

Substrate uptake into the cells was determined by following the accompanying O_2-uptake in the oxygen electrode.

7.5 mg dry weight of thymine-grown cells in a total volume of 3 cm^3 was pipetted into the electrode chamber of the oxygen electrode and allowed to equilibrate at room temperature for 5 minutes. 100 µl of substrate (0.15 M) was then introduced into the electrode chamber and the oxygen uptake recorded.

The rate of oxygen uptake remained constant at 1.1 µl/min. for the first 60 minutes. It then started to increase slowly and at 150 minutes the rate reached 2.9 µl/min.

The lag phase before the oxidation of succinate (as shown by the rate of oxygen uptake, above), exceeded

60 minutes, compared to a lag of only 30 to 40 minutes (Midwinter and Batt, 1960). This is possibly due to the lower temperature of incubation (20°C compared to 30°C of Midwinter and Batt).

B. Experimental Procedures

1. Long Term Labelling Experiment

a) Uracil-grown Cells

i) Incubation conditions

Cell suspensions were incubated with the [methyl-^{14}C] thymine as follows:

2.8 cm^3 cell suspension (containing 30 mg dry weight of cells); plus 2.8 cm^3 thymine (containing from 2µCi to 10µCi [methyl-^{14}C] thymine and sufficient carrier thymine to give a final concentration of 3.67 mM) were made up to a total volume of 14 cm^3 in 0.067 M phosphate buffer pH 7.2 and incubated at 30°C. A duplicate flask was set up without any labelled thymine.

Incubation time ranged from two to six hours.

Various ways were tried for aerating the cells to facilitate collection of samples and of labelled CO_2 evolved. These are described below (section iv).

ii) Sampling and Extraction Procedure

At appropriate times, 1.5 or 2 cm^3 aliquots were drawn from the incubation medium, the cells centrifuged in a Gallenkamp Junior centrifuge, setting 5 (1500g) for 5 minutes. The supernatant was carefully decanted and a drop of chloroform added to kill any remaining cells in solution.

To the residue, 5 cm^3 of hot 80% ethanol was added, then incubated at 80°C for 15 minutes. The ethanol extract was collected after centrifugation at 1500g for 5 minutes in the Gallenkamp centrifuge. This extraction procedure was repeated with another 5 cm^3 80% hot ethanol.

50 µl aliquots from the supernatant or ethanol extract were taken for the determination of radioactivity. The remainder was taken to dryness by passing an air stream over the solutions at room temperature.

iii) Determination of Radioactivity

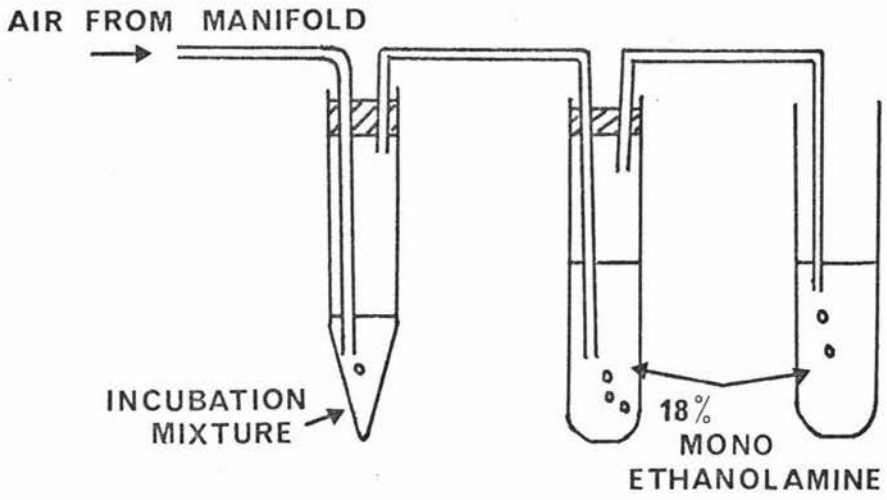
Radioactivity of samples was determined by scintillation counting on 50 µl aliquots as described in Section 5a.

iv) Determination and Absorption of Labelled CO_2

Various set-ups were tried to facilitate the absorption of labelled carbon dioxide.

At first, centre well flasks with 0.5 cm^3 hyamine hydroxide in the centre well were used. Hyamine hydroxide however turned jelly-like after being incubated at 30°C and this method of absorbing and collecting the labelled carbon dioxide was abandoned.

Next, a series of centrifuge tubes were used, each tube connected to a test tube containing 10 cm^3 18% monoethanolamine for collecting the CO_2, which was in turn connected to another test tube containing the same volume of ethanolamine, to ensure complete collection of the CO_2. The incubation medium was equally distributed amongst the centrifuge tubes and were aerated by means of air supplied through a manifold (see diagram below).



Samples were taken by disconnecting a complete unit (centrifuge tube plus test tubes) at each sample collection time. The centrifuge tube was immediately centrifuged as in (a) above, the supernatant decanted and the residue extracted with hot ethanol as described above. Sample collection was highly successful, but the drawback was the tendency of the Nocardia cells to stick to the walls of centrifuge tubes due to frothing. This slowed down the utilisation of [methyl-^{14}C] thymine.

Finally, flasks with side arms containing 10 cm^3 18% monoethanolamine were used. Each time samples from the incubation medium were collected, 50 µl samples of the monoethanolamine were drawn off for the determination of radioactivity as described in section 5a.

v) Diethyl Malonate as Inhibitor

0.02 M diethyl malonate (final concentration) was added to the incubation medium. A control, similar incubation medium without the diethyl malonate was set up. Samples were collected and treated as above.

b) 5-Methylbarbituric Acid-grown Cells and Cell-free Extracts

These cells, used whole, broken, as cell debris, or as cell-free extract were incubated at 30°C in mixtures of the following composition:

0.05 cm^3 substrate (0.02 M)

0.05 cm^3 2.5% bovine serum albumin

0.05 cm^3 CoASH (10 mM)

0.025 cm^3 Mg^{2+} (40 mM)

0.025 cm^3 Na_4ATP (40 mM)

0.05 cm distilled water

0.05 cm^3 cells (2 mg dry weight)

0.20 cm^3 Tris/HCl buffer pH 7.5 (0.05 M)

The substrate was either (i) freshly prepared [methyl-^{14}C] 5-methylbarbituric acid made up to the required concentration with carrier 5-methylbarbituric acid; or (ii) unlabelled 5-methylbarbituric acid.

Controls were set up without cells, or CoASH or ATP. At appropriate times, 0.05 cm^3 trichloroacetic acid was added to the incubation medium which was then centrifuged and decanted. Samples were taken from the supernatant either for the determination of substrate (spectrophotometrically) or for the counting of radioactivity (as in section 5a). The rest of the supernatant was freeze dried.

2. Short Term Labelling Experiment

a) Incubation Conditions

Cell suspensions were incubated with [methyl-^{14}C] thymine or [2-^{14}C] thymine as follows:

0.4 cm^3 cell suspension (containing 17 mg dry weight

of cells), 0.4 cm^3 thymine (containing 1 to 5 μCi labelled thymine plus sufficient carrier thymine to make a total of 0.17 μmole thymine) were made up to a total volume of 2 cm^3 with 0.067 M phosphate buffer, pH 7.2. Incubation time ranged from 5 seconds to 5 minutes; and incubation was at room temperature (20°C) or 4°C.

b) Sampling Procedures

At appropriate times, 0.5 cm^3 samples were removed by use of a syringe and immediately transferred to test tubes containing hot absolute ethanol and incubated at 80°C for 10 minutes. Then, the contents were centrifuged in a Gallenkamp Junior centrifuge, 1500g for 10 minutes. After samples had been removed for the determination of radioactivity, the ethanol extract was air dried and stored at -10°C.

Another sampling method involving Millipore filtration was used when separation of cells from the medium was desired. Samples (0.5 cm^3) were rapidly transferred to a Millipore filter, and filtered under vacuum. The residue was washed with 0.5 cm^3 distilled water and the filter with washed cells was immediately transferred to a beaker containing hot absolute ethanol (80°C) and heated for 10 minutes at 80°C. The time elapsed from taking the sample to transferring the residue to hot ethanol was from 15 to 30 seconds. The ethanol extract and the filtrate from the Millipore filtration were then separately air-dried and stored at -10°C.

c) Analysis of Samples

Samples were either air dried by directing an air stream on to them or freeze dried. The dried samples were taken up in 100 µl 60% ethanol and 10 or 25 µl used for chromatography to separate the labelled compounds.

These labelled compounds were tentatively identified by autoradiography and co-chromatography with authentic compounds.

3. Thin-layer Chromatography

a) Preparation of Plates and Sample Application

A slurry of cellulose MN 300 was spread on TLC (thin layer chromatography) plates at a thickness of 0.2 mm using a Desaga Heidelberg spreader. The plates (either 5 cm by 20 cm, or 20 cm by 20 cm) were left to dry at room temperature for at least 12 hours before being used.

For one-dimensional chromatography, 25 µl samples were streaked onto the plates and chromatographed. Two-dimensional chromatography was normally performed on 10 µl samples. Unlabelled standard compounds were sometimes included as markers.

b) Solvents

The following solvent systems were used:

1. n-Butanol : Acetic Acid : Water (2 : 1 : 1 v/v)
2. A. Tertiary-butyl Alcohol : Methylethyl Ketone : Water (40 : 30 : 25 v/v) in an atmosphere saturated with ammonia.

   B. Tertiary-butyl Alcohol : Methylethyl Ketone : Water : Ammonium Hydroxide (4 : 3 : 2 : 1 v/v).

3. Phenol : Water (72 : 28 w/w).
4. Amyl alcohol : Formic Acid : Water (2 : 2 : 1 v/v).
5. n-Propanol : Ammonia (3 : 2 v/v).
6. n-Butanol : Propionic Acid : Water (43 : 27 : 30 v/v)
7. n-Butanol : Pyridine : Water (2 : 2 : 1 v/v).

Solvents 1, 2A, 2B and 3 were used for the separation of pyrimidines and the unknown compounds. For the separation of dicarboxylic acids, solvents 4, 5 and 6 were used. Solvent 7 was used to separate amino acids from dicarboxylic acids which run with similar R_F values in both solvents 1 and 4. For two-dimensional chromatography solvent 3 was used in the first phase and solvent 1 in the second phase.

c) Detection of Compounds

i) UV lamp

The UV (ultraviolet) lamp was used for the detection of pyrimidines. These showed as dark areas when the chromatogram was irradiated with UV at 254 nm.

ii) Spray reagents

Dicarboxylic acids were detected using a universal indicator spray made alkaline with 0.1 cm^3 0.1 M NaOH per 20 cm^3 Universal indicator; or a solution of 1% methyl red in 95% ethanol made alkaline by the addition of 0.1 cm^3 0.1 M NaOH per 20 cm^3 methyl red. Acidic compounds show up as orange red spots against a blue, fading to yellow background.

For the detection of amino acids, ninhydrin from spray cans (Sigma) was used. The amino acids produced purplish blue coloured spots after the plates were heated for a few minutes at 60°C.

Urea showed up as yellow spots on being sprayed with

0.06 M p-dimethyl-amino-benzaldehyde in 1 M HCl. Barbituric acid gave an orange spot with this spray. This spray can also be used for the detection of dihydropyrimidines and β-ureido acids; the former must first be hydrolysed with a 0.5 M alkali spray (Fink et al., 1954).

iii) Scanning

Chromatographs were left to dry in a fume cupboard overnight after they were run in the above solvents. The 5 cm by 20 cm TLC plates were then scanned for radioactive bands using a Packard Radiochromatogram Scanner, model 7200. The gas used was Helium (98.7%) and isobutane (1.3%). Operating conditions were: gas flow 150 cm^3/min; slit width 2.5 cm; time constant 30; speed 0.5 cm/min; counts per minute 300, 1000 or 10,000 cpm; linear range.

iv) Autoradiography

TLC plates were individually placed in X-Ray film boxes in contact with sheets of Kodak rapid processing X-ray films. The X-ray film boxes were wrapped in aluminium foil to prevent light entering, and left in a cupboard for 10 days for low labelled compounds and 4 days for more highly labelled compounds.

X-ray films were developed using an X-ray developer for 30 seconds to 2 minutes (or until the radioactive regions were sufficiently dark); then washed in dilute acetic acid (10 cm^3 glacial acetic acid diluted to 500 cm^3). The film was then transferred to an X-ray fixer and washed until the background was clear, after which they were soaked in water for a few minutes.

4. Identification of Amino Acids (kindly performed by Dr. G.G. Midwinter).

a. High Voltage Electrophoresis

Ethanol extracts of cells were dissolved in 20 µl of 20 mM NH_4OH and applied as a 2 cm band across the bottom of a sheet of Whatman No. 1 chromatography paper (46 cm by 57 cm). 10 µl samples of amino acid markers were applied at the edge of each sheet.

The paper was moistened with pH 2.1 buffer (formic acid : acetic acid : water, 4 : 1 : 45) and excess buffer blotted off. Electrophoresis was carried out at pH 2.1 (Ryle and Sanger, 1955) in a Michl-type electrophoresis tank at 3 kv for 40 minutes.

After electrophoresis the paper was dried and exposed against a Kodak RP Royal X-Omat film for 48 hours. After exposure to the X-Ray plate the edges of each chromatogram were cut off and stained with ninhydrin. Those areas showing radioactivity and a positive ninhydrin reaction were eluted with 20 mM NH_4OH which was then applied to a second sheet of Whatman No. 1 paper. The basic and acidic amino acids were separated by electrophoresis at pH 6.5 in a pyridine : acetic acid : water buffer (100 : 4 : 900) at 3 kv for 40 minutes.

After exposure to an X-Ray plate followed by development of the edge markers with ninhydrin, the band containing the neutral amino acids was cut out and sewn onto a sheet of Whatman No. 1 paper. The backing strip was cut out, edge markers were applied and the neutral amino acids separated by electrophoresis at pH 2.1 for 40 minutes at 3 kv.

After drying, the electrophoretogram was exposed to an X-Ray plate for 72 hours and finally developed with ninhydrin.

b. Amino Acid Analyser

Samples containing amino acids were taken up in 0.2 cm^3 0.2 M Sodium citrate buffer and loaded onto the Beckman 120C amino acid analyser. The automatic loader was used and the sample was automatically analysed in Buffer 1 (0.2 M sodium citrate buffer, pH 3.25) for 65 min., then Buffer 2 (0.2 M sodium citrate buffer, pH 4.25) for 75 min., followed by Buffer 3 (1.0 M sodium citrate buffer, pH 6.65) for 165 min.

The position of the amino acid was then determined by comparison with standard markers.

5. Determination of Radioactivity

a) Aqueous samples (50 µl) were added to 7 cm^3 Triton/ Toluene scintillation fluid of the following composition: Triton 1 vol, Toluene 2 vol. PPO 4 g/l, POPOP 100 mg/l (Turner, 1968).

Efficiency of counting in unquenched samples with this scintillation fluid was about 69%.

Quenching was determined by the method of automatic external standardisation (A.E.S.).

b) The radioactive spots on chromatograms located by autoradiography were counted by two methods:

i) Direct counting with a Philips end-window counter. Efficiency of this method was established at 4.1% by use of standard [Methyl-^{14}C] thymine standardised in the scintillation counter against a [1-^{14}C] hexadecane standard. The thin layers were assumed to be of uniform thickness, hence no corrections for self-absorption were made.

ii) Radioactive spots were scraped from the TLC plates and put into counting vials containing 7 cm^3 scintillation fluid (as above). The radioactivity was then determined by scintillation counting in a Packard Tricarb 3375 scintillation spectrometer.

The efficiency of this radioactive counting was determined by the use of the same standard [Methyl-^{14}C] thymine as in method (i) above. The freshly shaken suspension had more radioactive counts than the sedimented sample and by counting at various times after shaking a curve was constructed relating efficiency to channels ratio. This correction curve was shown to be valid for a range of different compounds. The efficiency of counting after a settling time of 1 hour or more was generally about 46%.

Method (ii) was used wherever possible, but coloured samples (on sprayed chromatograms) were counted by method (i).

CHAPTER 3

I. GROWTH STUDIES

In the introduction, a possible pathway of the oxidative catabolism of thymine via 5-methylbarbituric acid and methylmalonate (or its CoA derivative) was discussed. Methylmalonate is most likely converted to succinate before being further broken down. This reaction could be by means of their respective CoA derivatives, catalysed by methylmalonyl CoA isomerase.

In this chapter, growth of N. corallina on the above compounds is described, as well as growth on uracil.

For these growth studies the inocula used had either been pre-adapted to growth on the substrate under study or had not been pre-adapted. Differences in length of lag phase were accordingly expected. Sampling and the determination of growth were discussed in the previous chapter.

Figure 3.1 shows the growth of variously adapted cells on methylmalonate.

Thymine, 5-methylbarbituric acid, methylmalonate, succinate and uracil at 0.2% concentration were found to support the growth of Nocardia corallina.

A lag phase of a few hours was commonly observed with the exception of thymine-adapted cells growing on methylmalonate, in which case, the lag phase exceeded 40 hours.

