PYRIMIDINE CATABOLISM IN NOCARDIA CORALLINA

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

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SECTION 1

INTRODUCTION

CHAPTER 1

PYRIMIDINE CATABOLISM IN MICRO-ORGANISMS WITH SPECIAL REFERENCE TO NOCARDIA CORALLINA

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

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

1. Oxidative catabolism of pyrimidines.

A. Barbituric acids as intermediates.

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

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

Lara (1952b) showed that cell-free extracts derived from

organisms grown on either thymine, uracil or barbituric acid were active towards thymine and uracil in oxidising these compounds to the corresponding barbituric acids.

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

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

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

Hayaishi and Kornberg (1952) working with crude enzyme preparations of uracil-thymine oxidase from strains of Mycobacterium and Corynebacterium, isolated and identified both the oxidation products from thymine and uracil as 5-methylbarbituric acid and barbituric acid respectively.

The oxidation of pyrimidines to the corresponding barbituric acids is shown as part of an overall scheme for pyrimidine catabolism in Figure 1.

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

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

* Homologous cells are those oxidising the pyrimidine on which they were grown.

Figure 1. Scheme for the oxidative catabolism of pyrimidines with barbituric acids as intermediates.

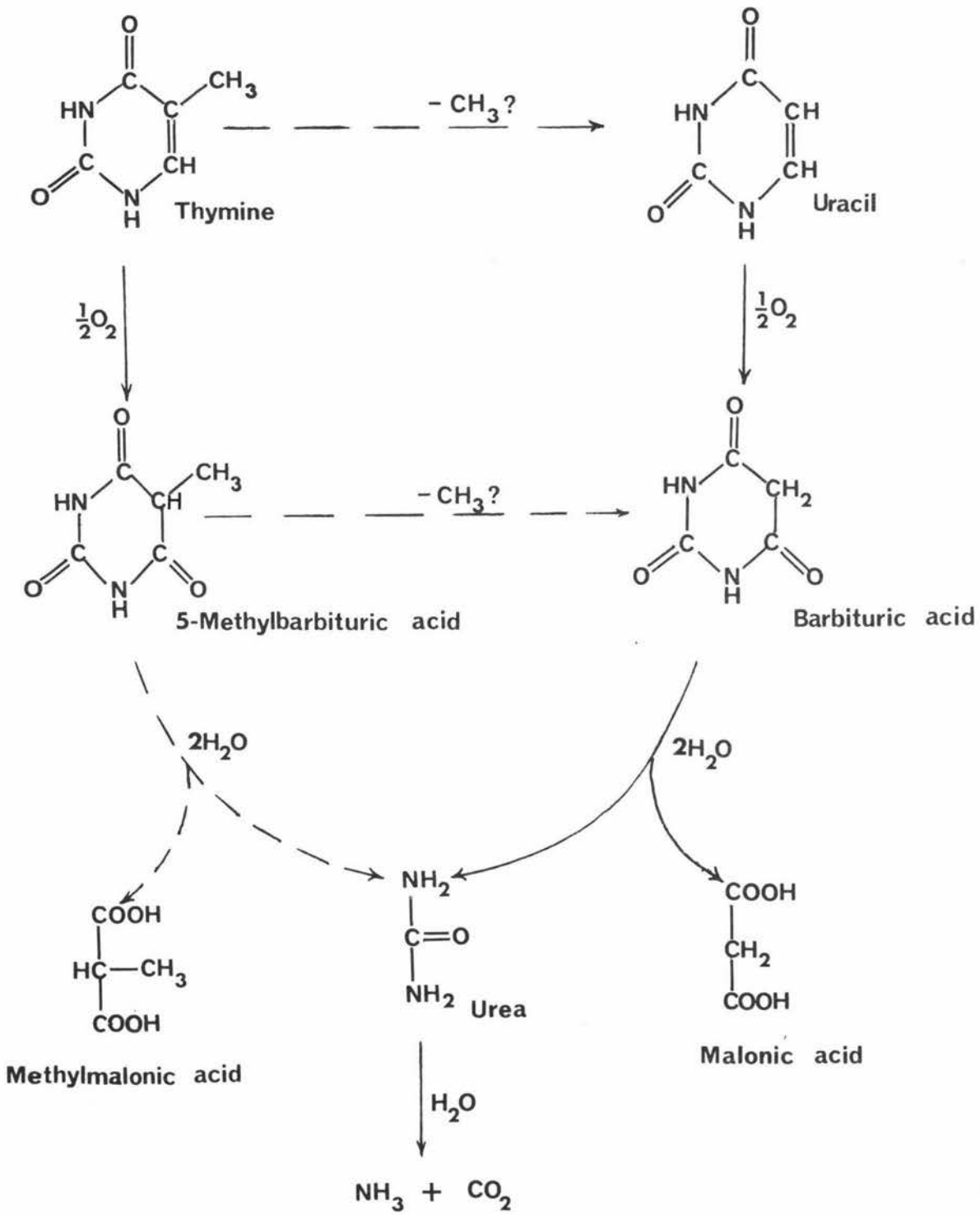


TABLE 1

Total oxygen consumption during oxidation of various pyrimidines.

(After Batt and Woods, 1961)

	Oxygen Uptake µmole/µmole pyrimidine for organisms grown on		Theoretical Oxygen Uptake µmole/µmole pyrimidine
Substrate	Uracil	Thymine	
Uracil	1.01	1.48	2.5
Barbituric acid	0.87	1.03	2.0
Thymine	2.07	2.05	4.0
5-methylbarbituric acid	1.47	0.88	3.5

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

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

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

Lara (1952) found that extracts obtained from N. corallina grown on thymine were active in hydrolysing barbituric acid with the formation

of 1 mole of CO_2, two moles of NH_3, and one mole of malonic acid/mole of substrate (Figure 1). Urea, which accounted for the total CO_2 and NH_3 production was established as a direct product of barbituric acid hydrolysis. Hayaishi and Kornberg (1952) working with extracts of uracil adapted organisms of a strain of Mycobacterium likewise established that barbituric acid was hydrolysed to malonic acid and urea. These workers partially purified the barbiturase enzyme using a technique of protamine sulphate fractionation, followed by cation exchange column chromatography.

Batt and Woods (1961) expressed doubt that free malonic acid was likely to be an intermediate in the breakdown of barbituric acid in intact cells. These workers showed that although barbituric acid could be converted anaerobically to malonic acid, CO_2 and NH_3 by enzyme preparations of N. corallina, malonic acid could not be degraded by whole cells under the same conditions. Such results were also supported by those of Hayaishi and Kornberg (1952) in which it was shown that whole cells of a uracil adapted strain of Mycobacterium could not attack barbituric acid under anaerobic conditions, as opposed to cell-free extracts which could.

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

From their results Hayaishi and Kornberg (1952) proposed that the absorption of barbituric acid into the cell was energy requiring,

and could only be facilitated under aerobic conditions. The poor utilisation of malonic acid by uracil adapted organisms was attributed to a permeability barrier. Thus malonic acid was not excluded as a likely product of barbituric acid degradation.

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

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

Because bacterial cell-free extracts were found to be inactive

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

Biggs and Doumas (1962) assessed the possible roles of 5-hydroxy-5-methylbarbituric acid and methyltartronylurea as intermediates of 5-methylbarbituric acid catabolism in Corynebacterium. Neither compound supported growth of the organism. Furthermore, urea, which is normally an end product of thymine catabolism, was not detected in the media. From such results it was considered unlikely that 5-hydroxy - 5-methylbarbituric acid or methyltartronylurea were intermediates of 5-methylbarbituric acid catabolism. It did not necessarily follow however, that these compounds could not be enzymatically formed from 5-methylbarbituric acid.

In 1963 Biggs and Doumas working with Corynebacterium provided evidence for 5-methylbarbituric acid being hydrolysed to methylmalonic acid and urea. When 5-methylbarbituric acid - 2 - C^{14} was used as the substrate, the isolated urea was not only radioactive, but also had the same specific activity as the starting material. The activity of the isolated methylmalonic acid was only 2% of that of 5-methylbarbituric acid - 2 - C^{14}. However it was found by chromatography that the methylmalonic acid was contaminated with other labelled compounds, and because of the small amount of acid isolated, a further purification was not attempted.

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

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

C. The oxidation of thymine to 5-hydroxymethyluracil.

5-hydroxymethyluracil has been established as a product of thymine oxidation in rat liver systems. (Fink, Cline, Henderson and Fink, 1956).

In microbial systems the only reported cases of thymine conversions to 5-hydroxymethyluracil have been shown to occur in Neurospora.

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

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

II. The reductive catabolism of pyrimidines

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

These studies were a continuation of earlier work on the nature of cytosine breakdown in yeasts. (Hahn and Haarmann, 1926; Chargaff and Kream, 1948; DiCarlo, Schultz and McManus, 1951.)

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

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

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

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

β-Aminopropionamide, β-ureidopropionic acid, hydro-orotic acid, and hydrouracil - 5 - carboxylic acid, were tested as possible degradation products of hydrouracil. Hydrouracil-5-carboxylic acid did not support the growth of either yeast and so was eliminated as a catabolic product of hydrouracil. β-ureidopropionic acid and β-Amino propionamide served as excellent nitrogen sources for T. utilis, but per-

mitted no growth of S. cerevisiae. It was suggested that both compounds were converted to β-alanine which could also be utilised by T. utilis. Dihydro-orotic acid was shown to be utilised by both yeasts, and this led DiCarlo, Schultz and Kent to suggest it was possibly produced from dihydrouracil.

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

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

Campbell (1957b) working with a strain of Clostridium uracilicum identified dihydrouracil, and β-ureidopropionic acid as intermediates in the conversion of uracil to β-alanine, CO_2, and NH_3. The scheme for the degradation of uracil according to Campbell is shown in Figure 3. Such findings would also offer support to the possibility that in T. utilis, β-ureidopropionic acid could serve as an intermediate in the reductive catabolism of uracil instead of dihydro-orotic acid.

Figure 2. Suggested scheme for the breakdown of cytosine in yeasts. (after DiCarlo, Schultz and Kent, 1952.)

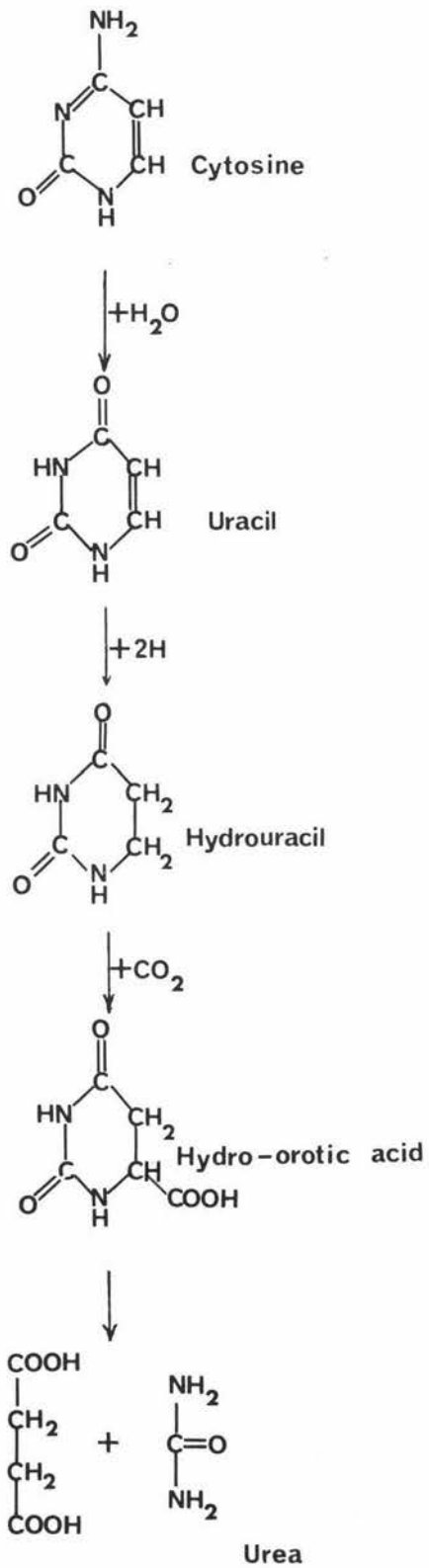
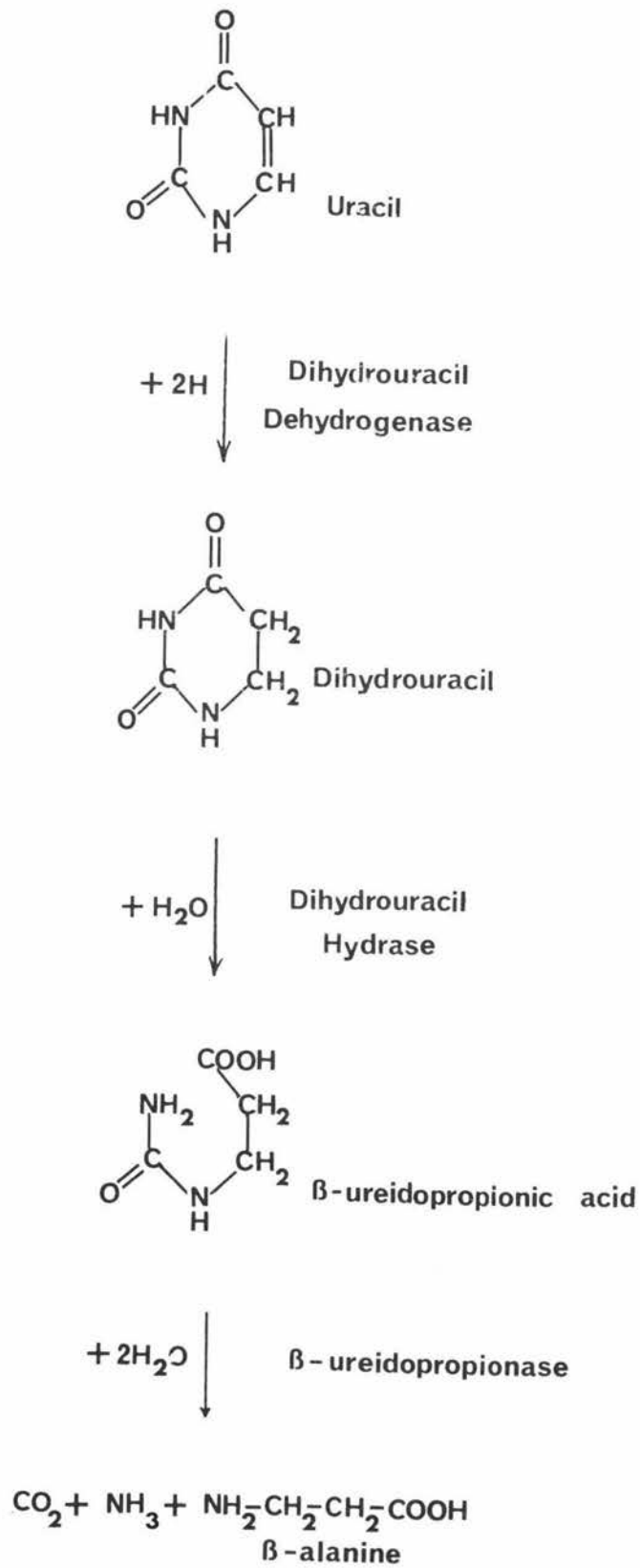


Figure 3. Scheme for the reductive catabolism of uracil.
(After Campbell, 1957; Kraemer and Kaltwasser, 1970.)



Campbell (1957c) isolated and partially purified the dihydrouracil dehydrogenase which catalysed the reductive step in uracil catabolism. The results of Campbell (1957) were further substantiated by the findings of Kraemer and Kaltwasser (1970a). These workers demonstrated with cell-free extracts of cytosine grown Hydrogenomonas facilis, that cytosine was converted to uracil, which in turn was converted to β-alanine, CO_2 and NH_3 via dihydrouracil and β-ureidopropionic acid. Barbituric acid and urea were not detected. Cytosine deaminase, dihydrouracil dehydrogenase, dihydrouracil hydrase, and 3-ureidopropionase but not uracil oxidase, were demonstrated in cell-free extracts.

Kraemer and Kaltwasser (1970b) carried out further studies on the metabolism of cytosine and uracil in wild type and mutant strains of H. facilis. Mutant strains unable to utilise uracil as a nitrogen source were derived by treatment of organisms with 1-methyl-3-nitro-1-nitroso guanidine and incubation at various concentrations of penicillin.

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

The results from these studies indicated that uracil and thymine

were utilised by a non-specific dehydrogenase and that both dihydro-uracil and dihydrothymine were acted upon by a non-specific hydrase.

SECTION II

EXPERIMENTAL

CHAPTER 2

THE AIM OF THE PRESENT INVESTIGATION

The present study was planned as both a confirmation and extension of an investigation initiated by Batt and Woods in 1951, which resulted in a publication in 1961 on the catabolism of pyrimidines in N. corallina. The investigation was directed mainly towards the study of thymine catabolism, and experiments were planned to elucidate the pathway or pathways by which the compound is degraded.

Emphasis was to be placed on an approach involving the use of metabolic inhibitors in accumulating intermediates of thymine catabolism with C^{14} labelled substrates. The effects of these inhibitors were to be examined with thymine-grown organisms, however it was intended that such studies should also be extended to uracil-grown organisms where appropriate.

CHAPTER 3

MATERIALS AND METHODS

1. Reagents.

All chemicals used in this investigation were obtained from British Drug Houses Ltd. (Poole, England), May and Baker Ltd. (Dagenham, England) or Koch-Light Laboratories Ltd. (Colnbrook, England) except for the following:

a. Diazomethane which was synthesised by the method of Schlenk and Gellerman (1960) (See Appendix IIA).

b. Diethyl methylmalonate and methylmalonic acid, obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.

c. 5-hydroxymethyluracil, synthesised from uracil by the method of Fink, Fink and Cline (1959) (See Appendix IIB).

d. 5-methylbarbituric acid, prepared by a method based on that used by Holmberg (1945) (See Appendix IIC).

e. 5-hydroxy-5-methylbarbituric acid which was synthesised from 5-methylbarbituric acid by the method described by Biggs and Doumas (1962) (See Appendix IID).

f. POPOP (1,4 bis [2 - (5-phenyloxazolyl)] - benzene, and PPO (2,5 diphenyloxazole), obtained from Nuclear Enterprises (G.B.) Ltd., Edinburgh, Scotland.

g. Thymine-methyl-C^{14} (specific activity 3.52 mc/mM) supplied by New England Radiochemical Centre, Boston, Mass., U.S.A.

II. Methods

A. Analytical techniques

1. Dry weight estimation

The dry weight content of a cell suspension was determined by reference to a standard curve calibrated in terms of dry weight against optical density (O.D.) at 490 nm. Separate standard curves were calibrated for glucose, thymine, and uracil grown cells respectively.

For the construction of the standard curve cells were taken at the termination of the log phase at growth, and harvested by centrifugation (2000x*g*:10 min). The pellets were washed in distilled water, and resuspended in an appropriate volume from which dilutions were made. The optical densities of a series of suspensions at different dilutions were recorded. For each suspension, dry weights were obtained by dehydrating cells in preheated, preweighed pyrex watch glasses and then weighing. Drying was effected at 100°C in a drying oven over-night, and samples were cooled in a dessicator before weighing.

2. Manometry

Standard manometric methods were used for the measurement of O_2- uptake by cell suspensions. (Umbreit, Burns and Stauffer, 1964; Batt and Woods, 1961).

Manometer vessels (approximately 15 ml) contained in the main compartment, 1.5 ml 0.1M phosphate buffer, 0.5 ml of bacterial suspensions equivalent to 5 mg dry weight, and in the side bulb, 0.5 ml of substrate solution (0.02 M unless otherwise stated) in phosphate buffer (pH 7.0; 0.1 M). Controls differed in containing

a. no substrate (for estimation of endogenous oxygen uptake).

b. no cells (to test stability of substrates).

3. Ultraviolet Spectrophotometry

Pyrimidine concentrations were determined from ultraviolet absorption spectra obtained from either a Unicam S.P. 800 Ultraviolet Spectrophotometer or a Beckman D.U. Spectrophotometer which was used for most routine estimations. Normally, samples were diluted in 0.1 M NaOH to give absorbance values between 0.1 and 1.0. For the determination of the concentration of a pyrimidine reference was made to a standard curve which showed a linear relationship between concentration and optical density.

4. Chromatography

a. Thin-layer chromatography (T.L.C.)

Preparation of plates

Glass plates (5 cm x 20 cm, 10 cm x 20 cm, or 20 cm x 20 cm) were spread with a slurry of cellulose MN 300 (according to Stahl), from Macheray, Nagal and Co. (Duren, Germany), at a thickness of 0.25mm with a spreader made by Desaga (Heidelberg, Germany).

The plates were left to dry at room temperature for a minimum period of 12 hr before use.

Solvent systems

For the separation of pyrimidines one or more of the following solvent systems were used. (Fink, Cline, Henderson and Fink, 1956).

i. N-butanol:acetic acid:H_2O (50:25:25 $^{V}/_{V}$)

ii. N-butanol saturated with H_2O in an ammonia atmosphere.

iii. Tert butyl alcohol:methyl ethyl ketone:H_2O (40:30:25 $^V/_V$) in an ammonia atmosphere.

For the separation of non volatile acids, (principally dicarboxylic acids) the following two solvent systems were normally used (Fink, Cline, Henderson and Fink, 1956; Nygaard, 1967).

i. N-butanol:acetic acid:H_2O (50:25:25 $^V/_V$)

ii. Amyl alcohol:formic acid:H_2O (40:40:20 $^V/_V$)

Detection of compounds on chromatograms

Ultraviolet absorbing compounds were located under a source of ultraviolet light.

Acids were detected by spraying the chromatograms with 1% solution of methyl red in 96% ethanol made slightly alkaline by the addition of 0.1M NaOH. The spray was found to be particularly useful in the detection of dicarboxylic acids.

b. Gas liquid chromatography

A column was selected, which was regarded as being particularly suitable for the separation of the methyl esters of dicarboxylic acids.

The separation of such compounds had been previously described by several workers (Alcock, 1965; Canvin, 1965; Dalglish, Horning, Horning, Knox and Yarger, 1966).

In this investigation the method of separation was based principally on that used by Alcock, 1965. Separations were carried out in a Packard Gas Chromatograph (Model 802) on a 4ft. x 3mm column of

12% DEGS (diethylene glycol succinate) supported on chromosorb W 60-70 mesh with argon as the carrier gas (25ml/min). The initial column temperature was 88°C and was programmed to increase at a rate of 1°C/min after injection of sample, to a final temperature of 167°C. Normally the temperature was held at 167°C for 15 minutes before being returned to the initial value.

5. Mass spectrometry

Compounds separated by gas liquid chromatography were further characterised by mass spectrometry. The compound to be examined was trapped in a glass collecting chamber, connected to the gas outlet of the G.L.C. apparatus, the collecting chamber containing glass wool soaked in methanol. After trapping, the compound was eluted with methanol into a flask, the methanol slowly evaporated off, under reduced pressure with some heating, and the residual material was used directly for mass spectral analysis.

This method provided only a partially purified compound and further purification would be essential to obtain clear-cut results from mass spectrometry.

6. Quantitative and qualitative measurement of radio-activity

a. Radio - Isotope counting

Normally a 300 µlitre aliquot of radioactive ethanol extract was added to a counting vial, followed by 10ml of Bray's Scintillation Solution. naphthalene (60g), PPO (4g), POPOP (200mg), methanol (100ml), ethylene glycol (20ml) and p-dioxane to make 1 litre .

Sample vials were counted through the preset C^{14} channels, in a Packard Tri-carb Liquid Scintillation Spectrometer (model 2002).

The counting efficiency of each sample was estimated by reference to a curve of observed counting efficiency vs. channel ratio. This curve was constructed by counting a measured amount of thymine - methyl-C^{14} with increasing amounts of non radioactive ethanol extract.

b. Radioisotope scanning

A developed chromatogram (on 5 cm x 20 cm thin layer plate) was scanned for radioactivity using a Packard Radiochromatogram Scanner, (Model 7200), with a mixture of 1.3% iso-butane - 98.7% helium as the carrier gas. Optimum conditions were as follows:

gas flow 110cc/min; voltage 1.15kv; time constant 30 sec; scale 0-300 cpm; speed 12cm/hr; slit width 2.5mm. The contributions of different compounds to the total radioactivity of the sample were assessed from the radio-chromatogram using a planimeter.

7. Autoradiography

Chromatograms which were scanned for radioactivity were normally further examined by autoradiography. Plates were placed in contact with X-ray film (Agfa-Gevaert, Belgium) and left in the dark for two or three weeks, and the film subsequently developed. Chromatograms in which radioactive compounds were separated by two-dimensional chromatography, were also examined by autoradiography.

B. Bacteriological methods

1. Organism

N. corallina is an aerobic, gram-positive, rod-shaped bacterium, which is neither motile nor spore-forming. When grown on different media, the organism exhibits considerable pleomorphism.

The strain of N. corallina used in this investigation was isolated by Batt and Woods (1951). Throughout the present study, the organism was maintained at 2°C on glucose yeast extract agar slopes (Appendix IC). Slopes were subcultured at monthly intervals onto fresh slopes which were incubated at 30°C for 24hr and then stored at 2-4°C.

The original isolate was identified by Professor Jensen as a strain of N. corallina (Batt and Woods, 1961). Further examination of the organism was carried out by Dr. Ruth Gordon (Rutgers, U.S.A.) in 1965, who described it as Mycobacterium rhodocrous but pointed out that it was reasonable to retain the name N. corallina in view of the taxonomic problems associated with the organism.

2. Growth in Liquid Media

Throughout the course of this investigation two types of liquid growth media were employed to give either cells induced or not induced for pyrimidine catabolism. Non-induced cells were grown in a glucose liquid medium (pH 7.0) which contained the following components in g/litre.

$(NH_4)_2SO_4$	3.0
$MgSO_4 : 7H_2O$	0.1

KH_2PO_4	13.6
Vitamin B_1	0.025
Glucose	7.5

Cells induced for pyrimidine catabolism were cultured in a medium containing either thymine or uracil and the sole source of carbon and nitrogen and inorganic salts.

The components of the medium are given in gm/L.

KH_2PO_4	13.6
$MgSO_4 \cdot 7H_2O$	0.1
Pyrimidine	2.0
Vitamin B_1	0.025

The method of preparation of both types of media is outlined in Appendix IA, IB. In all cases for inoculation, cells from an agar slope which had been stored for no longer than 2 days, were transferred using a sterile loop into 300ml of liquid medium held in a standard conical flask (1L). The flask containing the inoculum was then incubated at 30°C with shaking at 140 revs/min on a New Brunswick gyro-rotatory shaker (Model - G-25). Growth was followed spectrophotometrically by optical density measurement at 490nm in a Hitashi spectrophotometer.