No requirements for adaptation to any substrate was demonstrable except for methylmalonate. The absence of such a requirement was unexpected and contrasted with the results of Batt (1957) who observed a lag when cells grown in the

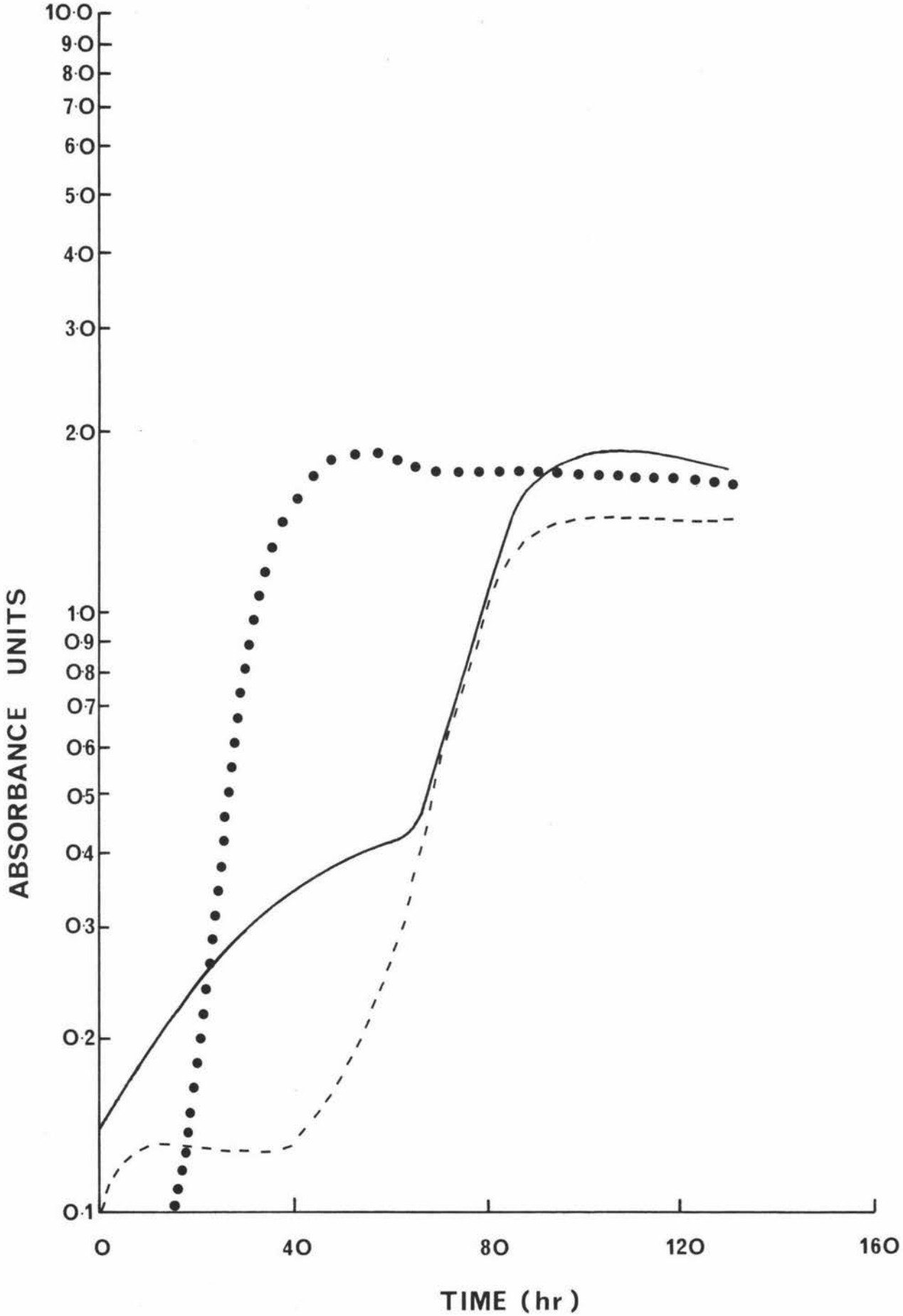


Figure 3.1. Growth of N. corallina on methylmalonate. Cells had been pre-adapted on —— glucose, --- thymine, and ● methylmalonate medium.

absence of pyrimidines oxidised pyrimidines as substrates, but no lag was observed when pyrimidine-grown cells oxidised pyrimidines.

II. CELL SUSPENSION STUDIES

The ability of Nocardia cells to oxidise the possible intermediates was further tested using cell suspensions.

A. Oxidation of Substrates by Cell Suspensions in the Oxygen Electrode

The ability of Nocardia corallina, grown on various substrates, to oxidise various possible intermediates of thymine catabolism was tested with the oxygen electrode as described in Methods, section II.A.8.

TABLE 3.I

The rate of oxygen uptake by N. corallina in nmoles/min. in a total volume of 6 cm^3.

Growth Substrate	Substrate in Resuspension Medium			
	Thymine	5-methyl-barbituric acid	Methyl-malonate	Succinate
Glucose	18	2	5	Negligible
Thymine	130	102	3	3
5-methyl-barbituric acid	64	150	10	7
Methylmalonate	0.7	4	132	20
Succinate	0.7	14	7	111
Uracil	n.d.	7	7	14
Barbituric acid	n.d.	32	n.d.	n.d.

n.d. = not determined.

Table 3.I shows the rates of oxygen uptake in nmoles per min. by Nocardia cells grown on different substrates when these same substrates were provided in the incubation mixture.

The following observations on this table can be made:

1. Oxygen uptake is dependent on
   i) substrate uptake into the region of the cell where metabolism can occur.
   ii) oxidative metabolism of the substrate. Cells grown on a particular substrate can catalyse the uptake and oxidation of that substrate. Thus thymine, 5-methylbarbituric acid, methylmalonate and succinate are each oxidised (or each promote oxygen uptake) by cells grown on the respective substrates.
2. 5-Methylbarbituric acid-grown cells oxidise thymine and thymine-grown cells oxidise 5-methylbarbituric acid, although the rates of oxygen uptake are in each case different from the corresponding rates with the substrate on which the cells were grown. 5-methyl-barbituric acid-grown cells presumably have no need for 'uracil-thymine oxidase' and a lower rate of thymine oxidation than with thymine-grown cells is not unexpected. Thymine-grown cells oxidise 5-methylbar-bituric acid at a rate approaching that of 5-methyl-barbituric acid-grown cells. The difference
   i) could reflect a slight permeability barrier in thymine-grown cells
   ii) might reflect an alternate pathway of thymine utilisation, so that thymine-grown cells do not require such a high level of 5-methylbarbituric acid oxidative enzymes.

3. The slight oxidation of 5-methylbarbituric acid by barbituric acid-grown cells is in accord with the finding (section B of this chapter) that 5-methylbarbituric acid is utilised by such cells after a short time lag.

4. Methylmalonate and succinate are not appreciably oxidised except by cells grown on the respective dicarboxylic acids. This probably reflects a requirement in each case for a specific permease. In the case of succinate, oxidation does occur in thymine-grown cells after a long lag (see results in Chapter 7). Midwinter and Batt (1960) on the basis of similar observations have postulated a common permease for succinate, malate and fumarate and a different permease for citrate. They found that cells of N. corallina grown on glucose, acetate or propionate oxidised these citric acid cycle intermediates only after a lag of 30-45 minutes, during which time induction of a permease system might occur.

From the results in this section, we cannot conclude that methylmalonate and succinate are or are not intermediates of thymine catabolism. The failure of thymine and 5-methylbarbituric acid-grown cells to oxidise methylmalonate and succinate is most likely due to a permeability barrier, as suggested by Midwinter and Batt (1960) for the case of succinate and other citric acid cycle intermediates. As mentioned above, a longer term incubation (Chapter 7) showed that succinate did stimulate oxygen uptake with thymine-grown cells after a 160 minute lag.

B. Appearance and Disappearance of Barbituric Acid and 5-Methylbarbituric Acid in Cell Suspensions

1. Uracil and Thymine-grown Cells Resuspended in Uracil and Thymine

In an attempt to isolate products of uracil and thymine catabolism, uracil and thymine-grown N. corallina were resuspended in uracil or thymine medium.

The resuspension medium was made up of

40 cm^3 0.02M phosphate buffer, pH 7.2

20 cm^3 0.02M substrate

20 cm^3 water

20 cm^3 cell suspension (containing 30 mg dry weight of cells).

Incubation was at 30°C with shaking in a shaker water bath, for 7 hours. Samples were removed at 30 minute intervals and treated as described in methods section. The amount of UV-absorbing material was determined and the samples were chromatographed in solvent 1.

a. Uracil-grown Cells

Figure 3.2.A is a graph of the amounts of thymine and 5-methylbarbituric acid remaining in the resuspension medium when uracil-grown cells were incubated with thymine. The 5-methylbarbituric acid concentration was at its maximum when almost all of the thymine had disappeared. No other UV-absorbing compounds, apart from thymine and 5-methylbarbituric acid were observed on chromatograms.

In a resuspension of uracil-grown cells on uracil, barbituric acid was formed. This was shown by the shift in the maximum absorbance from 285 nm to 258 nm in alkali.

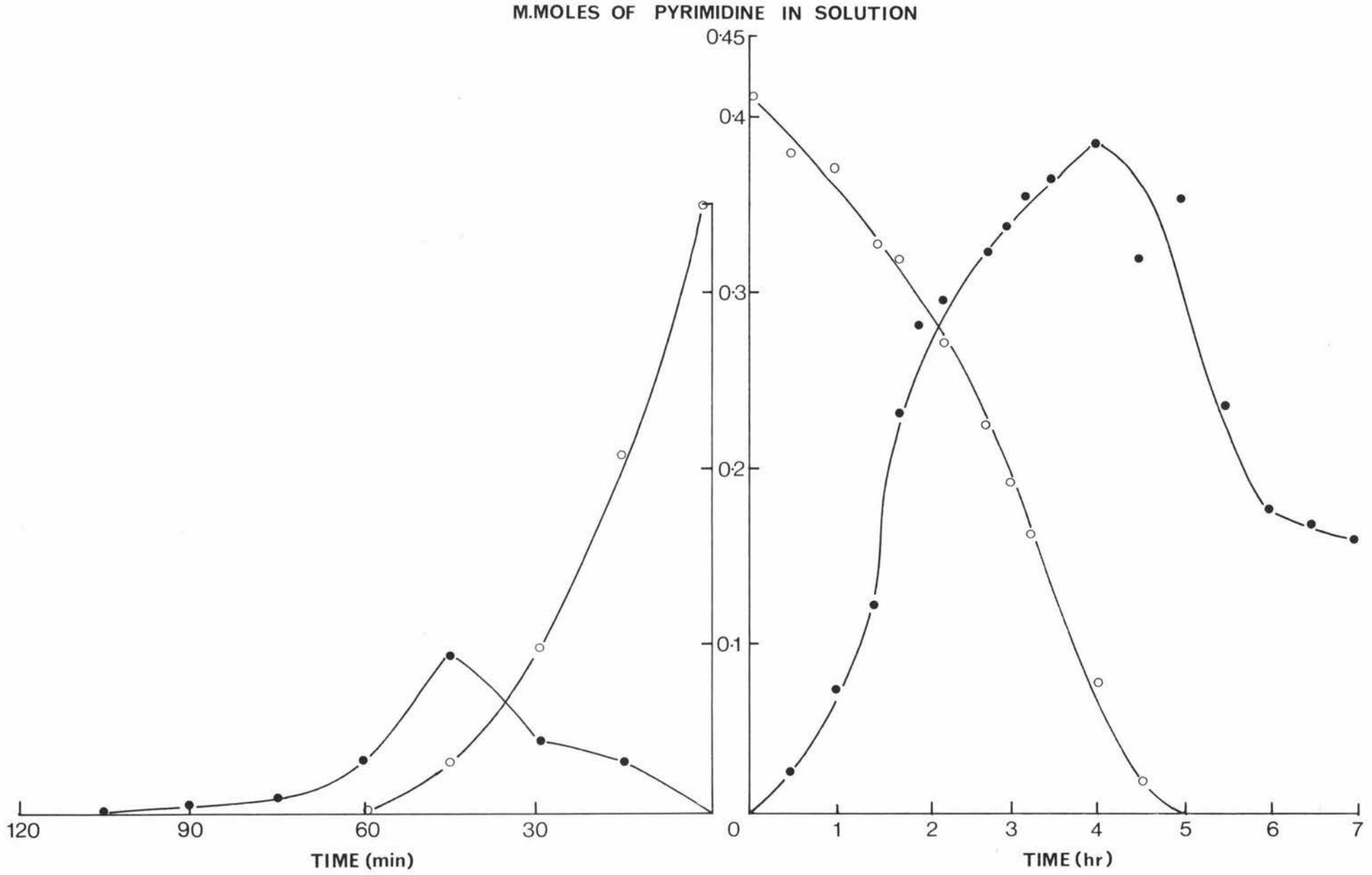


Figure 3.2.B. The metabolism of thymine by thymine-grown cells. Symbols as for Figure 3.2.A

Figure 3.2.A. The metabolism of thymine by uracil-grown cells. ○ amount of thymine and ● amount of 5-methylbarbituric acid in the resuspension medium.

As above, no other products were discernible on chromatograms of the samples.

b. Thymine-grown Cells

Similarly, thymine-grown cells were resuspended in thymine and uracil. Figure 3.2.B shows the disappearance of thymine and the appearance and disappearance of 5-methylbarbituric acid when thymine-grown cells were incubated with thymine. The increase to a maximum of the product coincided with the almost total removal of thymine. As in section (a), no other UV-absorbing products of uracil or thymine utilisation were discernible on chromatograms of the samples.

The accumulation of 5-methylbarbituric acid in this instance agrees with the findings of Batt and Woods (1961) (see Introduction, Figure 1.6) and with later results using radioactive substrates (Chapter 5, 6, 7).

2. Cells Resuspended in 5-Methylbarbituric acid

Cells grown on glucose, uracil, barbituric acid, thymine and 5-methylbarbituric acid were similarly resuspended on 5-methylbarbituric acid. Their utilisation of 5-methylbarbituric acid is shown in Figure 3.3. Neither glucose-grown nor uracil-grown cells were able to utilise 5-methylbarbituric acid, even after 3 hours incubation. Barbituric acid-grown cells on the other hand, started to utilise 5-methylbarbituric acid after a lag period of 45 minutes. This differed from the substrate utilisation by both thymine and 5-methylbarbituric acid-grown cells, which proceeded without any lag phase.

The behaviour of uracil-grown cells differed slightly

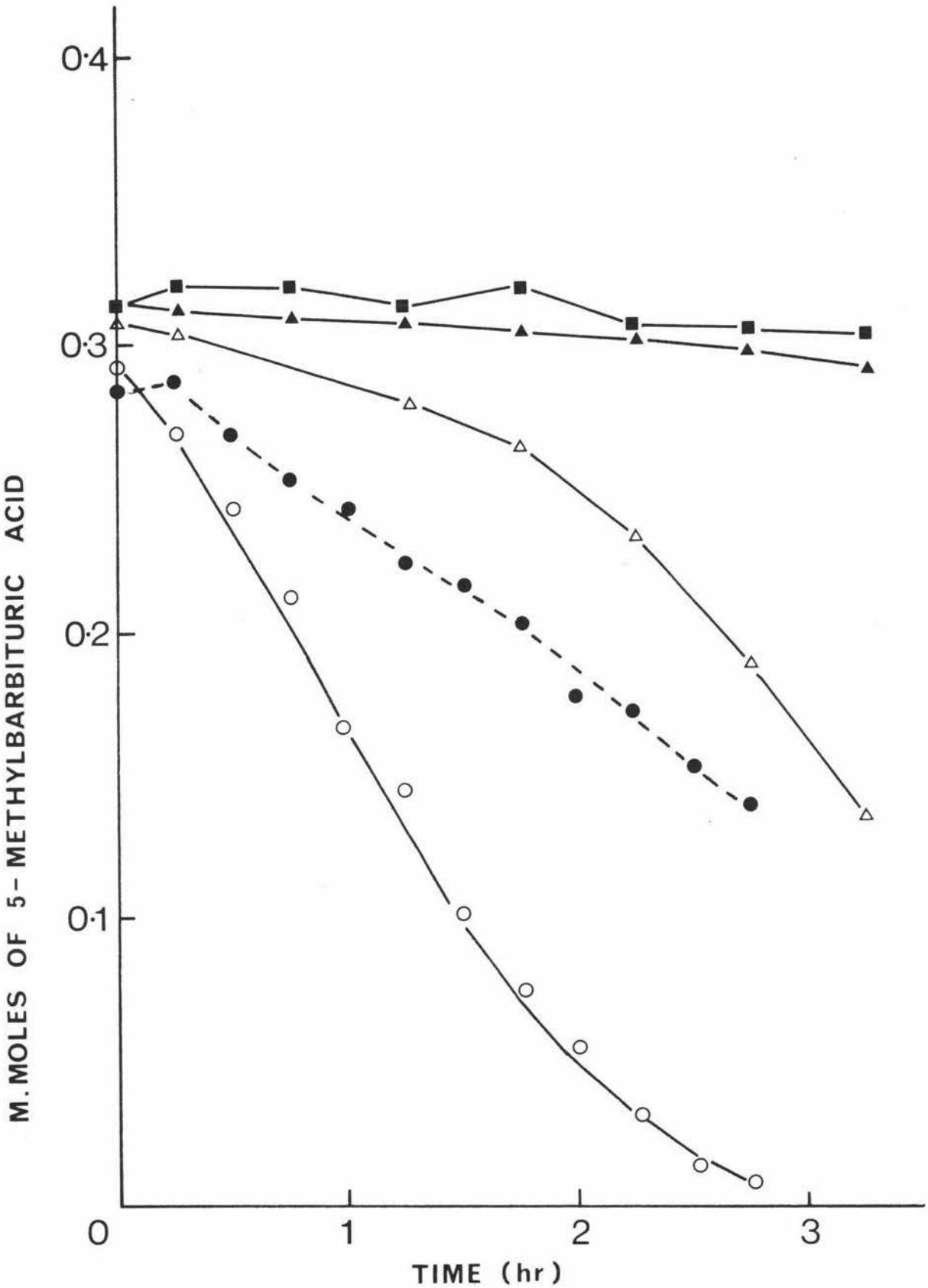


Figure 3.3. Metabolism of 5-methylbarbituric acid by ■ glucose-grown, ▲ uracil-grown, △ barbituric acid-grown, ● thymine-grown and ○ 5-methyl-barbituric acid-grown N. corallina. Resuspension conditions were as in section B.1.

from the results of Batt and Woods (1961). They found that oxygen-uptake by uracil-grown cells began after 2 to 3 hours incubation.

For further discussion of these results see Chapter 8 (Discussion).

C. Attempted Isolation of Succinate

Methylmalonate-grown cells were resuspended in buffer containing methylmalonate in an attempt to isolate succinate (the possible intermediate) from the medium. Malonate (final concentration 0.004M) was added to the resuspension medium to inhibit further oxidation of succinate.

However, chromatograms of the samples showed no signs of succinate. It is possible that malonate did not enter the cells, and the alternative use of diethylmalonate might have yielded more meaningful results.

Mountfort (1971) demonstrated the accumulation of succinate and methylmalonate in thymine-grown cells in the presence of diethylmalonate. Similar experiments with diethylmalonate are reported in the following chapter, where methylmalonate is shown to accumulate as a product of 5-methylbarbituric acid metabolism.

CHAPTER 4

CATABOLISM OF 5-METHYLBARBITURIC ACID

I. Preparation of 5-Methylbarbituric acid from Thymine Resuspension Medium Using Uracil-grown Cells

In order to determine the breakdown products of 5-methylbarbituric acid, it was initially planned to incubate Nocardia corallina with labelled 5-methylbarbituric acid. In view of the complicated procedure required for chemical synthesis of the labelled substrate (Biggs and Doumas, 1963), attempts were made to isolate 5-methylbarbituric acid from thymine medium on which uracil-grown cells had been resuspended. It was proposed to extend this to the isolation of the ^{14}C-labelled 5-methylbarbituric acid.