For organisms grown in a glucose medium, full growth of the culture was attained after approximately 50hr incubation shown by the establishment of maximum optical density at 490nm.

The period for full growth of pyrimidine grown organisms differed depending on whether thymine or uracil was used as the growth substrate. With thymine grown organisms full growth was established

after approximately 48hr incubation; with uracil grown cells, full growth was obtained in approximately 120hr.

Routine examination of morphology of organisms from cultures at full growth was carried out using light microscopy and gram staining.

3. Preparation of cells for experiments involving cell suspensions

In all cases, cells were harvested at full growth by centrifuging at 3000xg (Sorvall, Model RC-2B) for 10 min ($2^{\circ}C$) in screw capped polypropylene cups (250ml Nalgene) which had been sterilised by autoclaving at $121^{\circ}C$ for 15 min. The supernatant fluid was discarded and the pellets were re-suspended and recentrifuged in sterile phosphate buffer (pH 7.0 : 0.1M) then finally resuspended in the same buffer at the desired concentration.

C. Experimental procedures

1. General procedure in preliminary studies

Oxygen uptake

Except when otherwise stated the standard manometric procedure for the estimation of oxygen uptake was used in all experiments.

For the oxidation of pyrimidines the rate of oxygen uptake was normally followed until

a. it decreased to a value equal to that for the endogenous respiration of cells in the controls. and

b. no pyrimidine remained as shown by ultraviolet spectrophotometry.

In all cases the endogenous O_2 uptake was subtracted from the experimental values in the presence of substrate, to obtain correct values.

Examination of incubation media for pyrimidines and non volatile acids (non ultraviolet absorbing

Normally contents were removed from manometer cups at various stages of the incubation period and centrifuged at 3000xg for 10 minutes. The supernatants were discarded and stored at $0^{\circ}C$.

For the identification of pyrimidine products by ultraviolet spectrophotometry, supernatants were diluted 1:50 in 0.1M NaOH and 0.1M HCl.

For the identification of pyrimidines and non volatile acids by co-chromatography supernatants were spotted directly on to thin layer plates which were run using the appropriate solvent systems.

2. Experiments involving large scale incubation

a. Incubation media

Except where otherwise stated, incubation media including those in which cells were incubated in the presence of a metabolic inhibitor, each contained 0.25g thymine, and 3g wet weight of cells. Each medium was made up to a total volume of 150ml with sterile 0.1M phosphate buffer (pH 7.0).

b. Conditions for incubation

Flasks (500ml) containing 150 ml of medium, were plugged with cotton wool and incubated at $30^{\circ}C$ with shaking at 140rev/min in a New Brunswick gyro-rotatory shaker (Model - G-25).

c. Incubation procedure

Normally incubation was allowed to proceed until all the thymine had disappeared from the incubation medium. At various stages, aliquots (1ml) were removed from the incubation medium and centrifuged at 3000xg for 5 minutes. The supernatants, after being diluted 1:100 in 0.1M NaOH were examined by ultraviolet spectrophotometry for the presence of thymine and other pyrimidines.

When thymine had disappeared from the incubation medium, cells were removed by centrifugation (3000xg, 10 min) at 0°C, and the supernatant stored at the same temperature.

d. Extraction of ether soluble acids from the supernatant

This method was based on that used by Biggs and Doumas (1963) for the extraction of acids from the supernatant of incubation media, with ether.

The initial step in the procedure involved filtration of the supernatant using a Syke's filter. After the filtrate was adjusted to pH 10.0 with 5M NaOH, it was concentrated by evaporation under reduced pressure at 80°C, to a volume of approximately 30ml, and then acidified to pH 2.0 with 6M HCl. The acidified solution was extracted with ether (100ml) in a liquid-liquid extractor for 4 hr, and the ether extract was separated and stored. The aqueous solution was re-extracted with another 100ml of ether for a further 4hr and the ether extract was separated and combined with the first. The combined ether extracts were dried over anhydrous sodium sulphate ($Na_2 SO_4$) and evaporated to dryness under reduced pressure. The residue was redissolved

in a dry ether (approximately 5ml) in preparation for methylation.

e. Methylation of ether soluble acids

Methyl esters were prepared by the method of Schlenk and Gellerman (1960) using diazomethane generated from N-methyl-N-nitroso-p-toluenesulfonamide. The preparation of diazomethane is outlined in Appendix IIA.

For methylation, portions of diazomethane in ether were added to samples by a pasteur pipette. Sufficient diazomethane was added to each sample for the characteristic yellow colour to persist for at least 20 minutes. Boiling chips were then added to sample tubes which were placed in a beaker of hot water to evaporate off ether and excess diazomethane. All steps were carried out in a fume-cupboard. For gas liquid chromatography the methylated derivatives were redissolved in cold ether to a volume of 1ml and stored at $0^{\circ}C$.

3. Experiments with thymine-methyl-C^{14}

a. Incubation media

Incubation media, including those in which cells were incubated in the presence of diethylmalonate, contained 20 µmoles thymine-methyl-C^{14} (Specific Activity 0.05mc/mM), 50 mg dry weight of cells in sterile phosphate buffer (0.1M:pH 7.0) to make a total volume of 5ml.

b. Conditions for incubation

Flasks (25ml) each containing 5ml incubation medium, were plugged with cotton wool and incubated at $30^{\circ}C$ with shaking at 140 rev/min in a New Brunswick gyro-rotatory shaker (Model - G-25).

c. Procedure during incubations

Cells were incubated until all pyrimidine had disappeared from the incubation medium. The disappearance of pyrimidine was studied by removing aliquots (0.5ml) at regular intervals from a duplicate incubation medium containing non-radioactive thymine. Aliquots were centrifuged at 3000xg for 5 minutes and the supernatants, after being diluted 1:50 with 0.1M NaOH were examined by ultraviolet spectrophotometry.

During the incubation, aliquots (0.5ml) were removed from the thymine-methyl-C^{14} incubation medium at various intervals. Each aliquot was added to hot 80% ethanol (5ml) and the soluble components of the cells, extracted for 15 minutes.

d. Procedure for detecting radioactivity

Ethanol extracts were centrifuged at 12,000xg for 20 minutes at $0^{o}C$ (Sorvall - Model RG-2B). The supernatants were decanted and each made up to a total volume of 10ml with 80% ethanol. In each case a 0.3ml aliquot was taken from a 10ml volume, and counted in a scintillation Spectrometer, Packard (Model 2002). The rest of the 10ml volume was evaporated under reduced pressure with heating to about 0.5ml, and a 0.1ml aliquot was withdrawn to obtain an approximate count. The remaining radioactive material was kept for spotting on thin layer plates, which were developed for radioisotope scanning and autoradiography.

The amount of material spotted on each plate corresponded to 3,000 - 4,000 dpm.

4. Experiments with cell-free extracts

a. Preparation of cell-free extracts

Thymine grown cells were harvested by centrifugation (Sorvall, Model RC-2B) at 3000xg for 10 minutes at 0°C. The supernatant fluid was discarded and the pellets resuspended in phosphate buffer (pH 7.0: 0.1M). After recentrifugation, and decanting the supernatant, the pellets were made into a slurry by the addition of phosphate buffer. Normally 3g wet weight of cells were mixed with 4ml of cold phosphate buffer and the suspension stored at 0°C. Cells were disrupted using a French press. (Aminco, Maryland, U.S.A.). Normally two pressings at 7000lb/sq.inch were required to ensure a reasonable breakage of cells. Examination of crushings after two presses, by electron microscopy showed most of the cells to be disrupted. Cell-free extracts were obtained from crushings by two methods.

a. Crushings were centrifuged (Sorvall, Model RC-2B) at 3000xg for 10 minutes at 0°C. The supernatant was then carefully decanted and stored at the same temperature.

b. Crushings were sonicated (M.S.E. 100 watt ultrasonic disintegrator) for 2 minutes with 15 second bursts to break up gelatinous D.N.A. This was followed by centrifugation (Sorvall, Model RC - 2B) at 12,000xg for 20 minutes at 0°C. The supernatant was carefully removed and stored at 0°C.

b. Assay of cell-free extracts

To test the activity of cell-free extracts towards pyrimidines the following assay system was used:-

0.2ml extract

0.4ml phosphate buffer (0.1M; pH 7)

0.1ml pyrimidine (0.02M)

0.1ml B.S.A. (2.5%)

0.2ml H_2O

For assays at pH 6.5, phosphate buffer was used. Tris-HCl buffer was used for assays at pH 6.5-8.0. The activities of cell-free extracts towards barbituric acid and 5-methylbarbituric acid were measured from optical density readings at 255nm and 269nm respectively in 0.1M NaOH using a Beckman D.U. spectrophotometer.

Providing a linear relationship existed between substrate concentration and optical density the unit of enzyme activity was taken as that amount which brought about an optical density decrease of 0.01/minute.

CHAPTER 4

PRELIMINARY STUDIES

The procedure for the experiments in this section is outlined in Experimental procedure (1), Chapter 3. The experiments described here were carried out to confirm the results obtained by Batt and Woods (1961) for thymine oxidation by cells grown on both thymine and uracil. In addition, the oxidations of some compounds which were considered feasible as intermediates of thymine catabolism, were tested.

1. Thymine catabolism by uracil-grown organisms

A. Oxygen uptake during thymine oxidation

A steady increase in the rate of oxygen consumption with cell suspensions of N. corallina marked the first phase of thymine oxidation (Figure 4). At the end of the first phase i.e. when the oxygen uptake had reached a value of approximately 0.5 mole O_2/mole thymine, a decrease in rate occurred marking the beginning of a second phase of substrate oxidation. During the second phase, the rate of oxygen uptake again steadily increased and finally levelled off to endogenous values when all of the pyrimidine had been removed.

B. Identification of an oxidation product

Thin layer chromatography and ultraviolet spectrophotometry were used to identify a compound formed during the oxidation of thymine.

For thin layer chromatography, contents were removed from manometer cups at 0, 20, 40 and 60 minutes; 1.5, 2, 3, 5 and 6hr incubation. Aliquots (10 µlitres) of each supernatant were spotted on a 20 x 20cm

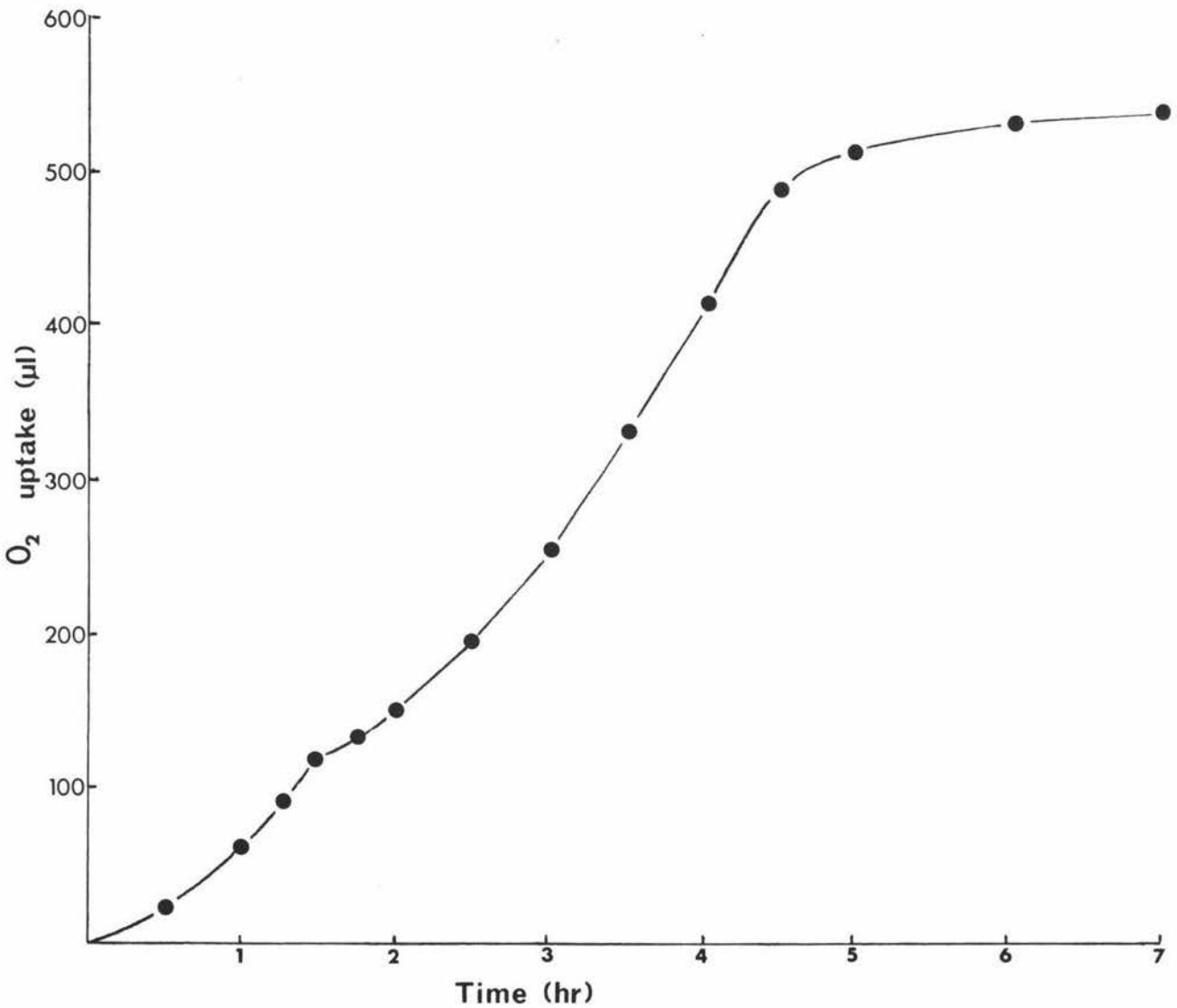


Figure 4. Uptake of oxygen by uracil grown organisms, incubated with thymine.
Values corrected for endogenous oxygen uptake.
Standard conditions as described for manometry in Methods section, Chapter 3.

thin layer plate. Thymine and 5-methylbarbituric acid were spotted as markers (1 and 10 µlitres of each). The plate was developed in an ammonia atmosphere with N-butanol saturated with water as the solvent.

The chromatogram (Figure 5) showed that after 20 minutes incubation, another ultraviolet absorbing compound was detected, the R_f of which closely corresponded to that for 5-methylbarbituric acid. After 90 minutes incubation which marked the end of the first phase of oxidation, thymine was no longer present. In the second phase of oxidation, the spot corresponding to 5-methylbarbituric acid was still detectable at an incubation time of 5hr, but had disappeared after 6hr which was about the time oxygen uptake figures approached endogenous values.

Ultraviolet spectrophotometry showed that over the first 1.5hr incubation, there was a shift in the spectrum given by thymine (λ max at 285nm in 0.1M NaOH) to one which was identical to that of 5-methylbarbituric acid (λ max at 269nm in 0.1M NaOH) (Figure 6). Further, the spectrum given by the ultraviolet absorbing compound in 0.1M HCl was similar to that for 5-methylbarbituric acid (Figure 6). In the second phase of oxidation the spectra for the ultraviolet absorbing compound gradually decreased until after 6hr none could be obtained.

C. Other intermediates

Attempts to detect principally dicarboxylic acids as possible intermediates of thymine catabolism were unsuccessful. Thin layer chromatography of supernatants from incubation media did not reveal the presence of acids when chromatograms developed with N-butanol:

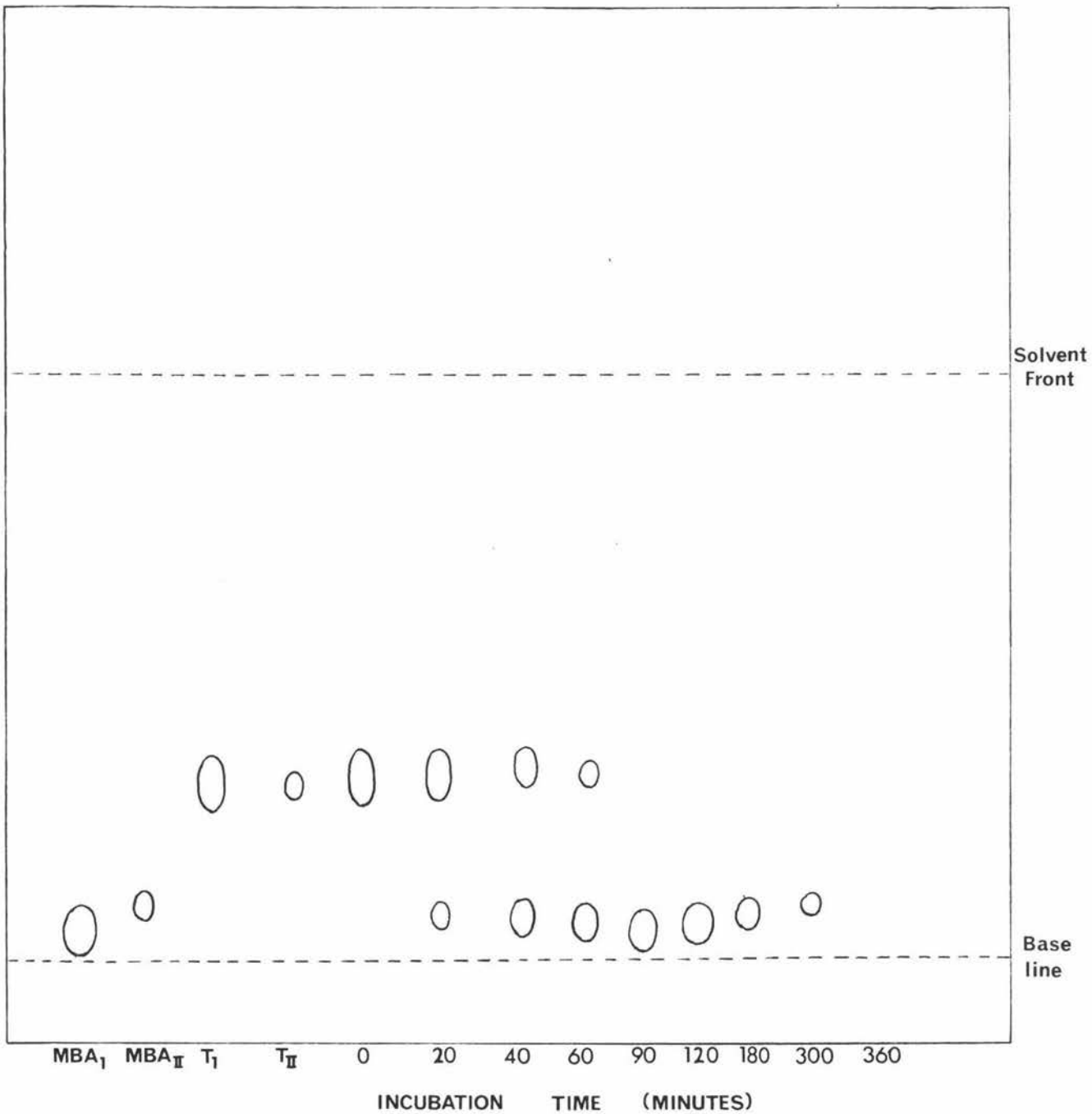


Figure 5. Chromatogram of samples removed at various times during the oxidation of thymine by uracil grown organisms. T and MBA represent thymine and 5–methylbarbituric acid markers respectively.
The chromatogram was developed with N–butanol saturated with H_2O in an ammonia atmosphere. Ultraviolet absorbing compounds were located under ultraviolet light.

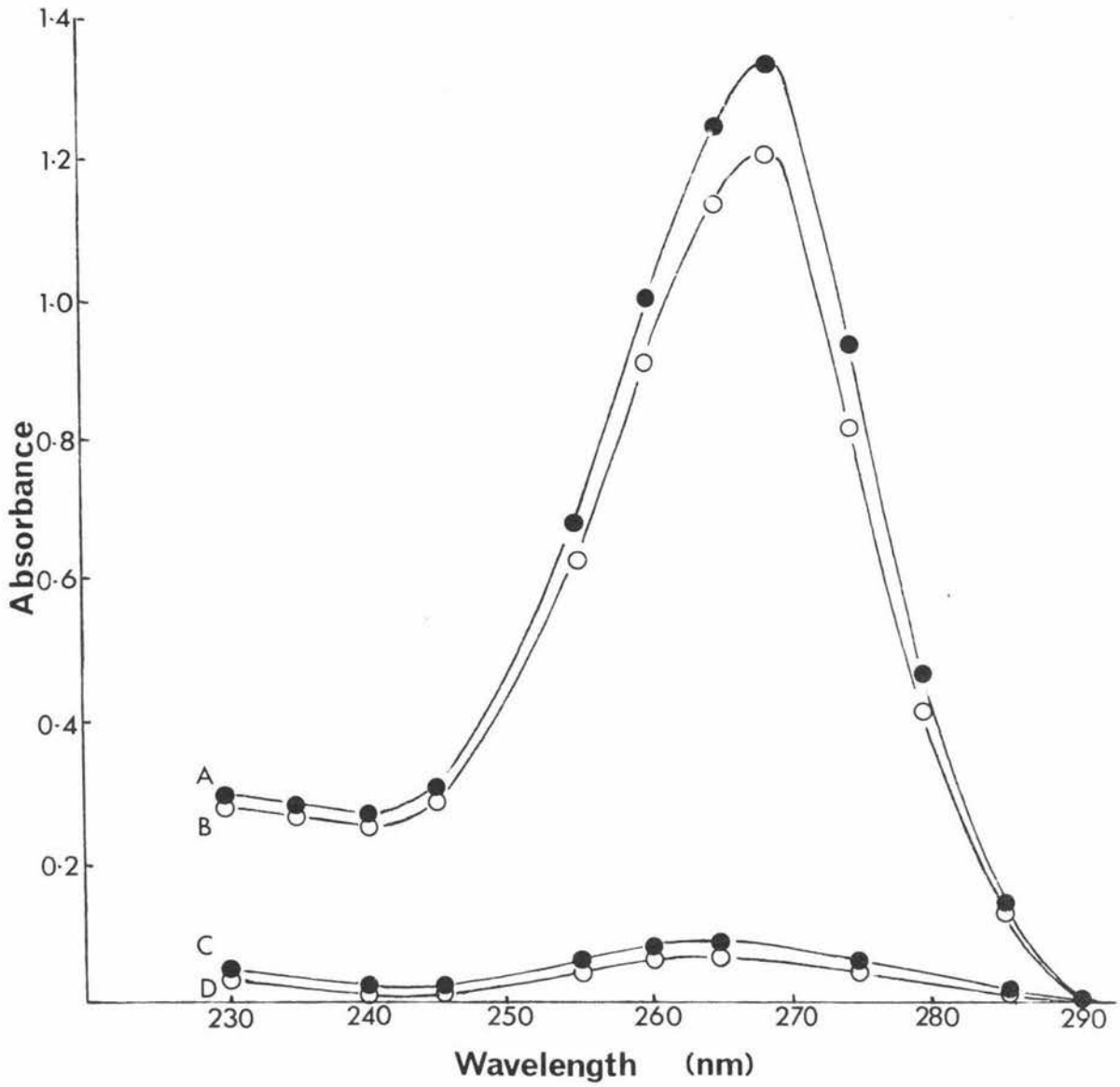


Figure 6. Comparison of spectra of thymine oxidation product and 5-methylbarbituric acid.
(A) and (C) 5-methylbarbituric acid (8×10^{-5}M) in 0.1M NaOH and 0.1M HC1 respectively.
(B) and (C) Thymine oxidation product in 0.1M NaOH and 0.1M HC1.
For examination of spectra of thymine oxidation product 0.5ml portions of supernatant were taken in the second phase and diluted 1/50 in 0.1M NaOH and 0.1M HC1 respectively.

acetic acid:H_2O (50:25:25 $^{V}/_{V}$) were sprayed with an alkaline solution of methyl red.

D. Stability of 5-methylbarbituric acid

It had been previously reported that 5-methylbarbituric acid is unstable in phosphate buffer at neutral pH and with adequate aeration is rapidly oxidised to 5-hydroxy-5-methylbarbituric acid (Batt and Woods, 1961; Biggs and Doumas, 1962). In this investigation 5-methylbarbituric acid (prepared by the method given in Appendix IIC) was found to be completely stable in 0.1M phosphate buffer (pH 7.0) during a 6hr manometry experiment, as shown by no observable oxygen uptake and no decrease in optical density (O.D. at 269nm in 0.1M NaOH) of aliquots removed at various intervals.

II. Oxidation of 5-methylbarbituric acid by N. corallina

Previous studies have shown that is is unlikely that 5-hydroxy-5-methylbarbituric acid is an intermediate in the further oxidation of 5-methylbarbituric acid by adapted cells of N. corallina. Batt and Woods (1961) found that whole cells of pyrimidine adapted N. corallina did not oxidise the compound. Biggs and Doumas (1962) obtained similar results with whole cells of Corynebacterium.

Little is known on the nature of 5-methylbarbituric acid decomposition in N. corallina and the following experiments were carried out to establish whether or not in the cell the initial step in the catabolism of the compound, was likely to be enzymatic.

Studies were carried out on the oxygen uptake by cells meta-

bolising 5-methylbarbituric acid and thymine in the presence of chloramphenicol (180ug/manometer).

Results showed that for cells metabolising 5-methylbarbituric acid in the presence of chloramphenicol, at the end of the 7hr incubation period, the oxygen uptake was 23% of that for cells incubated with 5-methylbarbituric acid in the absence of the inhibitor (Figure 7). Quantitative estimation of 5-methylbarbituric acid by ultraviolet spectrophotometry showed a comparable decrease in the concentration of the compound over the same period.

For uracil grown cells metabolising thymine in the presence of chloramphenicol the first phase of oxidation was almost unaffected by the inhibitor. However, the second phase was markedly depressed (Figure 8). Furthermore, at the end of the 7hr incubation period, a compound had accumulated, which was identified by ultraviolet spectrophotometry as 5-methylbarbituric acid. The concentration of the compound was comparable with that of the 5-methylbarbituric acid not utilised by cells metabolising it in the presence of chloramphenicol.