Cell suspension studies (Chapter 3) showed that uracil-grown cells give the highest yield of 5-methylbarbituric acid in resuspension medium with thymine as substrate, as earlier observed by Batt and Woods (1961). Uracil-grown cells had the 'thymine-oxidase' induced and this enzyme converts the thymine to 5-methylbarbituric acid. More detailed discussion is outlined in Chapter 1. Presumably in uracil-grown cells, the enzyme (if any) involved in the next step was not induced, hence the high transient accumulation of 5-methylbarbituric acid.

A. Stability of 5-Methylbarbituric acid

As discussed in the Introduction, 5-methylbarbituric acid may be autooxidised in air to 5-hydroxy-5-methylbarbituric acid. This oxidation is accompanied by the disappearance of the absorbance at 268 nm.

The absorbance of 5-methylbarbituric acid stored for a few days in water solution, in 2% EDTA solution and in 0.1M NaOH at room temperature either in the dark or exposed to light decreased after a few days. The decrease was not always to the same extent. The 5-methylbarbituric acid was perhaps more stable in 0.1M NaOH stored in the dark.

In one reading a solution of 5-methylbarbituric acid in water (exposed to light) has its absorbance reduced to 64% of the original value after 7 days, whereas in another reading, the value decreased to only 77% of the original value. In any case, under the conditions used here, 5-methylbarbituric acid was more stable than in the aerated phosphate buffer used by Batt and Woods (1961), where it disappeared in 30 minutes. And in the incubation of 5-methylbarbituric acid with cell-fractions reported in section IIb; 5-methylbarbituric acid was found to be perfectly stable over the 90 minute incubation period. Furthermore, Payakachat (pers. comm.) observed that neutral or basic solutions of 5-methylbarbituric acid stored at 25°C was quite stable over a period of 113 hours. He found that solutions of 5-methylbarbituric acid in 0.05M Tris/HCl buffer, pH 8.85 and in 0.1M NaOH were stable over the tested period, and that a solution of 5-methylbarbituric acid in 0.1M phosphate buffer pH 7.0, retained 78% of its absorbance value at the end of the 113 hours.

B. Preparation of Unlabelled 5-Methylbarbituric acid

Unlabelled 5-methylbarbituric acid was prepared chemically as described in Chapter 2, Section A.

As a preliminary to the preparation of labelled 5-methylbarbituric acid from thymine, it was necessary to do a control experiment with unlabelled thymine.

A suspension of uracil-grown cells was prepared as described in section IIA, of Chapter 2. The thymine resuspension medium was made up of the following:

20 cm^3 0.067M phosphate buffer, pH 7.2

10 cm^3 water

10 cm^3 0.02M thymine

10 cm^3 suspended cells (15 mg dry weight)

The incubation medium was poured into a 150 cm^3 flask plugged with cotton wool and incubated with shaking at 30°C in a shaker waterbath (Gallenkamp "Compenstat"). When all the thymine had disappeared (after one to two hours, checked spectrophotometrically) the contents were centrifuged and the supernatant collected. A few drops of chloroform were added to kill any remaining cells, and the amount of 5-methylbarbituric acid was determined spectrophotometrically and its identity verified chromatographically.

The absorbance of the 5-methylbarbituric acid in the supernatant at 268 nm, both in neutral solution and in 0.1M NaOH showed a 10 to 20% decrease over a period of three days.

Half of the supernatant was taken to dryness and taken up in 0.1M NaOH. Samples were applied to chromatograms, eluted with 0.1M NaOH, and scanned in the spectrophotometer to see if chromatographed 5-methylbarbituric acid

was recoverable.

TABLE 4.1

The absorbance at 268 nm of the 5-methylbarbituric acid (5MBA) from the supernatant

Treatment of Sample	Absorbance at 268 nm (Absorbance units)	% of initial amount of 5MBA
Initial absorbance (before application to chromatogram)	0.55	
Sample applied to chromatogram and eluted immediately	0.28	51
Sample applied to chromatogram, left overnight and eluted	0.27	49
Sample applied to chromatogram, chromatographed immediately and eluted	0.24	44
Sample applied to chromatogram, left standing for 2 hours, chromatographed and eluted	0.23	42
Sample applied to chromatogram, left standing overnight, chromatographed and eluted	0.24	44

Chromatography was in solvent 1 (n-butanol : acetic acid : water). Dilution factor was the same in all the above readings. The Spectronic 20, Bausch and Lomb Spectrophotometer was used.

Table 4.1 shows the absorbance at 268 nm of the variously treated 5-methylbarbituric acid. 42 to 44% of the 5-methylbarbituric acid was recovered from the supernatant by chromatography.

Doumas and Biggs (1962) state that 5-methylbarbituric acid is partially converted to 5-hydroxy-5-methylbarbituric acid on chromatography. Presumably some of the chromatographed 5-methylbarbituric acid had been converted to 5-

hydroxy-5-methylbarbituric acid, hence the 42 to 44% recovery of 5-methylbarbituric acid on chromatography.

It was not possible to recover any 5-methylbarbituric acid from phenol : water chromatograms (Chapter 7) unless the amount of 5-methylbarbituric acid applied to the origin was at least 100 µg.

C. Extraction of 5-Methylbarbituric acid from the Supernatant

An attempt was made to extract 5-methylbarbituric acid from the supernatant by the use of ethyl acetate, isopropanol and amyl alcohol. The supernatant containing the 5-methylbarbituric acid was acidified to pH 1.5 to 2 with 1M HCl, and the 5-methylbarbituric acid extracted with a volume of extraction solvent equivalent to the supernatant volume.

TABLE 4.II

Yield of 5-methylbarbituric acid during the extraction procedure, using amyl alcohol

Amount of 5-methylbarbituric acid	Yield in mmoles
Theoretical yield*	0.375
Found in supernatant	0.182
Extracted into amyl alcohol	0.094
Remaining after drying and redissolving in 0.1M NaOH	0.055
Chromatographed and eluted in 0.1M NaOH	0.011

* Equivalent to the amount of thymine used up.

Table 4.II shows the amount of 5-methylbarbituric acid extracted using amyl alcohol. The final amount of 5-methylbarbituric acid obtained after chromatography was only 6% of the 5-methylbarbituric acid in the supernatant, although

the amount extracted into amyl alcohol was 52%.

With ethyl acetate or isopropanol, the extracted 5-methylbarbituric acid was less than 10% of the amount in the supernatant.

Although amyl alcohol was a good extraction solvent, 5-methylbarbituric acid was very unstable in it. After one day, 84% of the 5-methylbarbituric acid was lost.

Extraction of 5-methylbarbituric acid from the supernatant as seen in the above results could not be achieved in satisfactory yields with organic solvents and was therefore abandoned. However, over 40% was shown to be recoverable after chromatography (Section B) and this method was therefore selected for isolation of ^{14}C-labelled 5-methylbarbituric acid as described below.

D. Preparation of [Methyl-^{14}C] 5-Methylbarbituric Acid

[Methyl-^{14}C] 5-methylbarbituric acid was prepared from [methyl-^{14}C] thymine, by incubating with a suspension of uracil-grown cells. The procedure was as outlined for unlabelled 5-methylbarbituric acid in section I.B.

The incubation medium was collected when the absorbance at 268 nm was at its maximum. The supernatant containing the [methyl-^{14}C] 5-methylbarbituric acid was evaporated to dryness, redissolved in 0.1M NaOH and chromatographed in solvent 1 (n-butanol : acetic acid : water). The band corresponding to 5-methylbarbituric acid was detected by autoradiography (see Figure 2.1.C) eluted with 60% ethanol and used directly for the experiments reported below.

II. Utilisation of 5-Methylbarbituric acid by Cells Grown on 5-Methylbarbituric acid

In order to determine the breakdown products of 5-methylbarbituric acid, whole cells and various cell-fractions of N. corallina were incubated with [methyl-^{14}C] 5-methylbarbituric acid. It was hoped that a cell-fraction might be obtained with high enzymic activity on 5-methylbarbituric acid so that the immediate products of the reaction might be identified.

A. Utilisation of Unlabelled 5-Methylbarbituric acid

Whole cells, cell-free extracts, cell debris, total disrupted cells and toluene-treated cells were used in the incubation with various co-factors. The incubation medium was made up of the following in each glass centrifuge tube.

0.05 cm^3 substrate (0.02M 5-methylbarbituric acid)
0.05 cm^3 2.5% bovine serum albumin
0.05 cm^3 CoASH (10 mm)
0.025 cm^3 Mg^{2+} (40 mm)
0.025 cm^3 NaATP (40 mm)
0.20 cm^3 Tris/HCl buffer pH 7.5 (0.05M)
0.05 cm^3 distilled water
0.05 cm^3 cells (2 mg dry weight)

The incubation was at 30°C and samples were collected at 0, 1, 5, 15, 30 and 60 min. At the appropriate times the reaction was stopped by adding 0.5 cm^3 15% trichloroacetic acid and the contents centrifuged. The amount of 5-methylbarbituric acid in the supernatant was determined spectrophotometrically and converted to µmoles using a standard curve (Chapter 2). Incubations without CoASH or NaATP or

cells were set up as controls. If methylmalonyl CoA were the immediate product of 5-methylbarbituric acid breakdown, then addition of ATP and CoASH might be expected to stimulate the reaction in cell-free extracts.

TABLE 4.III

The incubation of whole cells or cell-fractions with 5-methylbarbituric acid (5MBA) plus or minus various co-factors

Form of cells used (co-factors present unless otherwise stated)	µmoles 5MBA in the incubation medium at	µmoles 5MBA used up in
	'0' time	1 hour	1 hour
Whole cells	1.26	0.72	0.54
Total disrupted cells	1.46	0.53	0.93
Cell-free extract	1.14	0.42	0.72
Cell debris	1.45	0.63	0.82
Toluene-treated cells	1.29	0.53	0.76
Total disrupted cells (frozen for 4 days)	1.23	0.68	0.55
Whole cells (without co-factors)	1.26	0.63	0.63
Boiled whole cells	1.28	1.25	0.03
Incubation of 5MBA + co-factors (without cells)	1.11	1.09	0.02
Incubation of 5MBA (without co-factors or cells)	1.02	1.02	0

Conditions were as described in section II.A.

Table 4.III shows the amount of 5-methylbarbituric acid used up by the differently treated cells in one hour. Total disrupted cells, cell debris, cell-free extract and toluene-treated cells had appreciable activities towards 5-methylbarbituric acid, in the presence of co-factors. Cell-free extract and total disrupted cells were used in additional experiments with /methyl-^{14}C/ 5-methylbarbituric acids,

reported below.

B. Cell-free Extract and [Methyl-^{14}C] 5-Methylbarbituric acid

In a preliminary experiment, the cell-free extract was incubated with [methyl-^{14}C] 5-methylbarbituric acid under the conditions described in section A. Controls were set up, one without CoASH, another without NaATP and the third without any cell-free extract. Samples were collected at 30 minute and 90 minute, and the supernatant collected was freeze dried.

Autoradiographs of samples from the complete incubation mixture and that lacking ATP show, in addition to 5-methylbarbituric acid, an unidentified radioactive spot ('P' on Figure 4.1). This spot was lacking from the controls without CoASH or without cell-free extract.

C. Total Disrupted Cells and [Methyl-^{14}C] 5-Methylbarbituric acid

Further incubation work with [methyl-^{14}C] 5-methylbarbituric acid was carried out using total disrupted cells. The incubation mixture was as stated in section IIA of this Chapter. The sample collection time and the variation of the incubation mixtures were as shown in Table 4.IV.

Autoradiographs of the samples (Figure 4.1) showed the presence in samples C and D of the radioactive unknown (P) discussed above.

The unknown radioactive spot (P) in Figure 4.1 could be a product of the utilisation of [methyl-^{14}C] 5-methylbarbituric acid by N. corallina cells. Attempts were made

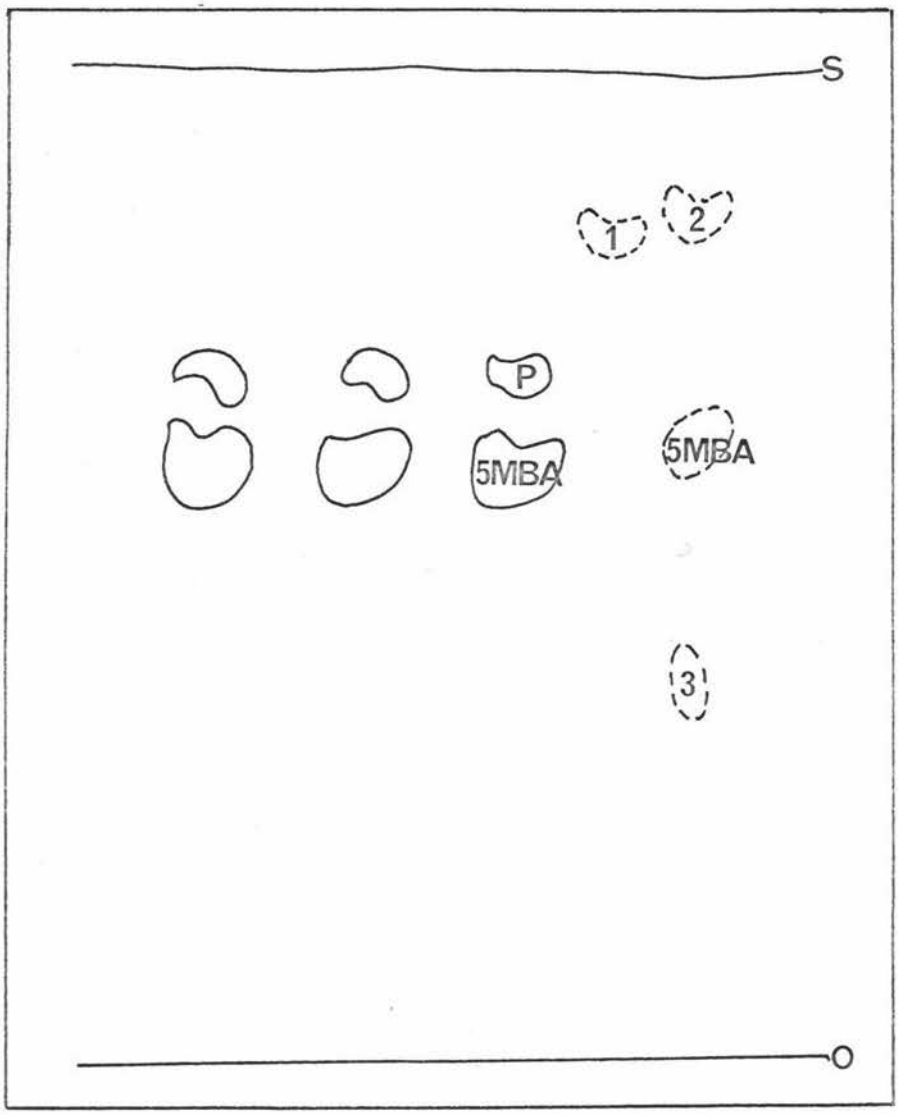


Figure 4.1. Autoradiographs of samples from the incubations of Cell-free Extract and Total disrupted cells with [methyl-^{14}C] 5-methylbarbituric acid.

Markers were run alongside the samples and the solvent used was n-butanol : acetic acid : water. P was the radioactive unknown; 5MBA, the position of 5-methylbarbituric acid; and 1, 2 and 3 were succinate, methylmalonate and CoASH markers, respectively.

(Radioactive compounds are circled in dark lines, the markers in dotted lines).

TABLE 4.IV

Incubation of total disrupted cells and [methyl-^{14}C] 5-methylbarbituric acid

Sample	Addition to Incubation mixture	Sample Collection Time (min.)
A	Complete (control)	0
B	Complete	1
C	Complete	15
D	Complete	60
E	Plus 0.02M Succinate	60
F	Minus NaATP	60
G	Minus CoASH	60

Incubation conditions were as in section IIA.

to identify this unknown compound by co-chromatography with authentic markers including 5-hydroxy-5-methylbarbituric acid, but so far, its identity remains unknown.

The work with ^{14}C 5-methylbarbituric acid was hampered by the difficulty in preparing the pure radioactive substrate. In some incubations, as little as 10% of the chromatographically purified 5-methylbarbituric acid was still present by the time the incubation mixture was set up. For this reason, further work with ^{14}C 5-methylbarbituric acid was abandoned in favour of whole cell experiments with ^{14}C thymine. These are reported in the next three chapters.

The experiments in this chapter have produced two tentative conclusions

i) metabolism of 5-methylbarbituric acid can be demonstrated in cell-free extracts. (This had not been observed by previous workers Hayaishi and Kornberg (1952), Lara (1952), Batt and Woods (1961), Biggs and

Doumas (1963), and Mountfort (1971).)

ii) There is evidence for the CoA-dependent formation of a specific unidentified radioactive compound in the cell-free extracts and whole disrupted cells when these are incubated with ^{14}C 5-methylbarbituric acid.

CHAPTER 5

CATABOLISM OF [^{14}C] THYMINE BY URACIL-GROWN CELLS

To study the breakdown products of 5-methylbarbituric acid, it was initially planned to feed N. corallina with labelled 5-methylbarbituric acid. However, the chemical synthesis of this labelled substrate was too complicated to be undertaken and problems were encountered with its preparation from the utilisation of labelled thymine by uracil-grown organisms (Chapter 4). The yield of 5-methylbarbituric acid extracted from growth medium was low (only about 40%) and its instability in solution further complicates matters.

For this reason, the use of ^{14}C thymine was decided upon. In the following experiments, N. corallina was fed [methyl-^{14}C] and also [2-^{14}C] thymine. Both long term and short term incubations with low and high specific activities respectively of [methyl-^{14}C] thymine were carried out. This chapter is concerned with long term incubations using uracil-grown cells.

It was hoped that uracil-grown cells would metabolise 5-methylbarbituric acid only slowly and that some of the intermediates of 5-methylbarbituric acid metabolism might be readily demonstrated, provided that they too were slowly metabolised.

I. Long Term Incubation of Cell Suspension with [Methyl-^{14}C] Thymine (Low Specific Activity)

An incubation (experiment 1) with 2μCi [methyl-^{14}C] thymine was set up as described in Methods Section (B.1) as a preliminary run. 1.5 cm^3 aliquots were removed immediately

and 2.5, 3.5, 4.5, 5.5 and 6.5 hours after incubation. Cell-extracts, cell-free supernatants and evolved CO_2 were collected as previously described in Methods.

In experiment 2, the same amount of labelled thymine as in experiment 1 was used in a system involving a series of seven centrifuge tubes as described in methods. Sample collection was done immediately and 1.5, 2, 3, 4, 5 and 6 hours after incubation.