III. Oxidation of thymine and 5-methylbarbituric acid by thymine grown organisms

A. Oxygen uptake

For the oxidation of thymine, the rate of oxygen uptake was both linear, and at a maximum rate over the first 1.5hr incubation (Figure 9). The rate of oxygen uptake then decreased, and after 4hr incubation when no thymine remained, there was no further uptake of oxygen. The pattern of 5-methylbarbituric acid oxidation was similar, except that

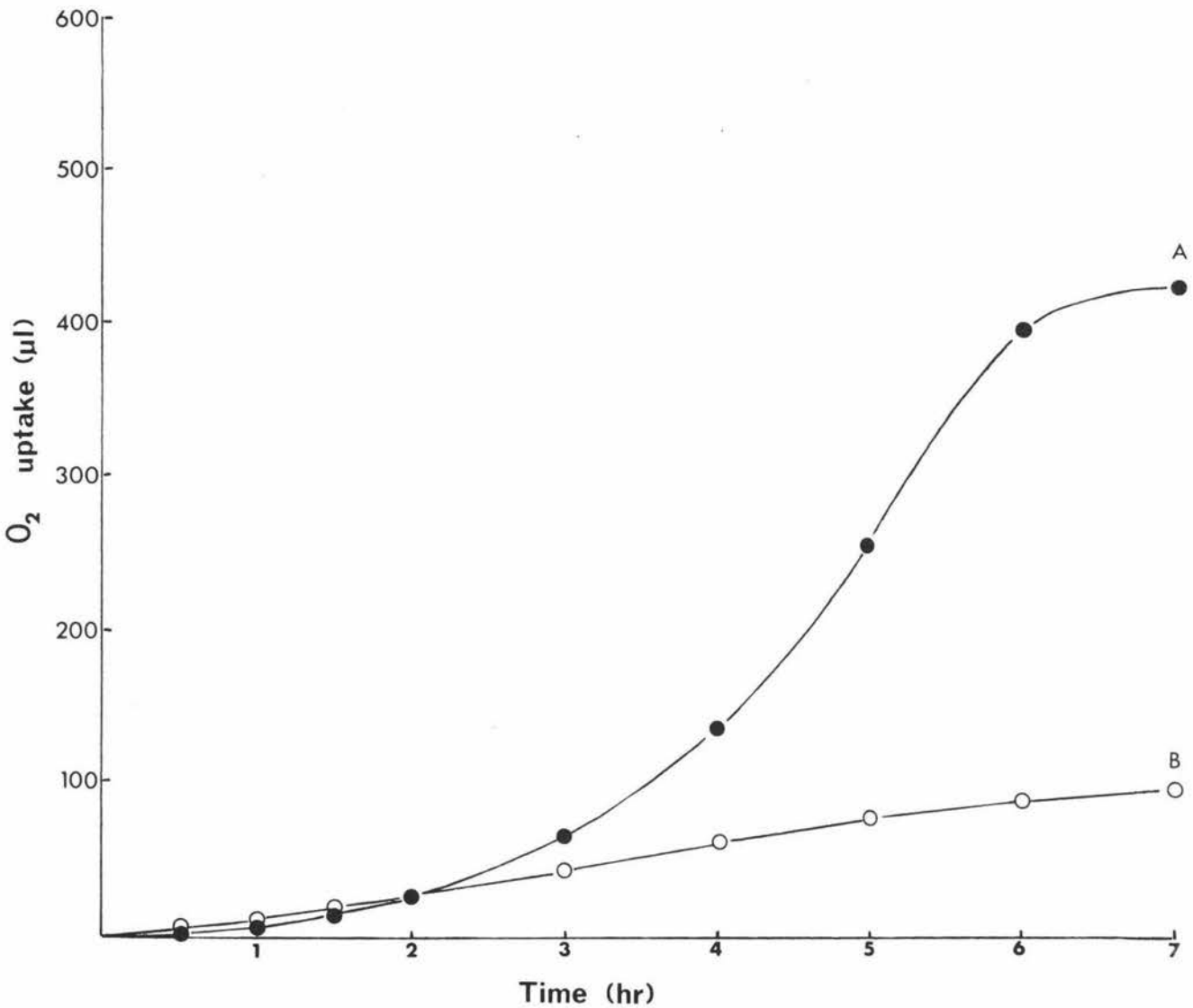


Figure 7 Oxygen uptake for uracil grown organisms metabolising 5-methylbarbituric acid.
(A) With chloramphenicol absent
(B) With chloramphenicol present (180ug/manometer)
Values corrected for endogenous 0_2 uptake.
Standard conditions as described for manometry in Methods section, Chapter 3.

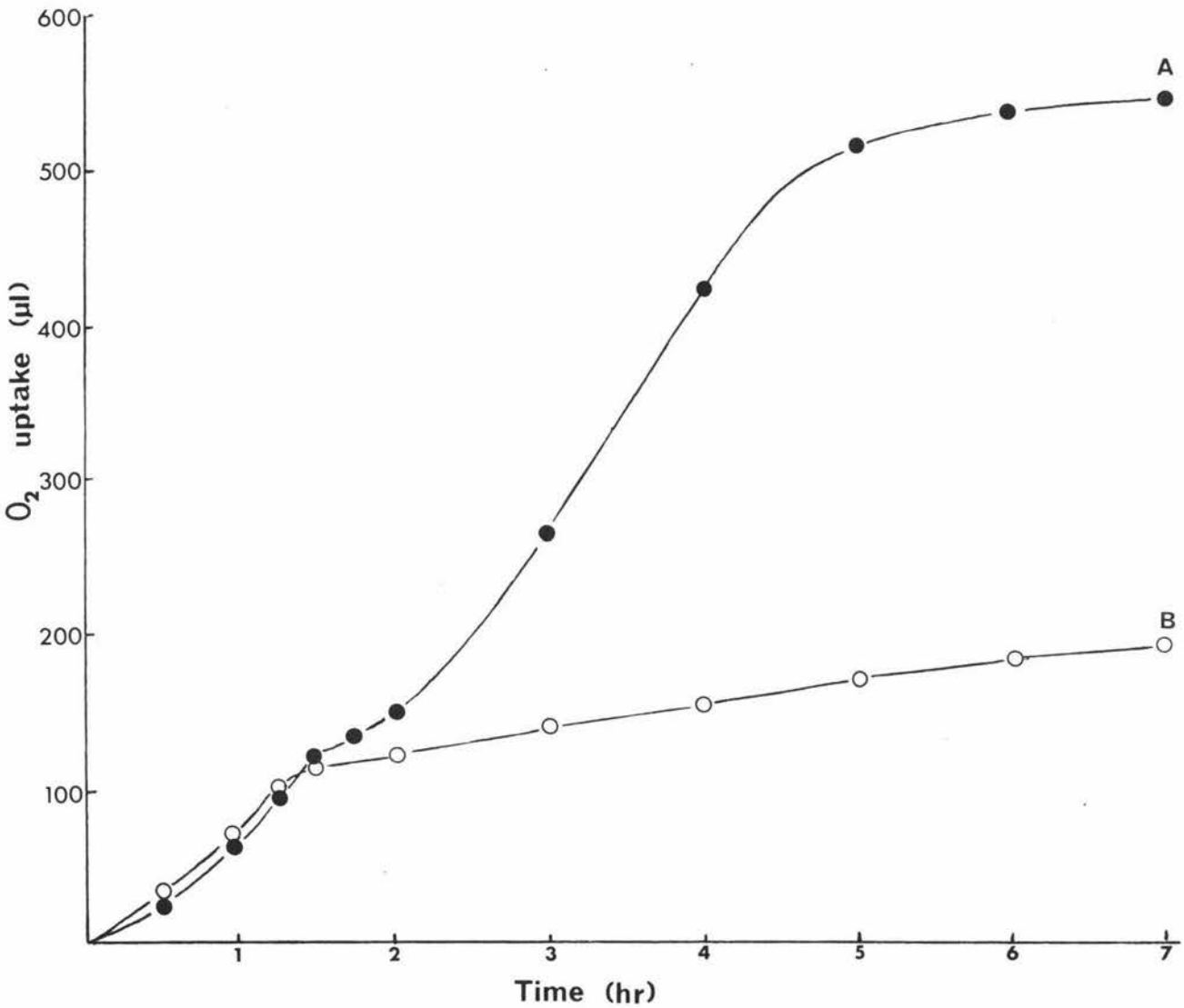


Figure 8. Oxygen uptake for uracil grown organisms metabolising thymine
(A) In the absence of chloramphenicol
(B) In the presence of chloramphenical (180ug/manometer)
Values corrected for 0_2 uptake.
Standard conditions as described for manometry in Methods section, Chapter 3.

the linear rate was evident for 1hr (Figure 9). The total oxygen uptake values for thymine and 5-methylbarbituric acid were 2.36 and 1.61 µmole O_2/µmole substrate respectively, giving a difference of 0.75 µmole O_2. The difference was not equivalent to the value for uracil grown cells which was 0.54 µmole O_2.

B. The Search for intermediates on thymine oxidative pathway

Thin layer chromatography of supernatants from thymine incubation media did not positively reveal the presence of 5-methylbarbituric acid when chromatograms developed with N-butanol:acetic acid:H_2O (50:25:25 $^{V}/_{V}$) were viewed under ultraviolet light. The same chromatograms when sprayed with an alkaline solution of methyl red did not show the presence of any other organic acids.

IV. Thymine grown organisms with 5-hydroxymethyluracil

5-hydroxymethyluracil was not oxidised by thymine grown organisms. The manometry run for the estimation of oxygen uptake was allowed to proceed for 6hr. Quantitative estimation of 5-hydroxymethyluracil in the supernatant, from a measurement of O.D. 286nm in 0.1M NaOH showed that there was no decrease in the concentration of the compound over the 6hr period.

V. Oxidation of methylmalonic acid by thymine and glucose grown oranisms

Methylmalonic acid utilisation by thymine and glucose grown cells was tested manometrically with 0.5ml of 0.05M substrate added /cup. Oxygen uptake was followed for 6hr.

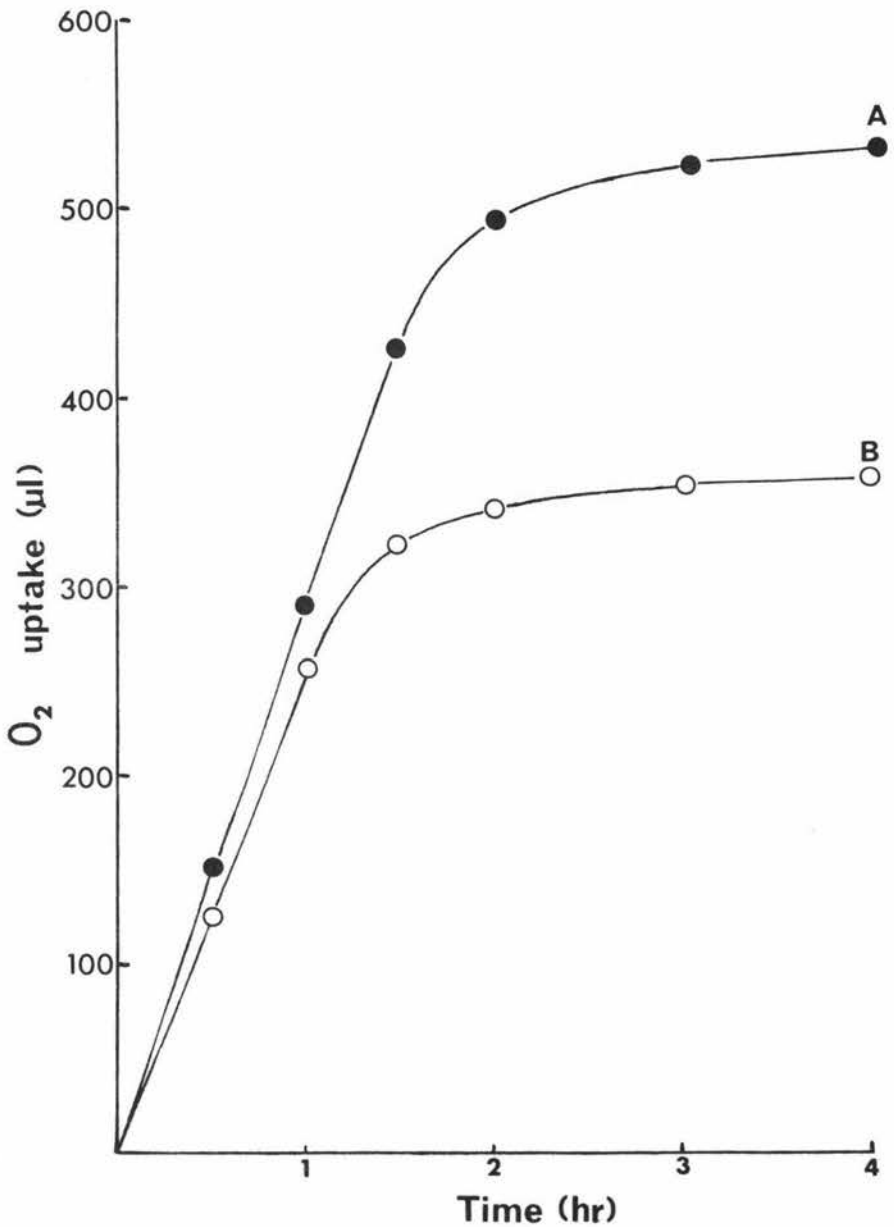


Figure 9. Oxygen uptake for thymine grown organisms metabolising
(A) Thymine
(B) 5–Methylbarbituric acid
Values corrected for endogenous 0_2 uptake
Standard conditions as described for manometry in Methods section, Chapter 3.