The third incubation (experiment 3) was made in a flask with a side arm containing ethanolamine. (This arrangement was the one used in subsequent long term experiments.) 10μCi of [methyl-^{14}C] thymine was used and samples were collected immediately and then 1.5, 2.5, 3, 3.5 and 4.5 hours after incubation. (Sample collection takes approximately 30 seconds and centrifugation of samples another 5 to 6 minutes. So, 'immediately' may be up to 7 or 8 minutes.)

In an attempt to extract labelled methylmalonate from the resuspension medium, an incubation was set up containing 0.004M diethylmalonate (final concentration). Samples were removed immediately and after 10 min., 30 min., 1 hour, 2 hours, 3 hours and 4 hours of incubation.

II. The Disappearance of Thymine and the Appearance and Disappearance of 5-Methylbarbituric acid

The amount of pyrimidines in the supernatant fractions was determined as described in Methods section II.A.7.

In experiment 1, thymine disappeared completely within 2.5 hours, at which time 5-methylbarbituric acid had already reached its maximum. 5-methylbarbituric acid then started to decrease and at 7.5 hours only 14% of 5-methylbarbituric

acid remained.

In experiment 2, thymine utilisation was very slow; 45% of the thymine remained in the resuspension medium after 6 hours, compared to the complete utilisation of thymine within 2.5 hours in experiment 1. Similarly, the increase in 5-methylbarbituric acid was slow; at 6 hours it was still increasing. The slow utilisation of thymine may be partly due to the decrease in the number of cells participating in the incubation; an appreciable proportion of cells having been blown onto the walls of the centrifuge tubes by the air stream provided for aeration.

In experiment 3, where a flask with a side arm was used, the thymine was totally used up after 3 hours. This coincided with the maximum production of 5-methylbarbituric acid. The decrease in the amount of 5-methylbarbituric acid was rather slow. After 4.5 hours incubation 92% of the total amount of 5-methylbarbituric acid remained.

The above results which showed the formation and accumulation of 5-methylbarbituric acid by uracil-grown cells utilising thymine agreed with that of Batt and Woods (1961) and Mountfort (1971). 'Uracil-thymine oxidase' had already been induced in uracil-grown cells, but the enzyme (if any) responsible for the degradation of 5-methylbarbituric acid had to be induced, hence the transient accumulation of 5-methylbarbituric acid observed (as discussed in Chapters 1 and 3).

III. The Collection and Radioassay of Carbon Dioxide (CO_2)

Hyamine hydroxide was initially used for the collection of CO_2. However, on incubation at 30°C, hyamine hydroxide

became jelly-like, making its sampling very difficult; hence the arrangement used in experiment 2. Collection of ethanolamine (which was used to absorb CO_2) was easy in experiment 2, but thymine utilisation was greatly reduced because of cells being blown onto the glass walls of the centrifuge tubes. Finally, the use of flasks with side arms containing ethanolamine was chosen as the most efficient system of CO_2 collection and cell incubation.

Measurement of the radioactivity in ethanolamine was as described in Methods section II.B.5. The incorporation of label into CO_2 is shown in Figure 5.1 as a percentage of the total radioactivity present in each sample. The incorporation of label into CO_2 in experiment 2 had a slower rate and the percentage radioactivity was also lower than that of experiment 3. This is consistent with the low rate of thymine utilisation observed in experiment 2.

The low percentage of radioactivity in the CO_2 suggests an incorporation process involving many intermediates. A possible incorporation route is through the Krebs cycle intermediates and their production of CO_2 from labelled carbon atoms (see Discussion, Chapter 8).

IV. Recovery of ^{14}C in Supernatant and Ethanol Extract of Cells

The radioactivity in the supernatant and ethanol extract of cells was determined by scintillation counting. Figure 5.1 shows the percentage recovery of ^{14}C in the supernatant and ethanol extract. The radioactivity in the supernatant decreased with time while that in the ethanol extract increased, although not to the same extent.

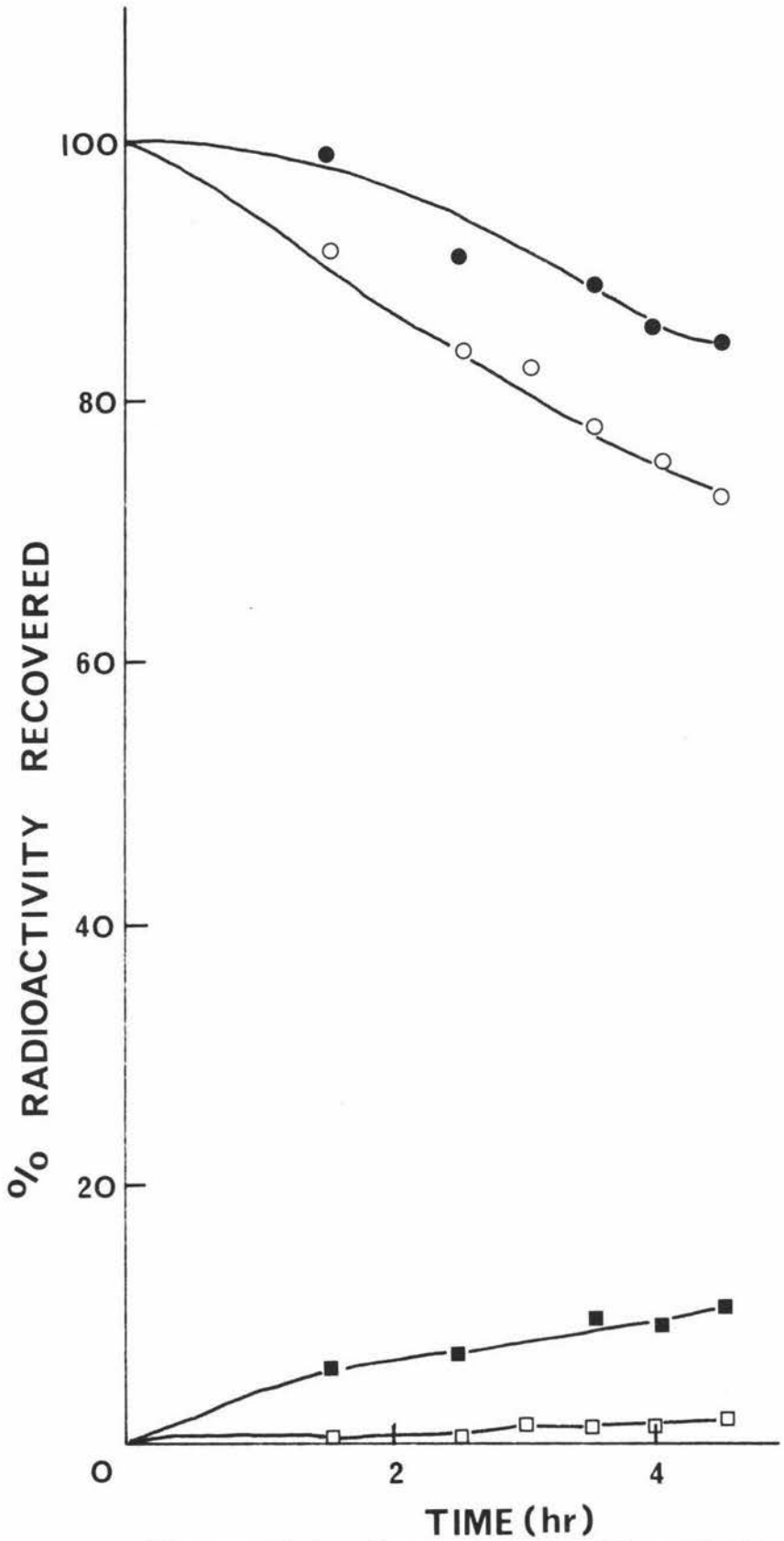


Figure 5.1. Percentage radioactivity recovered from ○ the supernatant, ■ the ethanol extract, □ in CO_2 and ● the total recovered (Experiment 3).

The total radioactivity recovered also showed a decrease with time. The decrease was greater than could be accounted for by production of CO_2. The explanation for this is not known.

V. Chromatography and Autoradiography

All samples collected were chromatographed; the supernatant fractions in n-butanol : acetic acid : water (2 : 1 : 1) and ethanol extract fractions in tertiary-butylalcohol : methylethyl ketone : water (45 : 35 : 25), in an attempt to separate and identify the radioactive products formed. The different radioactive compounds on the chromatograms were detected by scanning in a Radiochromatogram scanner and by autoradiography (see Methods).

Autoradiographs give a better picture of radioactive compounds, especially those running close together. Two or more radioactive bands with close R_F values would show up as a broad peak on radiochromatogram scans, whereas an autoradiograph would show them as distinct bands or spots. Autoradiographs of a series of samples taken at various times during experiment 3 are shown in Figures 5.2.A and B. Several radioactive compounds were formed from thymine and some of these were identified, as discussed in the next section.

All the major radioactive bands shown in Figures 5.2.A and B were eluted and the radioactivity counted. The percentage incorporation of radioactivity into the major products is shown in Figures 5.3.A and B. The incorporation of radioactivity into glutamate and the unknowns G and H increased with time.

Figure 5.2.A (top).

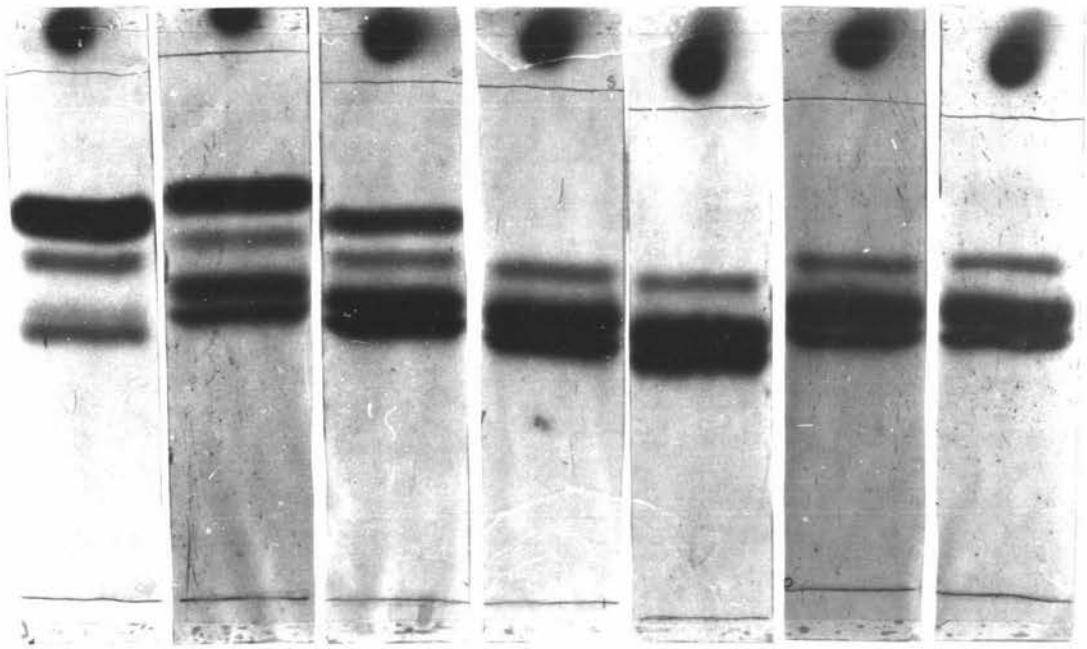
Autoradiographs of a series of supernatant samples taken at various times during Experiment 3. The solvent used was n-butanol : acetic acid : water.

0 is the sample removed at 'zero' time: 1 at 1.5 hr.; 2 at 2.5 hr.; 3 at 3 hr.; 4 at 3.5 hr.; 5 at 4 hr. and 6 at 4.5 hr.

Figure 5.2.B (bottom).

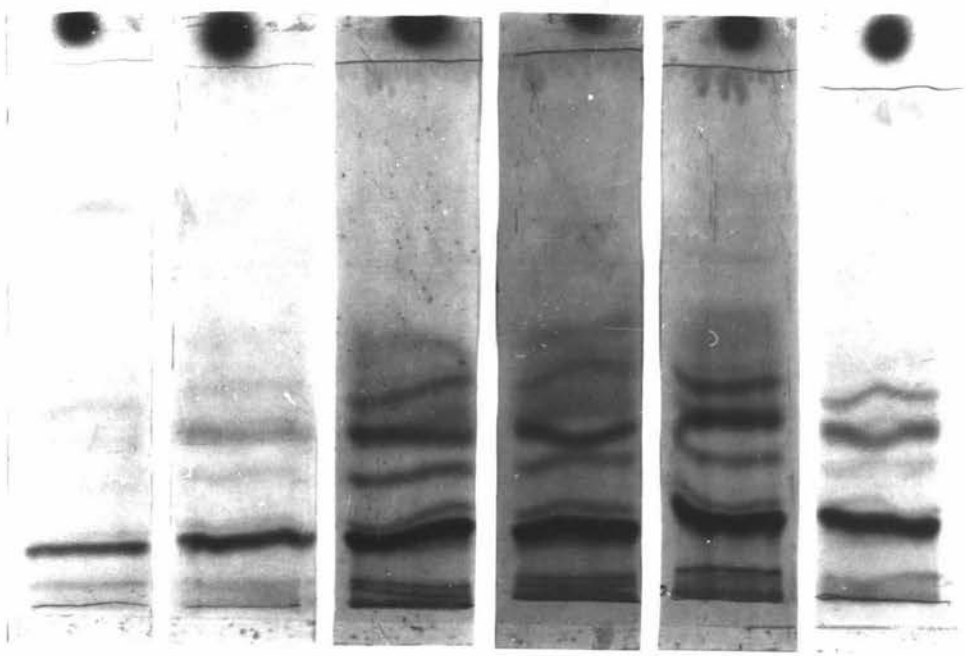
Autoradiographs of a series of ethanol extract samples taken at various times during Experiment 3. The solvent used was tert.butyl alcohol : methylethyl ketone : water. Sampling times are as in Figure 5.2.A.

3 S



O 1 2 3 4 5 6

3 E



1 2 3 4 5 6

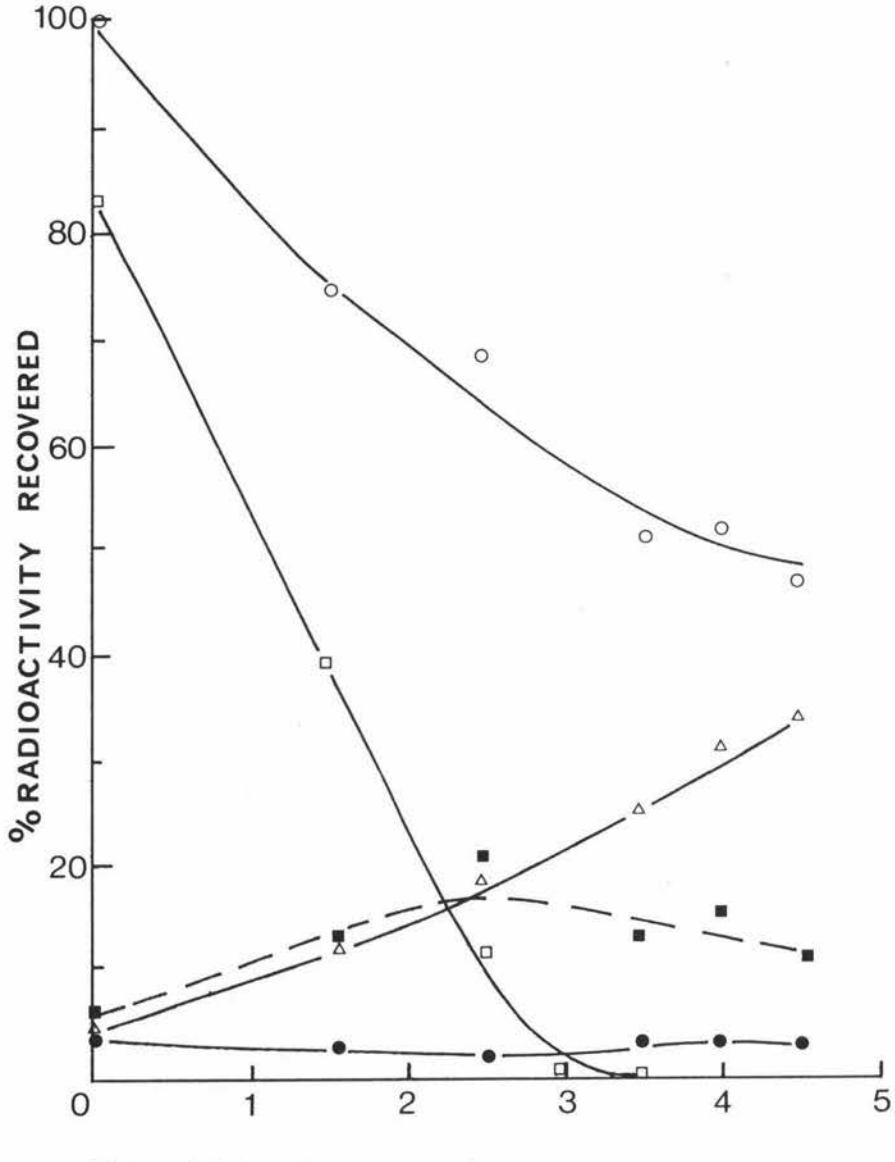


Figure 5.3.A. Percentage recovery of radioactivity in compounds in the supernatant fraction (Experiment 3). The compounds are □ thymine, △ 5-methylbarbituric acid, ● , ■ impurities B and D respectively. ○ total radioactivity recovered.

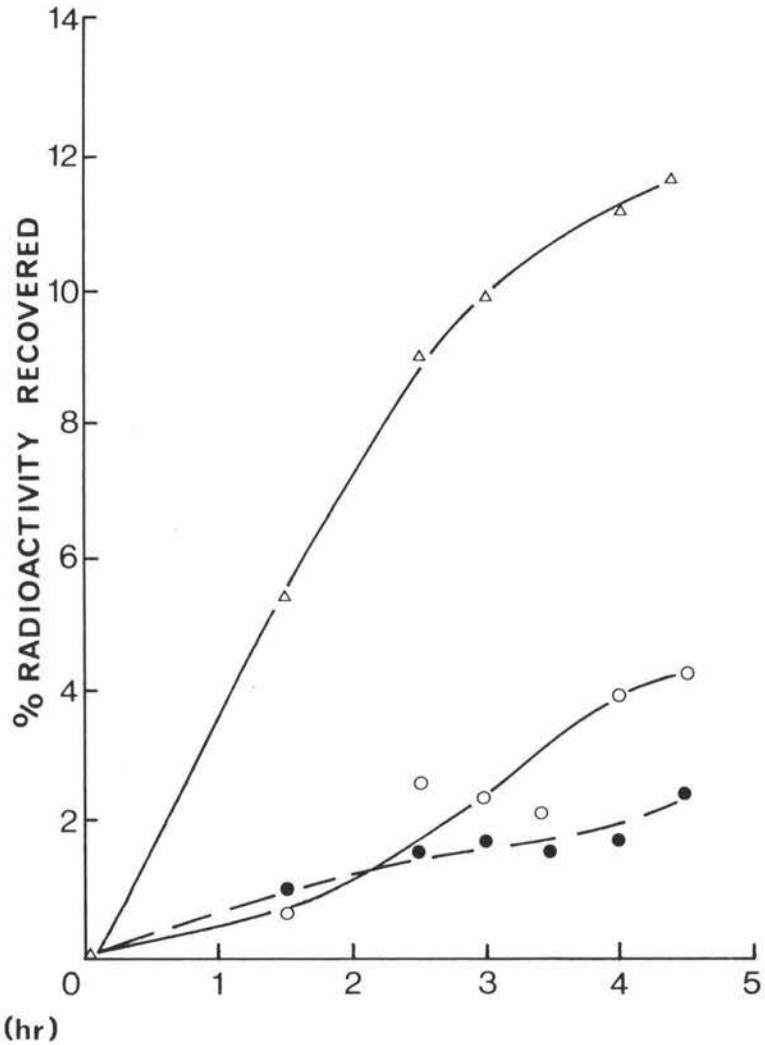


Figure 5.3.B. Percentage recovery of radioactivity in compounds in the ethanol extract (Experiment 3). The compounds are △ glutamate, ○ unknown H and ● unknown G (Figure 5.4).

VI. Identification of Unknowns

About eleven radioactive compounds are evident in the autoradiographs of ethanol extract in Figure 5.2.B. These group into seven major bands as shown in Figure 5.4. Some of these were identified as described below. Four bands are seen on autoradiographs of the supernatant (Figure 5.2.A).

The glutamate was initially identified by co-chromatography of the band J material (Figure 5.4) with authentic glutamate in solvents 1, 3 and 1 (2-D) and 2. A sample was also run on the Beckman amino acid analyser as described in Methods. There were 2 other radioactive compounds on chromatograms of ethanol extracts which were ninhydrin positive. Results from the amino acid analyser showed one to be aspartate. The other was probably glutamine (according to its R_F value on 2-D chromatography in solvents 3 and 1 respectively).

In an attempt to isolate radioactive methylmalonate, an incubation with diethylmalonate as inhibitor was set up. Mountfort (1971) had demonstrated accumulation of methylmalonate under such conditions. 2-D chromatography of the band corresponding to methylmalonate (I) the major radioactive spot (although it contained very low radioactivity, $< 0.5\%$ of the total) coincided with methylmalonate added as a marker.

The UV absorbing band F, corresponding to 5-methylbarbituric acid was eluted and shown to have a maximum absorbance in alkali at 268 nm.

The identity of some of the radioactive compounds are shown in Figure 5.4. Further attempts at identifying the

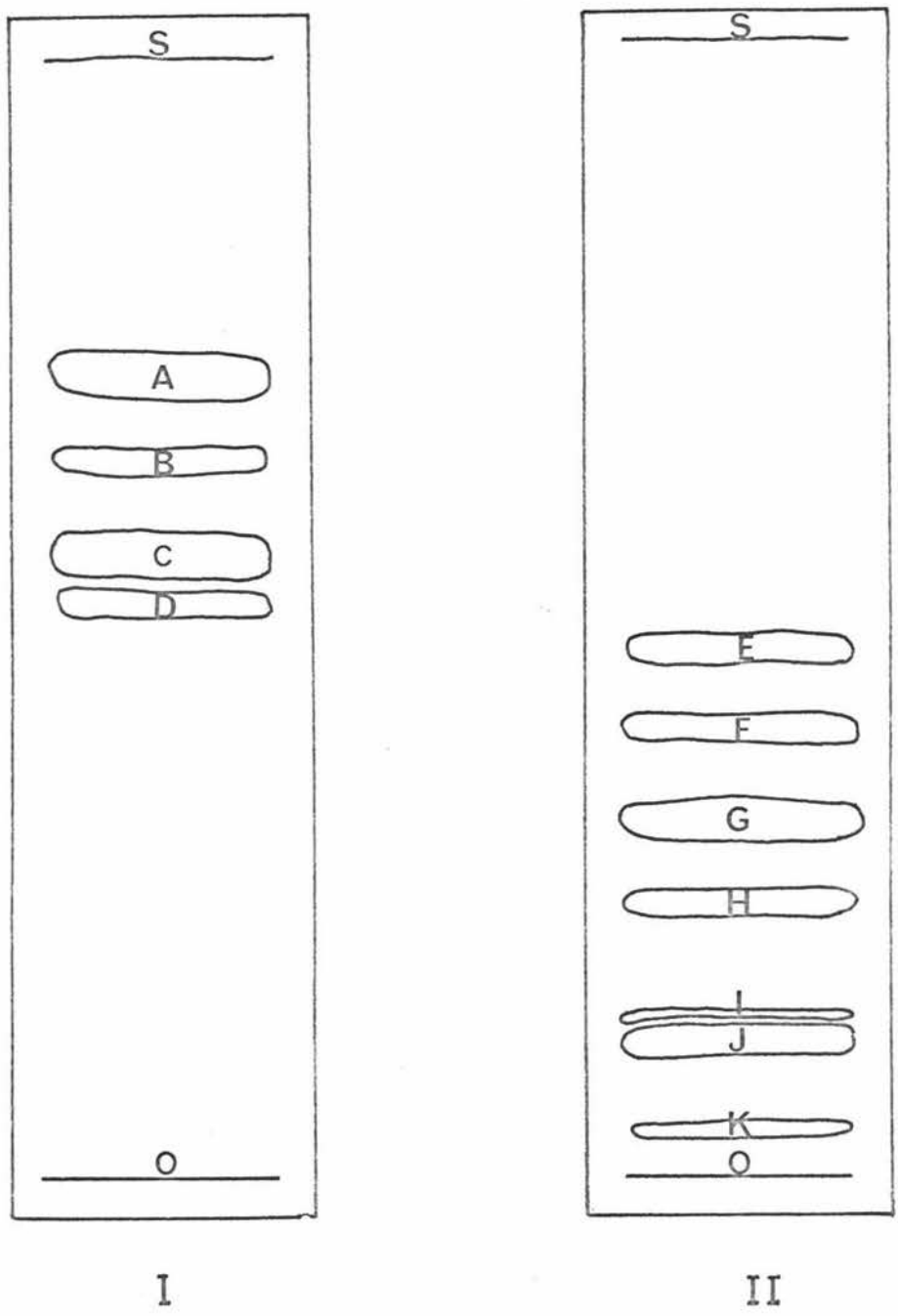


Figure 5.4. I is a typical autoradiograph of a supernatant sample in n-butanol : acetic acid : water solvent and II is that of an ethanol extract in tertiary-butylalcohol : methylethylketone : water solvent.

S is the solvent front and O is the origin.

A is thymine, C is 5-methylbarbituric acid and B and D are impurities present in the thymine sample.

F is 5-methylbarbituric acid, E, G, and K are unknowns (H is probably aspartic acid), I is methylmalonate and J is glutamate.

(Unknowns in Chapter 5 are denoted by different letters of the alphabet from those in Chapters 6 and 7.)

unknowns were done on samples from short term incubation studies, discussed in the following 2 chapters.

VII. Conclusions

In this Chapter methylmalonate has been demonstrated as a product of thymine metabolism in uracil-grown cells (in the presence of diethylmalonate as inhibitor) in confirmation of the results of Biggs and Doumas (1963) and Mountfort (1971). But the very low level of radioactivity recovered in methylmalonate ($< 0.5\%$ of the total radioactivity) suggests that an activated form of methylmalonate, rather than methylmalonate itself, may be the actual intermediate. The methylmalonate detected may in fact be the hydrolysis product of this activated derivative, during the extraction procedure. If an activated derivative of methylmalonate is on the major route of thymine catabolism, the low level of radioactivity in CO_2 reported in this Chapter is readily understood, since the label from the methyl group of thymine would appear in the methyl group of the methylmalonate derivative and thence probably in the methylene carbon atoms of succinate (or its activated derivative), from which it would emerge as CO_2 only slowly (for further discussion see Chapters 1 and 8).

In particular, the results suggest that there is little oxidation of thymine to uracil plus CO_2 (for example by the 5-hydroxymethyluracil pathway of Chapter 1).

A high proportion of the label in the ethanol extract was present in glutamate from the earliest times (90 minutes). Glutamate would not be expected to be one of the earliest labelled intermediates if the methylmalonyl CoA pathway

operates. Its high level of radioactivity may be explained by the very large glutamate pool size in N. corallina cells, this pool probably acting as a 'trap' for radioactivity from any source. Thus, identification of compounds in the experiments reported here is unlikely to contribute much to establishing a pathway for catabolism of thymine or 5-methylbarbituric acid. A semi-steady state had already been reached by 90 minutes, and short term experiments (with high specific activity thymine) must be used for following the appearance and disappearance of intermediates. This approach was adopted for the experiments reported in the next two chapters.

Some caution should be exercised in assessing the results from this Chapter in view of the impurity of the commercial ^{14}C thymine used. The impurities (B and D, in Figures 5.2 and 5.4) together constituted about 8% of the total radioactivity at "zero-time" and it could not be established from the autoradiographs that these compounds were not metabolised. For the major short-term experiments in Chapter 7, the /methyl-^{14}C/ thymine was purified before use, to eliminate these impurities.

CHAPTER 6

CATABOLISM OF [2-^{14}C] THYMINE BY THYMINE-GROWN CELLS

A minor pathway of the oxidative catabolism of thymine and 5-methylbarbituric acid could be via uracil and then barbituric acid, as discussed in the Introduction. Evidence for this pathway could be obtained with [2-^{14}C] thymine. Any label in the methyl group of thymine would presumably be lost as CO_2 in this pathway. [2-^{14}C] thymine on the other hand would allow the label to be traced in uracil and/or barbituric acid.

I. Short term Incubation of Cell Suspension with [2-^{14}C] Thymine (high specific activity)

Two experiments were carried out as described in Methods section II.B.2. In Experiment 1, two incubations were set up at 20°C, each with 1 µCi [2-^{14}C] thymine (and enough carrier thymine to make up to 0.17 µmoles total thymine) and 17 mg. dry weight cells. In one incubation, 15 µmoles barbituric acid was added one minute before the addition of thymine. Each incubation mixture was sampled at 5, 10, 20 and 40 sec. (Preliminary experiments with [methyl-^{14}C] thymine (Chapter 7) had shown that thymine was all gone in 40 sec. under these experimental conditions.)

In Experiment 2, four incubations, each containing 5 µCi [2-^{14}C] thymine (plus enough carrier thymine to make up to 0.17 µmoles thymine) and 17 mg. dry weight cells were set up at 20°C. Unlabelled substrates were added to three of the incubation mixtures, as shown in Table 6.I.

TABLE 6.I

Incubation of Thymine-grown Cells with [2-^{14}C] thymine, Experiment 2

Incubation Mixture	Unlabelled Compound added (15 μmoles)
A	-
B	5-Methylbarbituric acid
C	Barbituric acid
D	Urea

The unlabelled substrates were incubated with the cells at 30°C for three minutes before the addition of thymine. Samples were withdrawn from each incubation mixture at 5, 20 and 60 sec.

This experiment also included additional incubations with the same cell suspension but with [methyl-^{14}C] thymine and these are reported in Chapter 7. [2-^{14}C] thymine was not purified and contained trace amounts of impurities, notably a substance which co-chromatographs with 5-hydroxymethyluracil (see below).

Samples in each experiment were transferred (at the stated times) via a syringe to hot ethanol and incubated for 10 minutes at 80°C to extract the cell contents. No attempts were made to separate the cells from the supernatant.

Excess unlabelled barbituric acid was included in two incubations in order to trap any radioactive barbituric acid that might be formed. In Experiment 2, 5-methylbarbituric acid and urea, respectively were also included in two incubations to act as controls. 5-methylbarbituric acid and urea

are known products of thymine catabolism in N. corallina and addition of excess unlabelled 5-methylbarbituric acid or urea would be expected to lead to some accumulation of label in the respective compounds.

II. Identification of Products

In Experiment 1, 1-D chromatograms revealed three prominent radioactive bands in the first three samples with and without barbituric acid (Figure 6.1). The same three compounds were the principal radioactive spots observed on 2-D chromatograms of the samples from Experiment 2.A, and accounted for about 94% of the total radioactivity (in all three samples).

Co-chromatography with authentic unlabelled compounds established the identity of the three major radioactive compounds as thymine, urea and 5-methylbarbituric acid (in descending order of R_F values in solvent 1). The identity of urea was confirmed by co-chromatography in 2-D and by its disappearance on treatment with urease (Table 6.II). In the treated sample urease has reduced the urea level to 4% of its original level.

Uracil is not fully separable from urea in the chromatographic systems used, but we would expect uracil to be converted to barbituric acid. However, no radioactivity was detected in barbituric acid on co-chromatography. Hence, the residual activity in the urea region after urease treatment is likely to be unchanged urea rather than uracil.

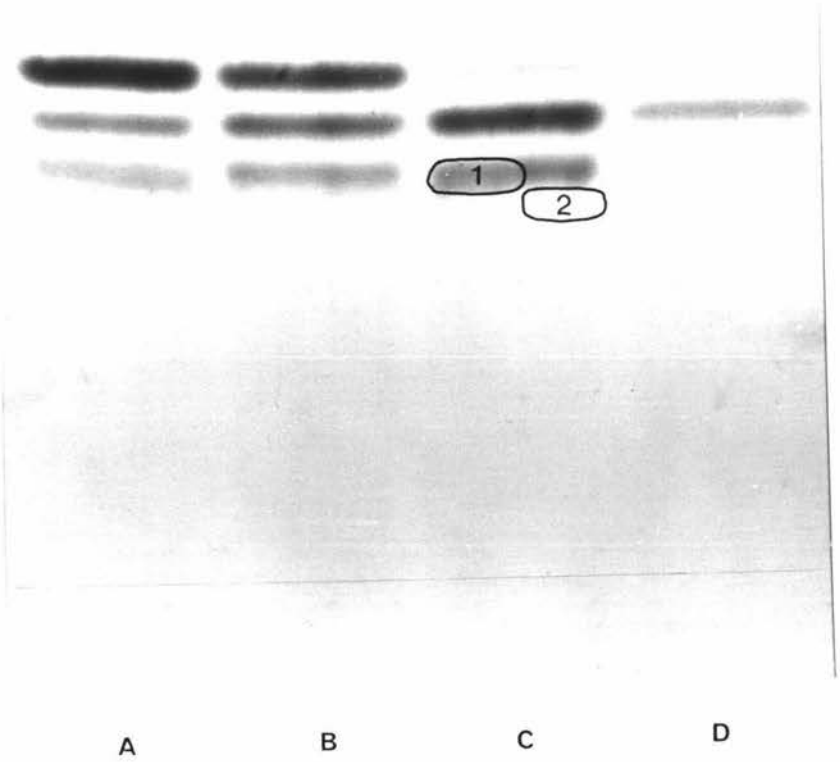


Figure 6.1. Autoradiographs of a series of samples removed at various times during Experiment 1 (in the absence of barbituric acid). A is the 5 sec. sample; B, 10 sec.; C, 20 sec. and D is the 40 sec. sample.

1 and 2 are 5-methylbarbituric acid and barbituric acid markers respectively.

TABLE 6.II

Comparison of the level of radioactivity in urease treated and untreated samples (20 sec. sample from Experiment 1)

Compounds	Treated Sample % of total radioactivity	Untreated Sample % of total radioactivity
Thymine	23	12
Urea	4	56
5-methylbarbituric acid	47	33

On 2-D chromatograms, 5-methylbarbituric acid ran principally as 5-hydroxy-5-methylbarbituric acid (identified by comparison with [methyl-^{14}C] 5-hydroxy-5-methylbarbituric acid, see Chapter 7) and on later 1-D chromatograms in solvent 1, both 5-methylbarbituric acid and 5-hydroxy-5-methylbarbituric acid were present (identified by co-chromatography). Evidently 5-methylbarbituric acid in cell extracts is converted to 5-hydroxy-5-methylbarbituric acid on storage in 60% ethanol at -10°C, as well as on chromatography in phenol : water solvent (see Chapter 8). Later chromatograms showed decomposition of the 5-hydroxy-5-methylbarbituric acid (as reported in Chapter 8).

Several minor spots were observed on autoradiographs. Some of these were deduced to be products of 5-methylbarbituric acid degradation. Two others appear to be impurities present in the [2-^{14}C] thymine while the others are products of the incubation. One of the impurities co-chromatographs with 5-hydroxymethyluracil. It was present in [2-^{14}C] thymine standard to the extent of 1.3% of the total radio-

activity (Table 6.III). 2-D chromatograms of samples taken at 5, 20 and 60 sec. from experiment 2.A showed 5-hydroxymethyluracil with approximately the same percentage of the total radioactivity in all samples (about 1-2%). This suggests that it was neither formed nor metabolised by the cells. Alternatively, but unlikely, it might be formed and used at the same rate. On incubation of cells with [methyl-^{14}C] thymine (Chapter 7) no 5-hydroxymethyluracil was detected. Hence it appears that the 5-hydroxymethyluracil observed in these experiments owed its origin to the impurity in the [2-^{14}C] thymine. But Batt *et al.* (1961) found that 5-hydroxymethyluracil was oxidised by pyrimidine-adapted *N. corallina*.

TABLE 6. III

Percentage radioactivity in [2-^{14}C] thymine and its breakdown products from experiment 2.A (The percentage radioactivity in standard [2-^{14}C] thymine is also included)

Compounds	Percentage Radioactivity			
	Samples			Standard
	5 sec.	20 sec.	60 sec.	[2-^{14}C] thymine
Thymine	81	33	21	98
5-methyl-barbituric acid	6	14	6	
Urea	8	49	74	
5-hydroxy-methyluracil	1.1	2	2	1.3
Unknown D	1.6	.4	.5	

One spot (accounting for approximately 0.1% of the total radioactivity in the 5 sec. sample from Experiment 2.A,

co-chromatographs on 2-D with aspartate. The incorporation into aspartate in 5 sec. is not more than 1% of the level found using [methyl-^{14}C] thymine in an identical 5 sec. incubation (Chapter 7).

Another very faint radioactive spot was found to co-chromatograph with glutamate in the 20 sec. and 60 sec. samples from Experiment 2.A.

A radioactive spot, running close to succinate in 2-D but not yet identified, accounted for most of the label not found in thymine, 5-methylbarbituric acid (or its non-enzymic products) or urea. This is referred to as 'D' in Table 6.III. Re-chromatography with markers shows that it is not succinate but its R_F in solvents 1, 2B and 3 suggests that it may be thymidine.