It was found that thymine grown organisms oxidised methylmalonic acid more rapidly than glucose grown organisms (Figure 10); at the end of the 6hr incubation period the total oxygen uptake for glucose grown organisms was 32% of that found with thymine grown organisms.

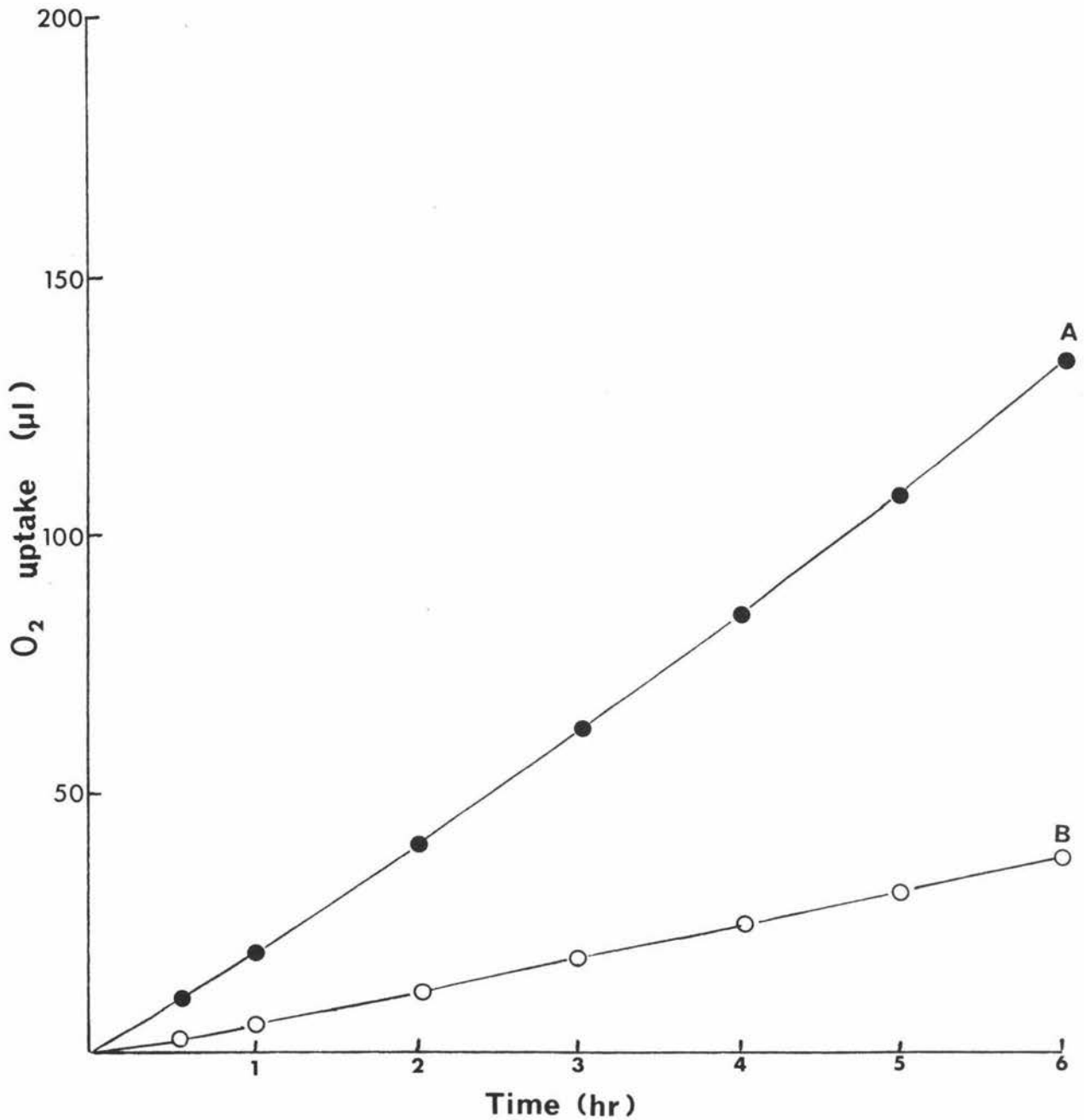


Figure 10. Oxygen uptake for (a) Thymine grown organisms and (b) Glucose grown organisms oxidising methylmalonic acid.
Values corrected for endogenous 0_2 uptake.
Standard conditions as described for manometry in Methods section, Chapter 3 except for the addition of 0.5ml of 0.05M substrate/manometer cup.

VI. Summary

a. The oxidation of thymine by uracil grown organisms occurred in two phases.

b. 5-methylbarbituric acid was identified as an oxidation product of thymine with uracil grown organisms.

c. The accumulation of 5-methylbarbituric acid was almost quantitative over the first phase of thymine oxidation with uracil grown organisms.

d. The pattern of oxidation of 5-methylbarbituric acid by uracil-grown organisms was similar to the second phase of thymine oxidation.

e. Thymine grown organisms oxidised thymine and 5-methylbarbituric acid more rapidly than uracil grown organisms.

f. The presence of an inducible enzyme system active upon 5-methylbarbituric acid was indicated from studies using chloramphenicol, with uracil grown cells acting on thymine and 5-methylbarbituric acid.

g. 5-hydroxymethyluracil was not oxidised by thymine grown organisms.

h. Thymine grown organisms oxidised methylmalonic acid more rapidly than glucose grown organisms.

CHAPTER 5

THYMINE CATABOLISM BY THYMINE GROWN ORGANISMS IN THE PRESENCE AND ABSENCE OF METABOLIC INHIBITORS

In the preliminary studies (Chapter 4) attempts to detect possible intermediates of thymine catabolism by thymine grown organisms were unsuccessful. It was considered that some intermediates and in particular, methylmalonic acid might be detected if the incubations with thymine were carried out on a larger scale.

The methods used in the following studies are outlined in Experimental Procedures 2, Chapter 3.

I. Incubation of cells with thymine in the absence of metabolic inhibitors

The oxidation of thymine was followed by ultraviolet spectrophotometry of aliquots removed from the incubation system; no other ultraviolet absorbing compounds other than thymine were detected.

After 6hr, when all the thymine had disappeared, the incubation was stopped, and the supernatant of the incubation medium was examined for the presence of ether soluble acids. Gas liquid chromatography of methylated derivatives in the ether extract of the supernatant, showed in only one experiment, the presence of a compound tentatively identified as the dimethyl ester of methylmalonic acid. In all other experiments this compound could not be detected with any certainty, and

no other compounds were detected.

II. Incubation of cells with thymine in the presence of metabolic inhibitors

A. Studies with mercuric chloride ($HgCl_2$)

In an attempt to promote the accumulation of intermediates in thymine catabolism, mercuric chloride ($HgCl_2$) was used. At concentrations of 1, 0.1, and 0.01 mM the compound potently inhibited the oxidation of thymine. In contrast to control experiments in which all the thymine had disappeared after 6hr, there was no observable decrease in the concentration of thymine over the same period, in the presence of mercuric chloride. At concentrations below 0.001 mM, $HgCl_2$ partially inhibited the metabolism of thymine. Examination of supernatant from the incubation media did not show the presence of any ultraviolet absorbing compounds, or ether soluble acids.

B. Studies with sodium arsenite

When sodium arsenite was used at a concentration of 5mM a partial inhibition of thymine oxidation similar to that observed with $HgCl_2$ was obtained. However again no intermediates were detected.

C. Studies with diethyl malonate

In an attempt to accumulate dicarboxylic acids as possible intermediates of thymine catabolism, diethyl malonate was used as a competitive inhibitor. The choice of the diethyl ester instead of the free acid was influenced by the observations of Batt and Woods (1961) who showed that malonic acid could not be oxidised by N. corallina, even

with cells which contained high barbiturase activity. One reason for this could be that the cells had a permeability barrier to this acid. The low permeability of cells to malonic acid was recognised by Webb (1966) who suggested that the diethyl ester could probably circumvent the permeability barrier.

In the following experiments diethyl malonate was employed at a concentration of 0.02M in incubation media.

Identification and estimation of an ultraviolet absorbing product of thymine catabolism

In control experiments, (no diethyl malonate present) after 6hr incubation all the thymine had disappeared, during which period, no other ultraviolet absorbing compounds were detected. When cells were incubated with thymine in the presence of diethyl malonate, the disappearance of the substrate, was accompanied by the accumulation of a compound having spectral characteristics identical to 5-methylbarbituric acid (Figure 11). At the end of 6hr incubation, the compound identified as 5-methylbarbituric acid was estimated by reference to a standard curve of O.D. 269 nm vs. concentration (mM). The quantity of acid which accumulated was equivalent to 30% of the original thymine added to the incubation system.

Gas - liquid chromatography of methyl esters of ether soluble acids

After cells had been incubated for 6hr with thymine in the presence of diethyl malonate, the supernatant of the incubation medium was tested for the presence of ether soluble acids. Gas liquid chromato-

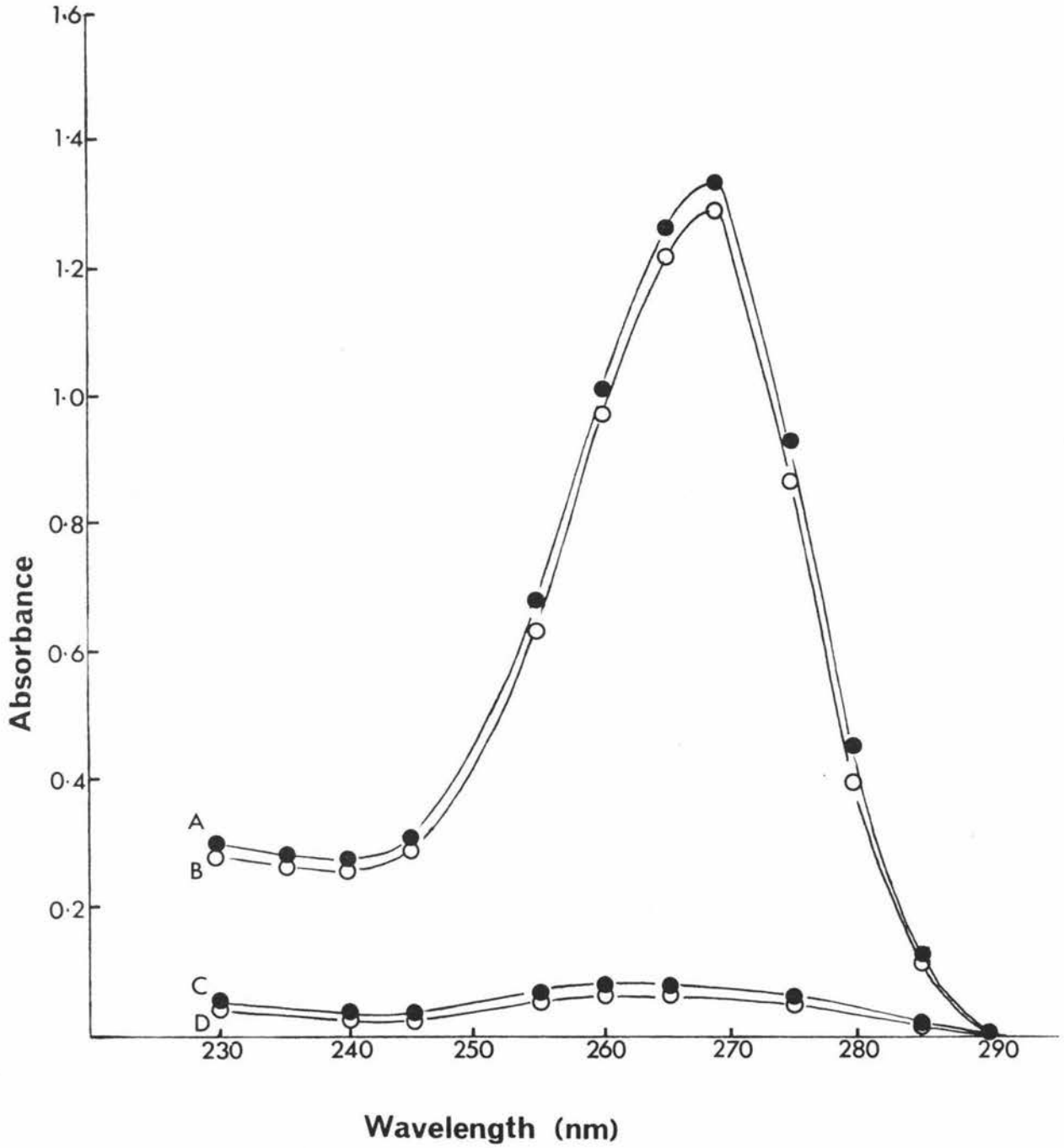


Figure 11.

Comparison of spectra of ultraviolet absorbing product and 5-methylbarbituric acid.
(A) and (C) 5-Methylbarbituric acid (8×10^{-5}M) in 0.1M NaOH and 0.1M HC1 respectively.
(B) and (D) Ultraviolet absorbing compound in 0.1M NaOH and 0.1M HC1
For the examination of spectra of ultraviolet absorbing compound 0.5 ml portions of supernatant were taken and diluted 1/50 in 0.1M NaOH and 0.1M HC1 respectively.

graphy of methylated derivatives from the ether extract of the supernatant, showed the presence of at least four compounds (Figure 12A). Three of these compounds were tentatively identified in having retention volumes corresponding to the dimethyl isosuccinate, dimethyl malonate, and dimethyl succinate standards respectively. The separation of the standards (Figure 12B) was carried out under the same conditions.