III. Kinetic Study

Radioactive bands from chromatograms of samples from Experiment 1, without barbituric acid (Figure 6.1) were scraped into scintillation vials and counted. A plot of the total radioactivity versus time in these samples is shown in Figure 6.2.

The graph and the ratio of counts in 5-methylbarbituric acid to urea (Table 6.IV) suggests the following pattern of labelling of the intermediates. Thymine $\longrightarrow$ 5-methyl-barbituric acid $\longrightarrow$ urea $\longrightarrow$ CO_2 (which is then lost to the atmosphere).

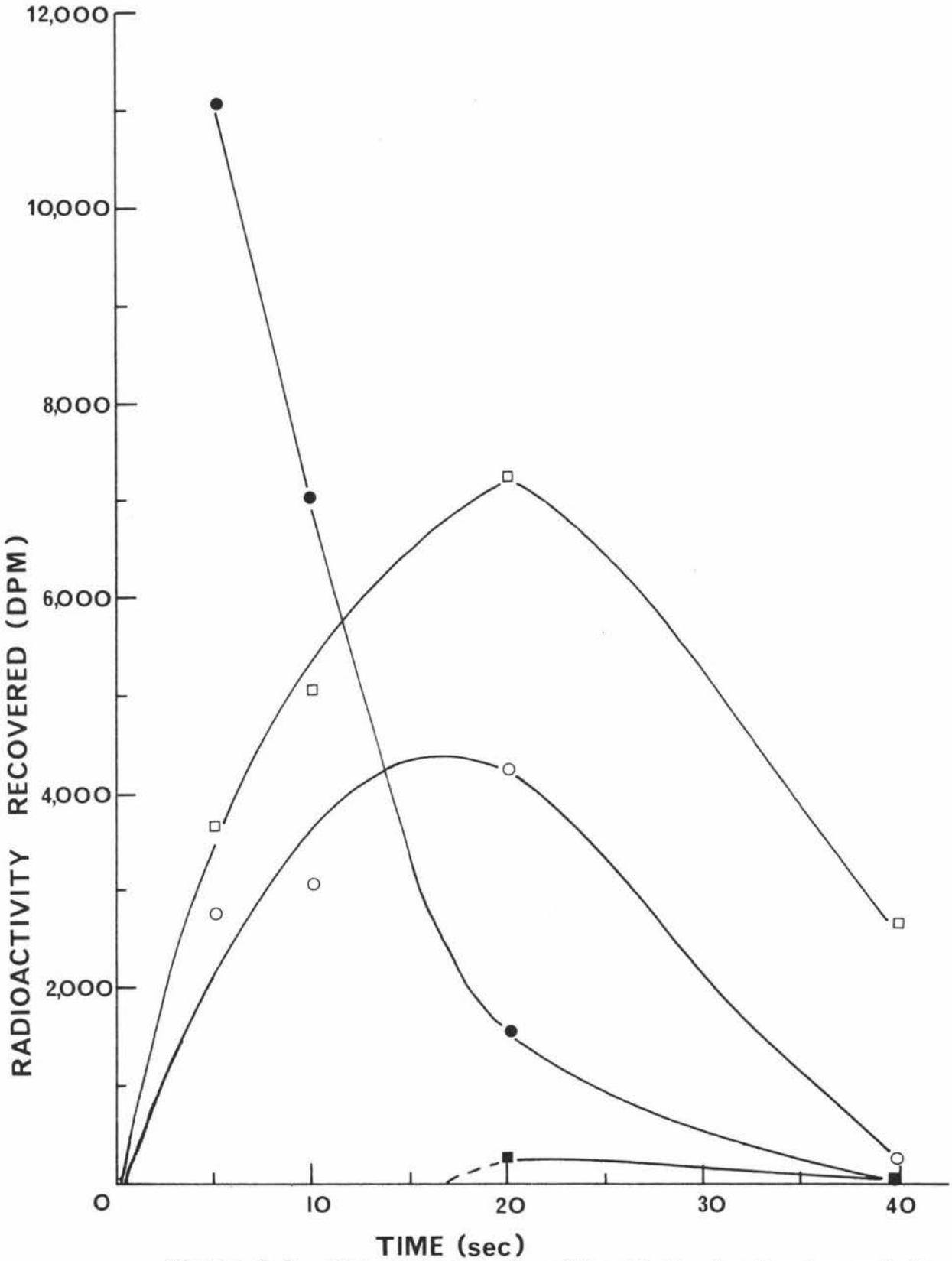


Figure 6.2. The recovery of radioactivity in thymine and its catabolic products from Experiment 1 (incubation in the absence of barbituric acid), in DPM. The compounds are ● thymine, ○ 5-methylbarbituric acid, □ urea and unknown D(■).

TABLE 6.IV

The ratio of radioactivity recovered in 5-methylbarbituric acid to that in urea from Experiment 1 (incubation in the absence of barbituric acid)

Sampling time (sec.)	5-methylbarbituric acid to urea ratio
5	.75
10	.60
20	.59
40	.09

The results of Experiment 2 (determined by end-window counting) are presented in Table 6.III. These support the results of Experiment 1 and they further show that the unknown D is rapidly metabolised. Unknown D becomes labelled from [methyl-^{14}C] thymine (Chapter 7) and is therefore possibly formed from the whole thymine molecule. It does not seem to be derived from 5-methylbarbituric acid, since it disappears before 5-methylbarbituric acid. It may represent an alternative pathway of thymine catabolism; but it is more likely to be thymidine, which might be expected to be in rapid equilibrium with thymine.

IV. Co-incubation with Unlabelled 5-methylbarbituric acid, Barbituric acid and Urea

Unexpectedly, the presence of 5-methylbarbituric acid in excess failed to act as a trap for the radioactive label. The percentage incorporation of label into 5-methylbarbituric acid in incubation 2.B (Table 6.I) at 60 sec. was 18% compared to 20% in incubation 2.A (at 60 sec.).

Similarly, barbituric acid, although it apparently caused a slight inhibition of thymine oxidation, did not affect the distribution of radioactivity between 5-methyl-barbituric acid and urea. No radioactivity was detected in barbituric acid.

On co-incubation with urea, the percentage of total measured radioactivity in urea was slightly elevated, but, since the radioactivity in CO_2 was not measured, it cannot be shown whether the added unlabelled urea was able to trap the radioactivity or not.

A possible explanation for the failure of 5-methyl-barbituric acid to act as a trap for radioactive label was that the 3 minutes pre-incubation had resulted in the total utilisation of the unlabelled substrate by the cells (for further discussion, see Chapters 7 and 8).

CHAPTER 7

CATABOLISM OF /METHYL-^{14}C/ THYMINE BY THYMINE-GROWN CELLS

Several long-term products of thymine catabolism had been determined in Chapter 5. Here, interest is focussed on the short-term products of thymine utilisation by thymine-grown cells. It was hoped that most of the intermediates in the oxidative catabolism of thymine would be isolated and identified.

I. Short-Term Incubation of Cell Suspension with /Methyl-^{14}C/ Thymine (high specific activity)

Freshly harvested thymine-grown cells were incubated with /methyl-^{14}C/ thymine and samples were taken at intervals as described in Methods section II.B.2.

Several preliminary experiments were done using 1μCi labelled thymine, to determine the length of time required for the complete utilisation of thymine. Audoradiographs of the chromatograms of samples showed that almost all of the radioactivity in thymine had disappeared by 40 seconds.

In subsequent experiments, 4-5μCi thymine was used. In Experiment 1, unpurified /methyl-^{14}C/ thymine was incubated with cells in the presence or absence of excess unlabelled succinate (15 μmoles added 1 min. before the thymine). Samples were withdrawn at 10, 20, 40 and 60 sec. and transferred to hot ethanol. It was hoped that radioactivity might be trapped in succinate if the latter was added in excess. The results (see Section IV) show very little difference in the pattern of radioactive incorporation between the samples with

and without succinate. This might be expected on the basis of the findings of Midwinter and Batt (1960) that succinate enters the cell only after a lag period.

In Experiment 2, therefore, one sample (D in Table 7.I) was pre-incubated with succinate until succinate uptake could be demonstrated by the use of the oxygen electrode as described in Methods section II.A.8. In Experiment 2, /methyl-^{14}C/ thymine was purified as described in Methods section I.B. Four incubation mixtures were set up, each with 4μCi thymine (and enough carrier thymine to make a total of 0.17 μmole thymine), and 17 mg. dry weight cells. The contents of the incubation mixtures are indicated in Table 7.I. Incubation was at 20°C.

TABLE 7.I

Incubation of thymine-grown cells with purified /methyl-^{14}C/ thymine, Experiment 2

Incubation mixture	Unlabelled compound added	Pre-incubation time with unlabelled compound
A	None	-
B	5-Methylbarbituric acid	3 min.
C	Succinate	3 min.
D	Succinate	160 min.

Samples were withdrawn from each incubation at 5, 20 and 60 sec. and transferred to hot ethanol.

Aliquots from samples in experiments 1 and 2 were subjected to 1-D chromatography in solvent 1 or 2-D chromatography in solvents 3 and 1 respectively, as described in Methods section II.B.2. Chromatograms were autoradiographed, the radioactive spots were identified as far as possible and

the radioactivity counted as in Methods section II.B.5.

Seven further incubation mixtures were set up with the same cell suspensions as in Experiment 2. Four of these (E-H) had [2-^{14}C] thymine and are described in Chapter 6 (Experiment 2). One (I) had 5μCi [2-^{14}C] thymine plus 2μCi purified [methyl-^{14}C] thymine. The remaining two (J,K) had unpurified thymine and the cells were separated from the supernatants at sampling times.

II. Identification of Labelled Products

Figure 7.I shows the approximate positions of the major radioactive spots on a typical autoradiograph from Experiments 1 and 2.

Tentative identification of as many spots as possible was made by comparison of R_F values with a wide range of markers in solvents 3 and 1. The cell extracts contained sufficient alanine, threonine, glutamine, aspartate and glutamate to give ninhydrin-positive reactions on chromatograms of cell-extracts. These provided internal reference points for comparison of R_F values.

Co-chromatography of radioactive samples with unlabelled carrier in solvents 3 and 1 was then used to establish the identity of these spots, and these are shown in Figure 7.2. Thymine, glutamate and aspartate were readily identifiable where they occurred. Malate was identified on only one chromatogram (5 sec. sample) where carrier malate was present. Its radioactivity was very low. Glutamine was weakly labelled after 60 sec. Others of importance are discussed below.

The identity of glutamate and aspartate was further

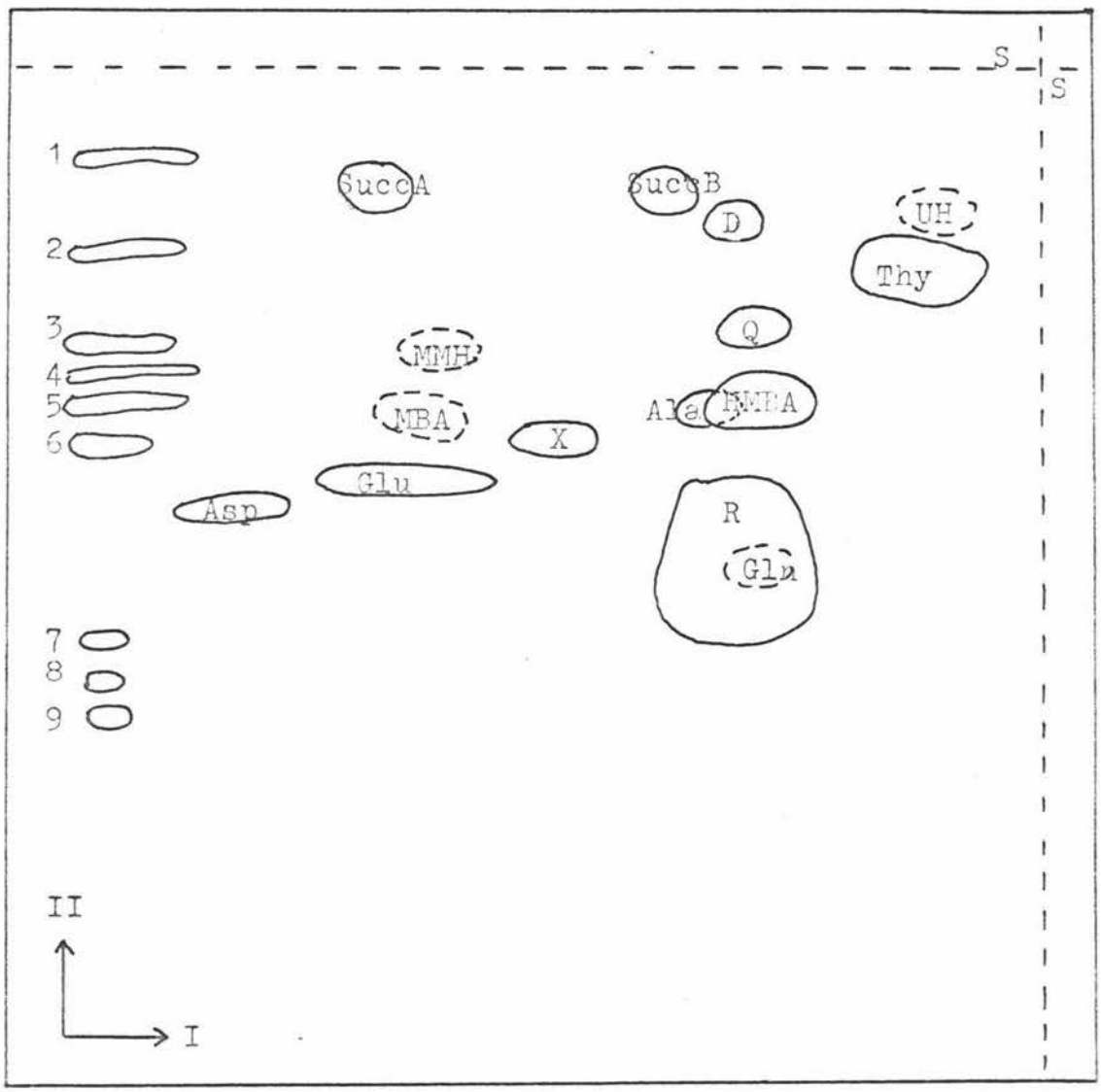


Figure 7.1. A typical autoradiograph of a 2-D chromatogram of the major radioactive spots from Experiments 1 and 2. The solvent in phase I was phenol : water and phase II was n-butanol : acetic acid : water.

Succ = succinate, Thy = thymine, HMBA = 5-hydroxy-5-methylbarbituric acid, MBA = 5-methylbarbituric acid, Asp = aspartate, Ala = alanine, Glu = glutamate and Gln = glutamine.

MMH and UH are $FeCl_3$ reactive spots on treatment of sample (5 sec.) from Experiment 2.A with hydroxylamine.

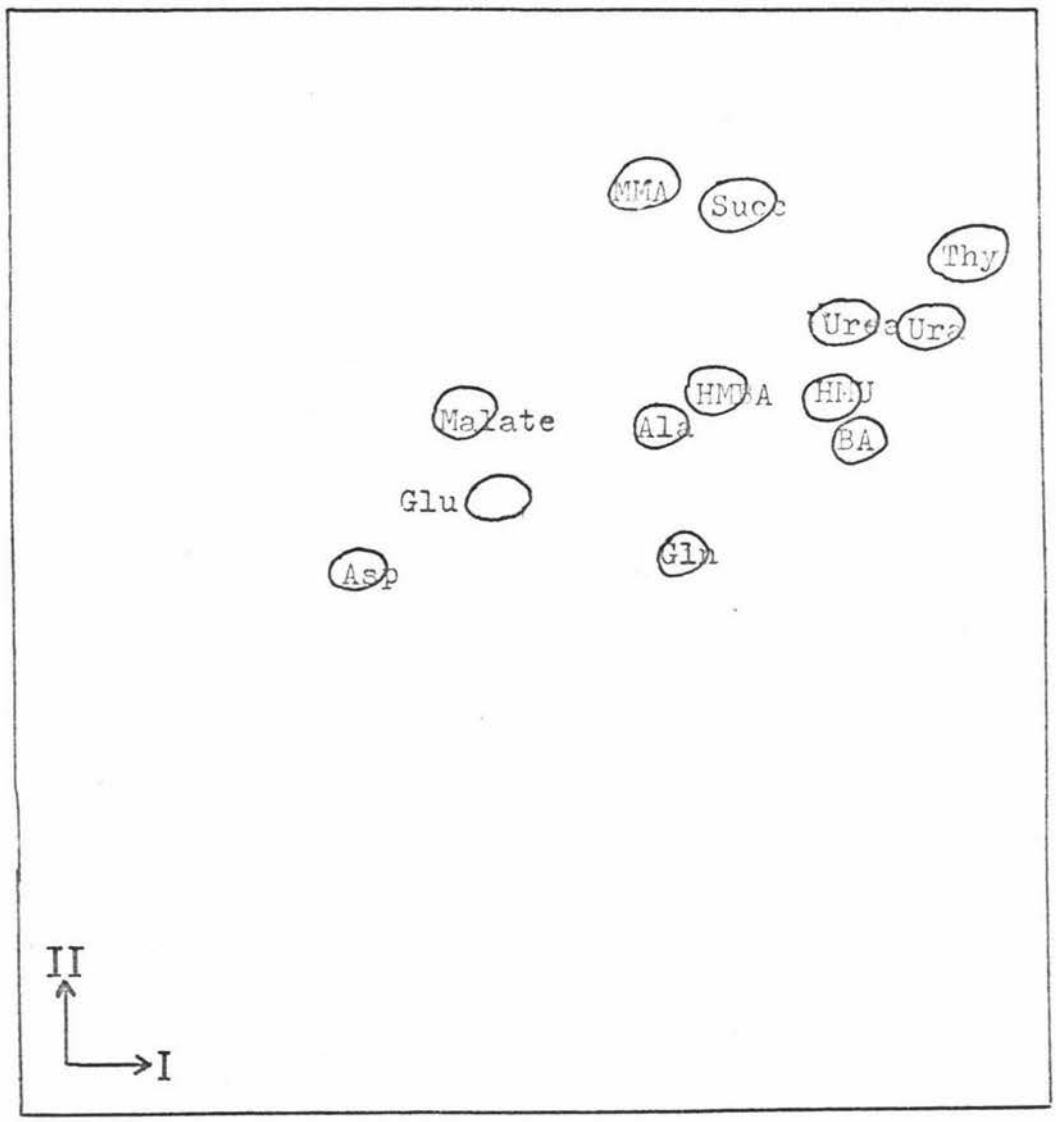


Figure 7.2. A typical 2-D chromatogram of some of the compounds used as markers. The solvent in phase I was phenol : water and phase II was n-butanol : acetic acid : water.

MMA=Methylmalonate, Ura = Uracil, HMU = 5-hydroxymethyluracil, BA = Barbituric acid. Other symbols are as in Figure 7.1.

confirmed by high voltage electrophoresis and passage through the amino acid analyser (Methods II.B.4).

Streaking in solvent 3 was commonly a problem with 2-D chromatography. Succinate and methylmalonate often streaked together but could sometimes be separated by eluting and re-chromatographing in solvent 3.

However, succinate sometimes ran as two distinct spots in solvent 3 (e.g. succ A and succ B on Figure 7.1) and complete separation of succinate and methylmalonate was not always achieved.

On some 2-D chromatograms, unknown D was not separated from succinate. Re-chromatography in solvent 2B provided efficient separation of unknown D from succinate (and methylmalonate).

Satisfactory separation of succinate, methylmalonate and unknown D was achieved only with the 5 sec. sample (experiment 2A). In Tables 7.II and 7.III radioactive counts for all three compounds are reported together as "succinate".

R_F values in solvents 1, 2B and 3 suggest that unknown D may be thymidine.

Alanine and 5-hydroxy-5-methylbarbituric acid were not fully separated on 2-D chromatograms. The combined spot (A) was therefore eluted and re-run in solvent 7, with alanine and 5-hydroxy-5-methylbarbituric acid markers. Chromatograms of spot A in this solvent showed alanine and 5-hydroxy-5-methylbarbituric acid well-separated. A further unidentified radioactive spot 'A_1' on such chromatograms was probably methyltartronylurea, a product of 5-hydroxy-5-methylbarbituric acid.

5-Methylbarbituric acid was unstable on chromatography in solvent 3, but relatively stable in solvent 1. It was identified on 1-D chromatograms run in solvent 1 shortly after the extraction of the cells, but was not detected on most 2-D chromatograms and appeared to be converted to 5-hydroxy-5-methylbarbituric acid on storage. Larger quantities (about 100 μg) of 5-methylbarbituric acid were more stable in solvent 3 and were still detected by their intense UV absorbance after 2-D chromatography (for further discussion, see Chapter 8).

The position of spot X on 2-D chromatograms corresponded approximately to that of 5-methylbarbituric acid. But when an aliquot from a sample in Experiment 2 was co-chromatographed with a large excess of authentic 5-methylbarbituric acid in solvents 3 and 1, the positions of X and 5-methylbarbituric acid did not coincide.

The proportion of X on different chromatograms of the same sample varied greatly and it appears that X was formed non-enzymatically from A on storage. The ratio of radioactivity in X to that in A (X/A) was about 1 : 12 in the earliest chromatograms and increased to 1 : 2 in most later ones.

A product formed non-enzymatically from 5-hydroxy-5-methylbarbituric acid under mild conditions <u>in vitro</u> is methyltartronylurea (Doumas and Biggs, 1962). Spot A was scraped from a chromatogram, taken up in 0.1 cm^3 water with universal indicator, neutralised with K_2CO_3 and left at 30°C overnight. The drop in pH overnight indicated that a reaction had occurred. The mixture was acidified (HCl) to about pH 2

and extracted with acetone, then methanol, then 60% ethanol. Subsequent 2-D chromatography with amino acid markers and autoradiography indicated complete conversion of the 5-hydroxy-5-methylbarbituric acid to two other spots whose R_F values corresponded to X (96% of the total radioactivity) and Q (4%).

When X was eluted from a chromatogram and re-run 2-D with amino acid markers, 95% of the label corresponded in R_F to X, while 5% corresponded to Q. When Q was similarly re-run, it was recovered unchanged. Thus, apparently, we have the reaction sequence 5-hydroxy-5-methylbarbituric acid ⟶ X⟶ Q. Preliminary evidence (Dr. I.G. Andrew) from co-chromatography with authentic methyltartronylurea suggests that compound X is methyltartronylurea. In this case, Q might be a further breakdown product such as lactylurea (Doumas and Biggs, 1962).

To test whether any of the early-labelled compounds were activated derivatives (CoA or otherwise) a sample from Experiment 2.A (taken at 5 sec.) was incubated with hydroxylamine (Methods section EF) and then chromatographed 2-D. The resulting autoradiograph differed from the standard one (without hydroxylamine) in the absence of spot R and the presence of two other spots (MMH and UH on Figure 7.1). When the treated sample was co-chromatographed with impure unlabelled methylmalonyl hydroxamate, the major $FeCl_3$ reactive spot coincided with MMH, while UH showed a minor $FeCl_3$ reaction. To obtain evidence on the identity of UH, it was scraped off a plate, heated 10 min. in 1M HCl at 100°C and the mixture then extracted with ether. On chromatography of the ether extract in solvent 3, all the radioactivity co-chromatographed with succinate. Thus UH is likely to be succinyl hydroxamate.

Spot R appeared to be double on some chromatograms (R_1 and R_2) and since it seemed to give rise to two hydroxamate spots, it is possible that R contained both methylmalonyl CoA and succinyl CoA. UH and MMH contained about equal proportions (each about 3-4%) of the total incorporated radioactivity. Hence methylmalonyl CoA and succinyl CoA may each account for about 3-4% of the total incorporation in the 5 sec. sample.

For further evidence on the nature of R, the region of spot R was scraped off two plates, heated 10 min. in 1M NaOH at 100°C, acidified with HCl (to pH 2) and extracted with ether. On chromatography of the ether extract in solvent 3, the major portion of the radioactivity co-chromatographed with methylmalonate, supporting the hypothesis that R contained methylmalonyl CoA. Succinate was not detected. (The work on R was done by Dr. I.G. Andrew.)

III. Kinetics of Labelling

Aliquots from samples removed at 5, 20 and 60 sec. from Experiment 2.A were chromatographed 2-D in solvents 3 and 1 respectively and autoradiographed. Radioactive spots from these chromatograms were scraped into scintillation vials and the radioactivity counted (see Methods section II.B.5).

The incorporation of radioactivity from [methyl-^{14}C] thymine into different products was expressed as a percentage of the total radioactivity excluding thymine (Table 7.II). Figure 7.3 is a graph of the percentage incorporation of radioactivity into some of the products versus time, from Experiment 2.A.

TABLE 7.II

Percentage incorporation of label from [methyl-^{14}C] thymine, expressed as percentage of the total radioactivity excluding thymine. Samples were from Experiment 2.A and were removed at 5 sec. (1), 20 sec. (2) and 60 sec. (3)

Compounds	Samples		
	1 (%)	2 (%)	3 (%)
'5-methylbarbituric acid'	36	25	1.6
R	8.4	6.8	1.4
'Succinate'	14.5	13.3	1.5
Aspartate	17	25	47
Glutamate	2	5	28
Alanine	1.5	2.9	6.7
Unknowns 1	0.7	0.9	-
2	2	0.7	-
3 + 4	4	4	0.3
5	5	3	4
6	4	4	1
7	2	2	3
8	-	0.4	2

The values for 'succinate' include succinate, methylmalonate and unknown D. The values for '5-methylbarbituric acid' include its non-enzymic breakdown products.

TABLE 7.III

Percentage incorporation of label from [methyl-^{14}C] thymine expressed as a percentage of the total radioactivity excluding thymine. (The radioactivity in thymine was a percentage of the total.)

	Samples							
	2A			2B		2C	2D	
	5 sec.	20 sec.	60 sec.	20 sec.	60 sec.	60 sec.	20 sec.	60 sec.
'5-methylbarbituric acid'	37	23	7	13	25	5	9	6
R	8	6.8	1.4	-	-	-	9.5	-
'Succinate'	15	13	2	58	24	8	42	8
Aspartate	17	25	46	12	16	34	28	42
Glutamate	2	5	27	7	27	41	2	33
Thymine	64	2	0	64	19	4	46	4

The values for 'succinate' include succinate, methylmalonate and unknown D. The values for '5-methylbarbituric acid' include its non-enzymic breakdown products and alanine.

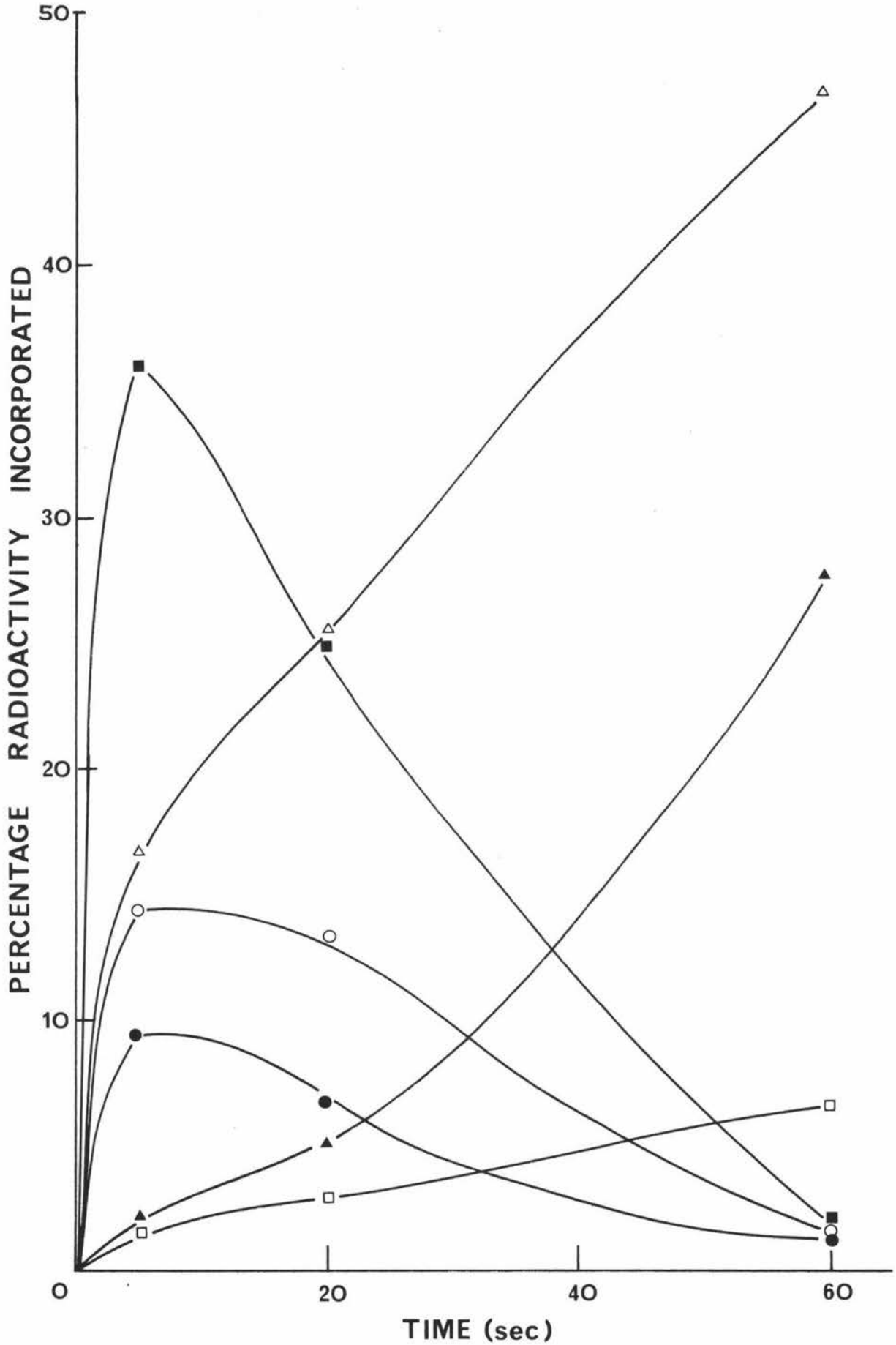
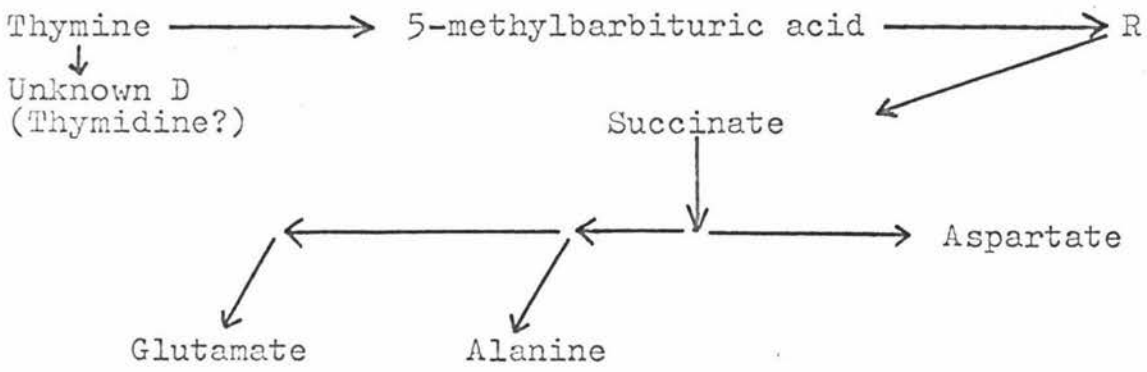


Figure 7.3. Percentage incorporation of radioactivity from [methyl-^{14}C] thymine expressed as a percentage of the total, excluding thymine- into ■ 5-methylbarbituric acid, ○ 'succinate' (plus methylmalonate), △ aspartate, ▲ glutamate, □ alanine and ● unknown R.

Since spots A_1, X and Q are all derived non-enzymatically from 5-methylbarbituric acid, and their relative radioactivities changed with time on storage, their combined radioactivities are presented in Tables 7.II, 7.III and Figure 7.3 as 5-methylbarbituric acid. Succinate, methylmalonate and unknown 'D' are combined as 'succinate' in Tables 7.II, 7.III and Figure 7.3, because of the problems associated with their chromatographic separation. Such separations as were achieved suggest that the radioactivity in methylmalonate was low compared to that in succinate in all samples, while evidence from $[2\text{-}^{14}C]$ thymine incorporation suggests that the level of radioactivity in unknown D was low in all but the 5 sec. samples. Actual counts for the 5 sec. sample indicated that unknown D had about 6% of the incorporated radioactivity, succinate about 7% and methylmalonate 2%.

From the values in Tables 7.II and 7.III, ratios of incorporation of radioactivity into different compounds can be determined, suggesting that incorporation takes place most rapidly into 5-methylbarbituric acid, then R, then succinate and much more slowly into aspartate, alanine and glutamate (in that order). Of the minor compounds, 2 is labelled very early, 1, 3 and 4 more slowly (compared to succinate) and 5, 6, 7, 8 and 9 are later.

Since experimental error has not been evaluated and since the contributions of unknown D and methylmalonate have not been quantitated, the order of labelling is not rigorously established. The results are, however, consistent with the metabolic sequence:



Label accumulates in the amino acids, especially glutamate, since these form a sizeable pool within the cell (Midwinter, 1964).

Evidence above suggests that at least a significant portion of R may be methylmalonyl CoA. The amount of methylmalonate detected was very low, and it is not possible yet to establish whether methylmalonate or its activated (CoA) derivative is formed first.

The above sequence is discussed further in Chapter 8 (Discussion).

IV. Incubation in the Presence of Excess Unlabelled Compounds (which may be intermediates)

In an attempt to trap the radioactive label, incubations with an excess of compounds (which are suspected intermediates) were set up. Cells were incubated with 5-methylbarbituric acid and succinate prior to being used (Experiments 2B, 2.C, 2.D in Table 7.I).

A. Incubation with an excess of 5-Methylbarbituric acid (Experiment 2.B)

The incorporation of radioactivity into all compounds except succinate in this experiment slowed down.

Although there was a large excess of 5-methylbarbituric acid, there was only a very slight inhibition of thymine

utilisation by the cells. In 20 sec. 64% of the original thymine was still present, and 19% after 60 sec. (Table 7.III). Without 5-methylbarbituric acid (Experiment 2.A) the level of thymine was down to 64% in 5 sec. and 2% in 20 sec. Unexpectedly, 5-methylbarbituric acid did not prove to be an effective trap for label, since only 13% of the label incorporated in 20 sec. was in 5-methylbarbituric acid and its non-enzymic derivatives, while 58% was in succinate (identified by co-chromatography and re-chromatography with carrier succinate and methylmalonate). At 60 sec. 24% was still present in succinate.

5-methylbarbituric acid was added to the cells 3 min. before the addition of ^{14}C thymine and it is possible that its metabolism to succinate was largely complete in this time, so that unlabelled succinate was present in excess in the cells, hence providing a trap for radioactive label.

B. Incubation with an excess of Succinate (Experiment 2.C and 2.D)

Again, the incorporation of radioactivity from thymine was slowed down. Succinate did trap the radioactivity to some extent, but less than expected from the excess present. Notably, the succinate trap was less efficient than in Experiment 2.B where unlabelled 5-methylbarbituric acid was the source of succinate (See Table 7.III) and it is possible that the intracellular level of succinate was much lower in 2.C and 2.D due to the permeability barrier of the cell to succinate. In Experiment 2.D, 160 min. pre-incubation with succinate evidently induced some succinate transport but probably most of the succinate still remained outside the cells.

V. Separation of Cells from Supernatant

In those incubations where cells were separated from the supernatant at sampling, supernatant fractions contained radioactivity only in thymine and 5-methylbarbituric acid (plus its non-enzymic degradation products), but no new data emerged from the examination of cell extracts.

CHAPTER 8

DISCUSSION

Growth studies show that N. corallina were able to utilise uracil, barbituric acid, thymine, 5-methylbarbituric acid, methylmalonate and succinate as growth substrate. However, no requirement for adaptation to any substrate was demonstrable except in the case of methylmalonate. This was unexpected and contrasted with the results of Batt (1957) and Batt and Woods (1961). The reasons for the differences are not known.

Oxygen uptake studies failed to show the oxidation of methylmalonate or succinate by pyrimidine-adapted cells. But this may be due to a permeability barrier, since longer term incubation showed that succinate did stimulate oxygen uptake with thymine-grown cells after a 160 minute lag.

The accumulation of 5-methylbarbituric acid was found to be almost quantitative when uracil-grown cells were incubated with thymine, but was rather low when the thymine-grown cells were used. This agrees with the findings of Batt and Woods (1961) and suggests that in uracil-grown cells the enzyme responsible for the breakdown of 5-methylbarbituric acid was not induced, unlike that in thymine-grown cells.