Values of retention time and retention volume for standards and unknown are given in Table 2.

TABLE 2

Retention times and retention volumes for methyl esters of dicarboxylic acid standards and unknowns.

		Ret. time (min)	Ret. volume (cc)
Dimethyl isosuccinate		38.0	950
Dimethyl malonate		48.2	1,200
Dimethyl succinate		61.2	1,530
Unknowns	1	37.6	940
	2	47.3	1,185
	3	60.5	1,512
	4	79.3	1,980

Mass spectrometry

This method was used to further characterise the compound tentatively identified as dimethyl isosuccinate.

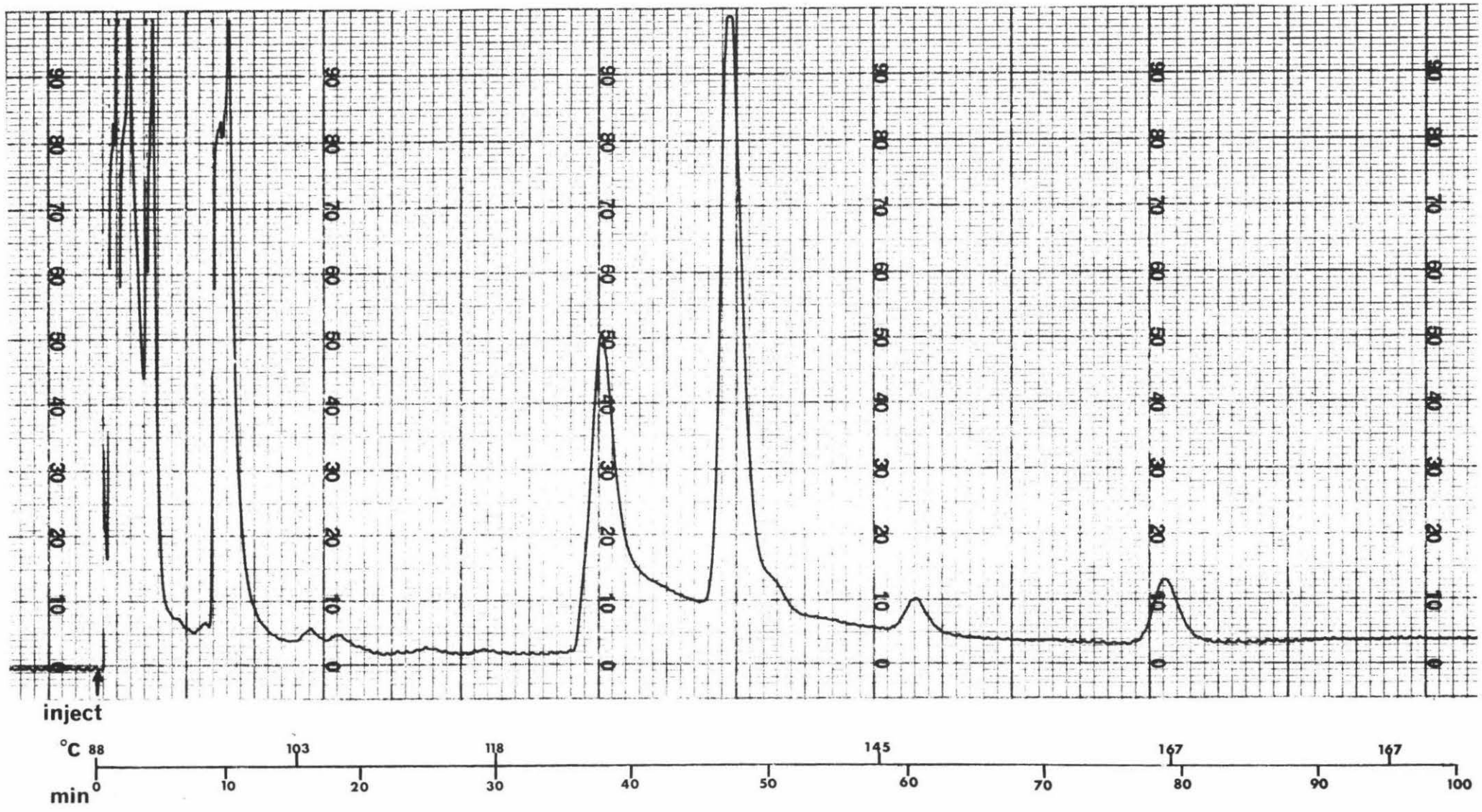


Figure 12A. Gas Liquid chromatogram of methyl esters of ether soluble acids from supernatant.
Separation was achieved using 10% diethylene glycol succinate (DEGS) on chromosorb W 60 – 70 mesh with argon as the carrier gas (25 ml/min). The temperature of the column was programmed from 88°C – 167°C at 1°C/min and held at 167°C for 15 min before being returned to the initial value.

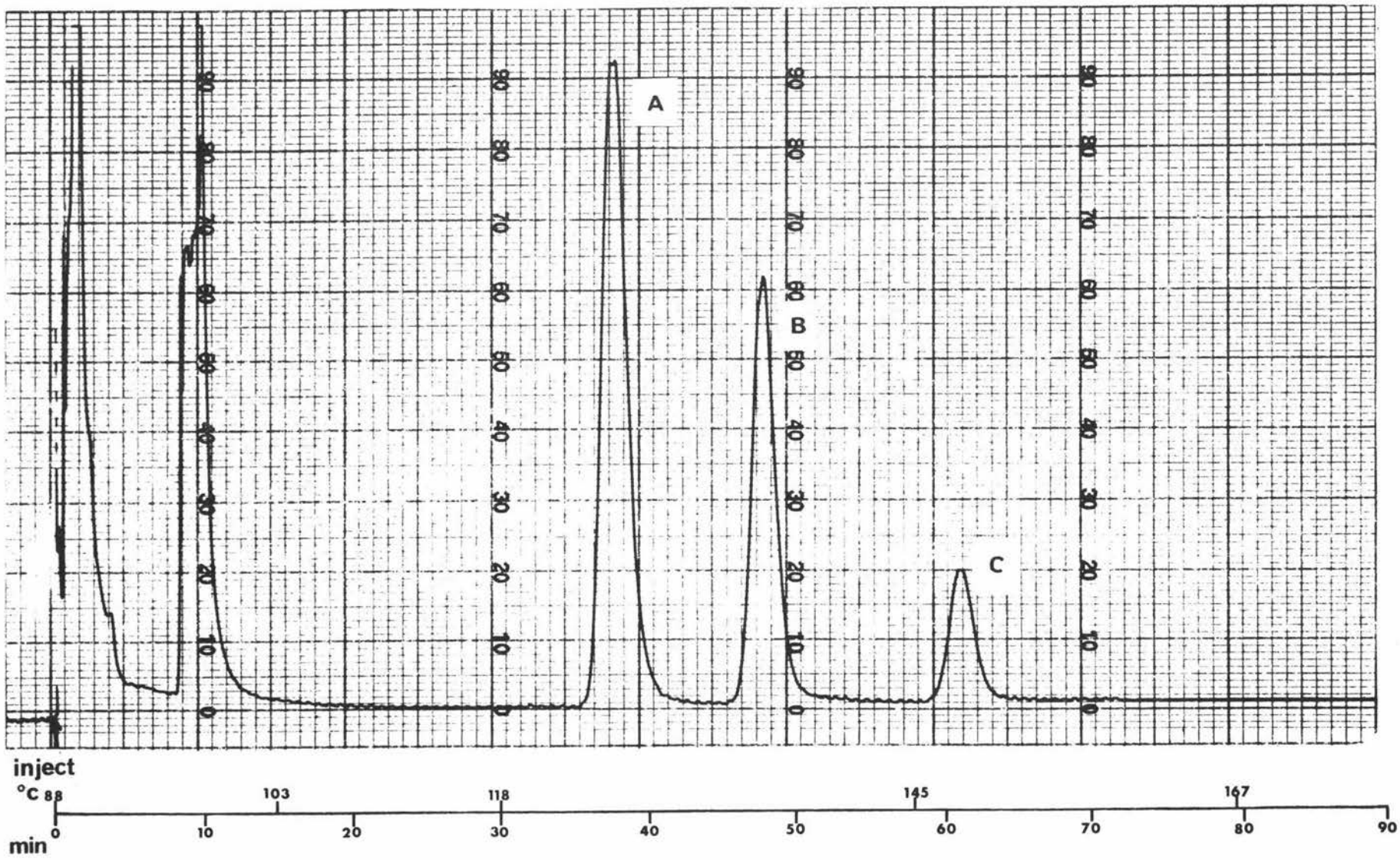


Figure 12B. Separation of dimethyl esters of methylmalonic, malonic, and succinic acids by gas-liquid chromatography. Conditions for separation the same as outlined in Figure 12A.

Key:
A. Dimethyl isosuccinate
B. Dimethyl malonate
C. Dimethyl succinate

A glass collecting chamber was connected to the gas outlet of the G.L.C. apparatus at the initial stage in the recording of the first peak corresponding to that of dimethyl isosuccinate. The material once collected, was eluted into a flask with methanol (redistilled). The methanol was evaporated off under reduced pressure with some heating, and the residual material used directly for mass spectral analysis.

Mass spectral data showed the presence of an ion with $^{lm}/e$ = 146.0587. The dimethyl isosuccinate ($C_6H_{10}O_4$) ion required an $^{m}/e$ value of 146.0578. Other compounds were shown to be present in the sample, but further purification to obtain more clear cut results was not attempted.

III. Summary

a. When cells were incubated with thymine in an inhibitor free system, no ultraviolet absorbing products were found, and in only one experiment, was the presence established of a compound tentatively identified as methylmalonic acid.

b. Possible products of thymine catabolism were not detected in incubation media in which arsenite or mercuric chloride partially or completely inhibited the utilisation of thymine.

c. A compound identified as 5-methylbarbituric acid accumulated when cells were incubated with thymine in the presence of diethyl malonate.

d. The extent of 5-methylbarbituric acid accumulation was equivalent to 30% of the original thymine concentration.

e. Three compounds, tentatively identified by gas liquid chromatography of their methylated derivatives, as isosuccinic, succinic, and malonic acids, were present in the incubation media of cells metabolising thymine in the presence of diethyl malonate.

f. The compound tentatively identified as the diethyl ester of isosuccinic acid was further characterised by mass spectrometry.

CHAPTER 6

STUDIES ON THE CATABOLISM OF THYMINE-METHYL-C^{14}

In Chapter 5 the effect of diethyl malonate in accumulating methylmalonic acid and 5-methylbarbituric acid was described.

Although, in this section diethyl malonate was used in later experiments, the approach was to initially study the catabolism of thymine by both thymine and uracil grown organisms in a free system.

I. The catabolism of thymine-methyl-C^{14} by thymine grown organisms in a free system

Organisms were incubated with thymine-methyl-C^{14} for 1hr, during which after the first 30min all the substrate had disappeared as established by ultraviolet spectrophotometry.

Samples (0.5ml) were removed from the incubation medium at 0, 15, 30 and 60min. The recoveries of radioactivity in the ethanol extract from samples removed at various times are shown in Table 3.

TABLE 3

Recoveries of radioactivity in ethanol extracts of samples removed when thymine grown organisms were incubated with thymine-methyl-C^{14}

Incubation time (min)	Dpm	% recovery
0	6,600	100
15	5,700	87
30	4,800	73
60	3,600	55

Radiochromatograms were developed using N-butanol:acetic acid: H_2O (50:25:25 $^{V}/_{V}$) as the solvent.

For the sample removed after 15min incubation, the radiochromatogram scan showed apart from the major peak corresponding to thymine, one other significant peak (Figure 13A). This peak was found to account for 25% of the total radioactivity of the material scanned and appeared to be given by a single radioactive compound which was referred to as unknown A. Unlike thymine, this compound could not be detected under ultraviolet light.

Two other peaks which could not be discerned with any certainty corresponded to 5-methylbarbituric acid and methylmalonic acid respectively.

The contributions of these peaks towards total radioactivity were minor. There was no radioactivity corresponding to the 5-hydroxy-5-methylbarbituric acid marker.

For the sample removed after 30min incubation the radiochromatogram scan showed one major peak (Figure 13B). From autoradiography it appeared that the peak was given by two compounds, one of which was tentatively identified as unknown A, on the basis of its R_f value. The other was referred to as unknown B. Another small peak appeared to be given by a compound referred to as unknown C. None of the unknown compounds could be detected under ultraviolet light.