Utilisation of 5-methylbarbituric acid was rapid in thymine and 5-methylbarbituric acid-grown cells but slower in barbituric acid and uracil-grown cells; both the latter showing induction (Figures 3.2A, 3.3). Barbituric acid-grown cells utilised 5-methylbarbituric acid much more readily initially than uracil-grown cells. Perhaps this observation may be related to a common transport system for barbituric

acid and 5-methylbarbituric acid which is not induced in uracil-grown cells. This might also account in part for the slower utilisation of 5-methylbarbituric acid by thymine-grown than by 5-methylbarbituric acid-grown cells.

Much of the early part of this work is concerned with finding conditions that will stabilise 5-methylbarbituric acid in solution. Although 5-methylbarbituric acid is stable in the solid state, its stability on storage varied greatly. Batt and Woods (1961) showed a hundred percent disappearance of 5-methylbarbituric acid (10 μmoles in 0.08M phosphate buffer, pH 7.2) when it was shaken aerobically. But they found it to be relatively stable in the presence of cells under the same conditions.

Results on the stability of 5-methylbarbituric acid in this thesis showed a gradual decrease in absorbance values after several days at room temperature. Solutions tested were made up in water, 0.1M NaOH and a 2% solution of EDTA (which Doumas and Biggs, 1962, suggested might stabilise 5-methylbarbituric acid) and one half of the solutions were stored in the dark while the other half were exposed to day-light. 5-methylbarbituric acid is perhaps more stable in 0.1M NaOH stored in the dark. Payakachat (personal communi-cation) found that 5-methylbarbituric acid was stable in 0.1M NaOH, 0.1M phosphate buffer, pH 7.0, 0.05M Tris/HCl buffer pH 8.85 at a concentration of 5.72×10^{-5}M over a period of 5 days, but that in 0.1M HCl, the absorbance values showed a slow decrease.

Results from Chapter 5, 6 and 7 showed a gradual decrease over several days in the amount of 5-methylbarbituric acid in the cell extracts in 60% ethanol stored at -10°C.

Early 1-D chromatograms of the samples showed only 5-methylbarbituric acid whilst 5-methylbarbituric acid and 5-hydroxy-5-methylbarbituric acid were detected in later chromatograms. Still later chromatograms showed only 5-hydroxy-5-methylbarbituric acid.

Although 5-methylbarbituric acid was found to be stable on 1-D chromatography in n-butanol : acetic acid : water solvent, when chromatographed in phenol : water solvent, it was completely oxidised to 5-hydroxy-5-methylbarbituric acid. This oxidation is apparently concentration dependent, since at high concentrations, on chromatography in phenol : water solvent, there was only a slight oxidation to 5-hydroxy-5-methylbarbituric acid, whereas at low concentrations, all of the 5-methylbarbituric acid was oxidised. Batt and Woods (1961) made a similar observation with acidic chromatography solvents. It is possible that this concentration dependence may account for the variability in the results on the stability of 5-methylbarbituric acid obtained by the various workers (above).

In the course of this work, an attempt was made to extract 5-methylbarbituric acid from growth medium of N. corallina oxidising thymine, by using organic solvents. But 5-methylbarbituric acid was found to be very unstable in organic solvents and the attempt had to be abandoned. Instead it was found that elution of 5-methylbarbituric acid from chromatograms with 60% ethanol yielded better results. So, this method was used to obtain labelled 5-methylbarbituric acid from the incubations of uracil-grown cells with [methyl-^{14}C] thymine.

The instability of 5-methylbarbituric acid on chromatography makes its identification very difficult. On 2-D chromatograms of cell-extracts, all the [^{14}C] 5-methylbarbituric acid decomposed in the first dimension (phenol : water), unless a great excess of unlabelled 5-methylbarbituric acid was present to overcome the concentration effect. The principal decomposition product is 5-hydroxy-5-methylbarbituric acid (identified by co-chromatography in three solvents), but an additional product (unknown X) also appears. The latter was shown to be formed from 5-hydroxy-5-methylbarbituric acid on storage or incubation at pH 7.

At 38°C, pH 7.15 (Doumas and Biggs, 1962), 5-hydroxy-5-methylbarbituric acid is readily converted to methyltartronyl urea. Chromatographic evidence suggests that unknown X is methyltartronyl urea. Unknown X was itself partially converted, on storage, to another unknown, Q.

As mentioned in the Introduction, Batt and Woods (1961) showed discrepancies in the oxygen uptake of thymine-grown cells utilising thymine and 5-methylbarbituric acid, and suggested that an additional pathway of thymine catabolism may operate in thymine-grown cells.

One aim of this project was in determining whether the oxidative catabolism of thymine in pyrimidine-adapted N. corallina operates solely via the 5-methylbarbituric acid pathway. The approach chosen was the utilisation of [2-^{14}C] thymine by thymine-grown cells. Results from these studies showed 5-methylbarbituric acid (and its oxidation product, 5-hydroxy-5-methylbarbituric acid) and urea as the principal radioactive products from [2-^{14}C] thymine. A radioactive compound which was chromatographically indistinguishable from

5-hydroxymethyluracil was detected on 2-D chromatograms of samples from the incubation of [2-^{14}C] thymine. But this appeared to be an impurity in the [2-^{14}C] thymine. The relative amount of radioactivity was found to be the same as that detected on a 2-D chromatogram of standard [2-^{14}C] thymine. Furthermore, no label was found in 5-hydroxymethyluracil when [methyl-^{14}C] thymine was used as the substrate. Hence, 5-hydroxymethyluracil is not a metabolite of thymine under the experimental conditions reported here. No uracil or barbituric acid were detected on co-chromatography with authentic marker compounds. Hence it appears that neither the 5-hydroxymethyluracil pathway nor any other pathway involving uracil or barbituric acid operates in thymine-adapted N. corallina.

No evidence was found for radioactivity in dihydrothymine or β-ureidoisobutyrate when extracts were co-chromatographed with authentic markers. Thus it is unlikely that the reductive pathway for thymine catabolism was operating in the cells.

Another pathway of thymine metabolism is suggested by the presence of a radioactive compound (unknown D) which showed a more rapid turnover than 5-methylbarbituric acid. This cannot be an intermediate in the formation of 5-methylbarbituric acid, since no such intermediate exists in the reaction catalysed by thymine oxidase. The chromatographic properties of this unknown compound suggest that it may be thymidine. This would presumably be formed by the action of thymidine phosphorylase. The rapid subsequent disappearance of thymidine (see Chapter 6) would be explained by the ready reversibility of this reaction or by the subsequent removal

of the thymidine from the ethanol-extractable pool by phosphorylation and assimilation into DNA.

The time-course experiment with [2-^{14}C] thymine suggests the following order of labelling of intermediates: Thymine ⟶ 5-methylbarbituric acid ⟶ urea ⟶ CO_2. Very little label remained after the disappearance of urea, suggesting that urea was converted to CO_2 which was then lost to the atmosphere. Trace levels of radioactivity were found to co-chromatograph with aspartate and glutamate, possibly as a result of fixation of CO_2 derived from the urea.

From the above evidence, it seems likely that the pathway via 5-methylbarbituric acid is the sole pathway of thymine oxidative catabolism in N. corallina. And so, the results of Batt and Woods (1961) on the discrepancies of oxygen uptake by thymine-grown cells oxidising thymine and 5-methylbarbituric acid cannot be explained by invoking another pathway.

The next part of this investigation is concerned with the products of 5-methylbarbituric acid catabolism. The use of cell-extracts in incubations with [methyl-^{14}C] 5-methylbarbituric acid resulted in the formation of an unidentified radioactive compound (Chapter 4). This unknown is the only labelled compound observed as the product of the incubation of cell-extracts with 5-methylbarbituric acid, and this process is CoA dependent. In these preliminary studies, non-enzymic breakdown products of 5-methylbarbituric acid could not be eliminated from the incubation mixtures.

No further work was done with cell-extracts, since initial experiments with [methyl-^{14}C] thymine and whole cells indicated that this approach might be more profitable.

Following the approach of Mountfort (1971), uracil-grown cells were incubated with low specific activity [methyl-^{14}C] thymine, and studies of long-term products carried out. The rationale for this approach was that uracil-grown cells would have uracil-thymine oxidase and hence should rapidly convert thymine to 5-methylbarbituric acid, but that enzymes for 5-methylbarbituric acid utilisation would not be highly induced (see Introduction). Hence, intermediates might accumulate and be observed.

In these long-term studies, the major radioactive products were 5-methylbarbituric acid, aspartate and glutamate. A large pool of the two amino acids exist in N. corallina and this serves as a 'trap' for the radioactive label, hence the relatively high amounts of radioactivity recovered in both aspartate and glutamate. Only a trace was found of a radioactive compound which co-chromatographs with methylmalonate. If methylmalonate is the immediate product of 5-methylbarbituric acid metabolism, then it should be found amongst the earlier labelled products of thymine or 5-methylbarbituric acid metabolism.

It seems likely that the metabolism of the immediate products of 5-methylbarbituric acid was too rapid for any to accumulate at significant levels. The pattern of accumulation in these long-term experiments probably approached a steady-state condition, the extent of incorporation into any compound reflecting its pool size. No changes in the relative incorporation with time were apparent, apart from the disappearance of thymine and the accumulation of others (see Figures 5.2A and 5.2B). It therefore seemed advisable to study incorporation over a much shorter time, using a pulse

of high specific activity thymine. Such an experiment is reported in Chapter 7. Thymine-grown cells were used in this case to ensure rapid metabolism of the 5-methylbarbituric acid, and sampling was made as rapid as possible in order to follow the kinetics of appearance of label in different compounds.

Of the products of [methyl-^{14}C] thymine catabolism; aspartate, glutamate, alanine, 5-methylbarbituric acid, 5-hydroxy-5-methylbarbituric acid, succinate and methylmalonate were tentatively identified by co-chromatography (and in the case of aspartate and glutamate, also co-electrophoresis) with authentic unlabelled compounds. Malate was also identified on one chromatogram (5 sec. sample), and glutamine was found to be labelled at a very low level (60 sec. sample). Other radioactive products were unidentified and these are discussed below.

The time course experiments show that, apart from unknown D, which may be thymidine, the label from thymine appears first in 5-methylbarbituric acid (chromatographed 2-dimensionally as spots A, X and Q, Figure 7.1), spot R (possibly methylmalonyl CoA and succinyl CoA, see later) and a further unidentified spot (unknown 2). Also rapidly labelled, but apparently following the above are succinate and methylmalonate and three other unidentified spots (unknowns 1, 3, 4). Other compounds all show slower incorporation of label. It is noteworthy that for the incorporation of label into amino acids, that into aspartate was most rapid, then alanine, followed by glutamate.

The above order of labelling may be explained by the scheme outlined in Figure 8.1.

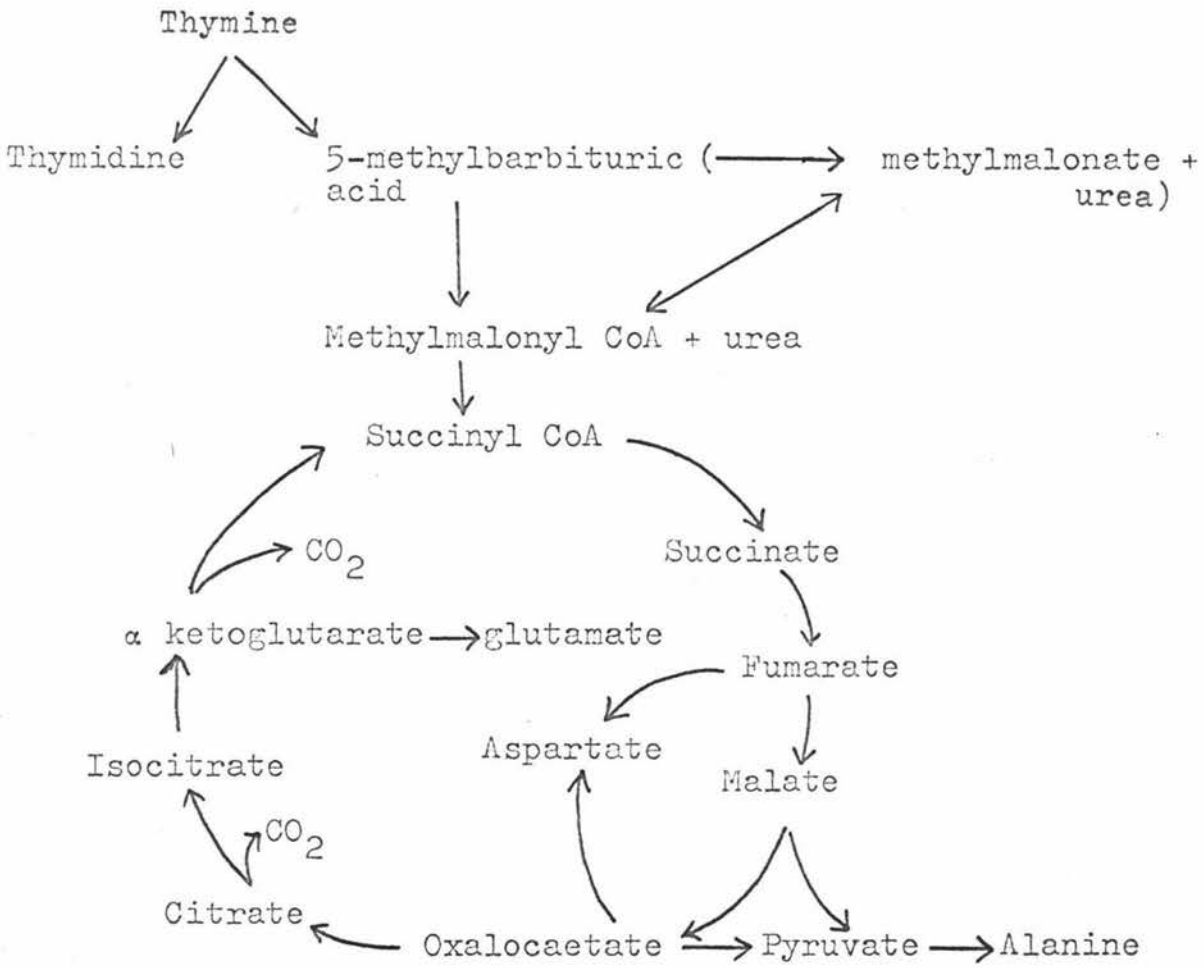


Figure 8.1. A tentative scheme of labelling of products formed from [methyl-^{14}C] thymine during its catabolism by pyrimidine-adapted N. corallina.

This scheme involves the participation of the intermediates; fumarate, oxaloacetate, citrate, isocitrate and α-ketoglutarate, which were not identified, as well as the other identified products.

The above representation explains the order of the incorporation of label into some of the identified compounds; for example: methylmalonate and succinate were labelled before the amino acids; aspartate labelled before glutamate; and it also explains the low levels of radioactivity recovered in CO_2 (Chapter 5).

The finding that methylmalonate and succinate were amongst the early labelled products of thymine catabolism agreed with the results of Biggs and Doumas (1963) and Mountfort (1971) and supports the participation of 5-methylbarbituric acid, methylmalonate and succinate (or their CoA derivatives) as intermediates in the oxidative catabolism of thymine.

In the case of the breakdown of barbituric acid, it has been suggested (Pearce, 1974) that the *in vitro* formation of malonate may be an artifact of the cell extraction procedure, while malonyl CoA would be the normal *in vivo* product. Indeed, Pearce (1974) did demonstrate some malonyl CoA formation *in vitro* and it is also possible that the immediate *in vitro* product is malonyl CoA but that this is rapidly hydrolysed to malonate and CoA. The situation may be similar in the catabolism of 5-methylbarbituric acid and in fact methylmalonyl CoA rather than methylmalonate may be the metabolite formed from 5-methylbarbituric acid *in vivo*.

In the search for methylmalonyl CoA, samples were treated with hydroxylamine, to produce the hydroxamate.

2-D chromatography of these treated samples showed the disappearance of a diffuse radioactive spot R (which possibly included two spots R_1 and R_2); and the appearance of two other radioactive spots, which gave a positive reaction with $FeCl_3$ spray (a positive hydroxamate reaction). One of these hydroxamate spots co-chromatographed with methylmalonyl hydroxamate (synthesised in this laboratory by Dr. I.G. Andrew). The other, on acid hydrolysis, yielded a spot which co-chromatographed with succinate. Thus it seemed probable that R contained active forms of methylmalonate and succinate, possibly methylmalonyl CoA and succinyl CoA, respectively. On alkaline hydrolysis of R, a radioactive spot was obtained which co-chromatographed with methylmalonate. But before R (or its constituents R_1 and R_2) could be positively identified as methylmalonyl CoA and/or succinyl CoA, a comparison with authentic methylmalonyl CoA and succinyl CoA will be necessary.

In the co-incubation with cold succinate, the accumulation of radioactive label in succinate was not as high as expected (although it was greater than that in the control). If succinate is an intermediate, then the label should be 'trapped' by the presence of a large excess of succinate in the incubation mixture. The cells used in this experiment appeared to be oxidising succinate (from oxygen electrode results) but the amount entering the cells may have been insufficient to trap the radioactive label effectively.

In the co-incubation with cold 5-methylbarbituric acid, there is a slight inhibition of thymine utilisation and the incorporation of radioactivity in all products except succinate slowed down. This may be due to some form of competition of thymine with 5-methylbarbituric acid.

Evidently, not all of the added 5-methylbarbituric acid competed effectively with thymine, otherwise a much lower ^{14}C uptake would have been observed (see Chapter 7). The increased accumulation of label in succinate (and methylmalonate) and the decreased accumulation in 5-methylbarbituric acid or its breakdown products observed in this experiment may be explained in the following manner. It may be that the conversion of unlabelled 5-methylbarbituric acid to succinate was virtually complete in the 3 minutes prior to thymine addition and the succinate so formed then acted as a trap for the label.

The evidence presented above seems to point to the possibility of the scheme outlined in Figure 8.1 operating during oxidative catabolism of thymine by pyrimidine-adapted N. corallina. It now awaits the identification of some of the unknowns (shown in Figure 7.1) either to support or to disprove this possibility. With the present identification and the order of labelling in 5-methylbarbituric acid, methylmalonyl CoA (?), succinate, aspartate, and glutamate known, the above scheme seems a logical representation of the in vivo formation of intermediates in the oxidative catabolism of thymine.

The evidence of this thesis strongly suggests that the pathways of thymine catabolism involving dihydrothymine and β-ureidoisobutyrate (Figure 1.1), 5-hydroxymethyluracil (Figure 1.3), or uracil or barbituric acid (Figure 1.2) do not operate in thymine-adapted N. corallina under the experimental conditions used here, and that catabolism of thymine is almost entirely via 5-methylbarbituric acid and an activated derivative of methylmalonate. Further support for the suggested pathway could be obtained by measuring specific activities of the suspected intermediates.

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