The radiochromatogram scan obtained for the 60min incubation

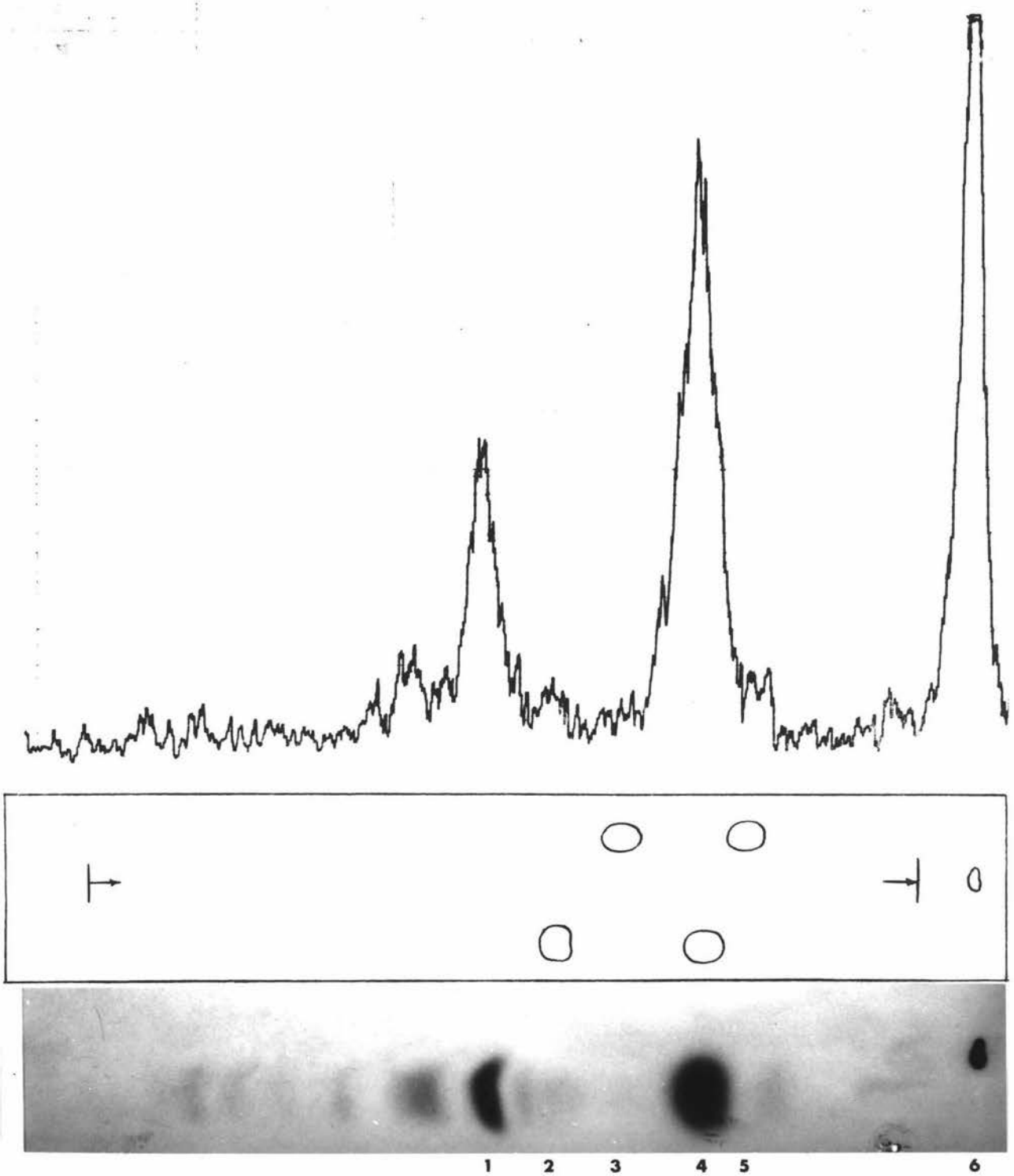


Figure 13A.

Radiochromatogram scan of ethanol extract obtained for the sample removed at 15 min, when thymine grown cells were incubated with thymine–methyl–C^{14}.

Key:
1. Unknown A
2. 5–Methylbarbituric acid
3. 5–Hydroxy–5–methylbarbituric acid.
4. Thymine
5. Methylmalonic acid
6. Marker

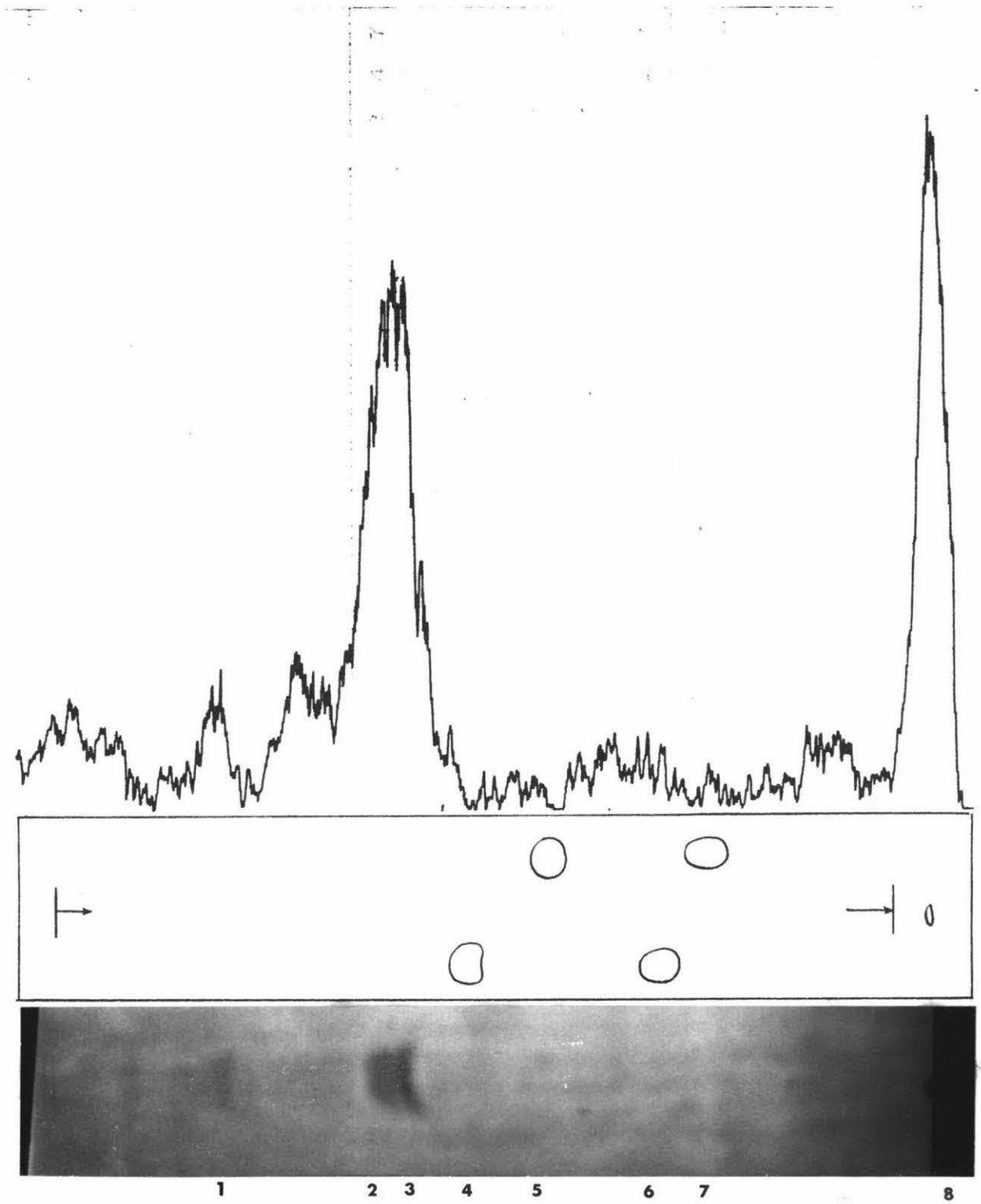


Figure 13B.

Radiochromatogram scan of ethanol extract obtained for the sample removed at 30 min, with thymine grown cells incubated with thymine-methyl-C^{14}.

Key:

1. Unknown C
2. Unknown B
3. Unknown A (tentative)
4. 5–Methylbarbituric acid
5. 5–Hydroxy–5–methylbarbituric acid
6. Thymine
7. Methylmalonic acid
8. Marker

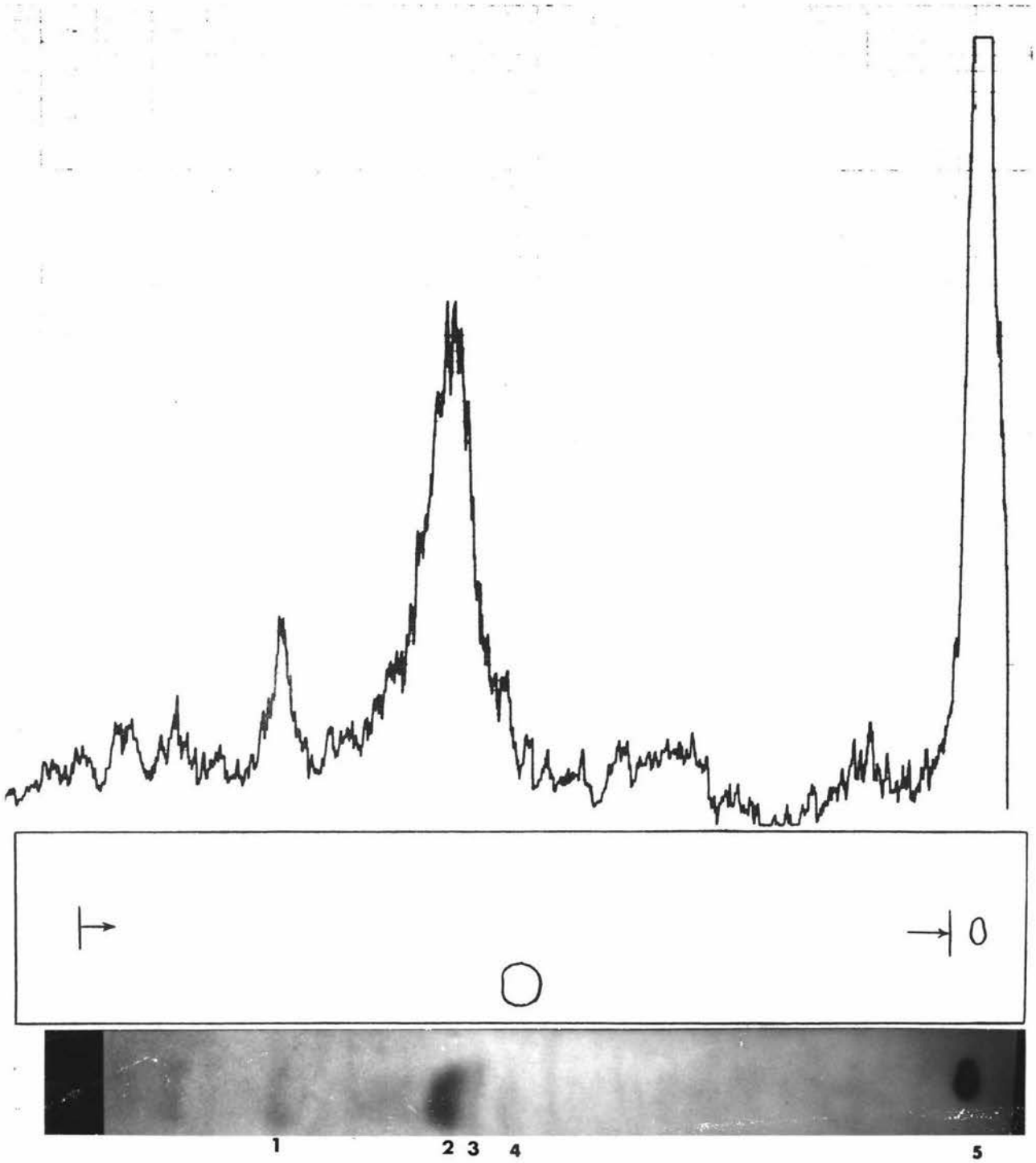


Figure 13C.

Radiochromatogram scan of ethanol extract obtained for sample removed at 60 min, after thymine grown cells had been incubated with thymine methyl-C^{14}.

Key:
1. Unknown C (tentative)
2. Unknown B (tentative)
3. Unknown A (tentative)
4. 5-Methylbarbituric acid
5. Marker

period showed the distribution of radioactivity to be similar to that for 30min (Figure 13C). However autoradiography suggested that most of the activity came from unknown B.

II. The catabolism of thymine-methyl-C^{14} by uracil grown organisms in a free system

Organisms were incubated with thymine-methyl-C^{14} for 5hr. Samples (0.5ml) were removed from the incubation medium at 0, 1, 2, 4, and 5hr. The recoveries of radioactivity in the ethanol extract for samples removed at these times are shown in Table 4.

TABLE 4

Recoveries of radioactivity in ethanol extracts of samples removed when uracil grown organisms were incubated with thymine-methyl-C^{14}

Incubation time (hr)	Dpm	% recovery
0	6,600	100
1	6,400	97
2	6,200	94
4	4,700	71
5	3,900	59

Radiochromatograms were developed using N-butanol:acetic acid:H_2O (50:25:25 V/V) as the solvent.

The radiochromatogram scan obtained for a sample removed after 1hr incubation showed peaks corresponding to thymine and 5-methylbarbi-

turic acid. (Figure 14A). The scan for 2hr incubation, showed all the radioactivity corresponding to the 5-methylbarbituric acid marker. For 4hr incubation the scan showed apart from the peak corresponding to 5-methylbarbituric acid, another which occurred in the position of unknown A (Figure 14B). For the final sample obtained after 5hr incubation the scan indicated that almost all of the radioactivity occurred in the position of unknown A. Unlike 5-methylbarbituric acid the unknown compound could not be detected under ultraviolet light.

III. The catabolism of thymine-methyl-C^{14} in the presence of diethyl malonate with thymine grown organisms

Diethyl malonate was employed at a concentration of 0.02M in the incubation medium.

Organisms were incubated for 1hr at which time all the thymine had disappeared from the medium as established by ultraviolet spectrophotometry.

Samples (0.5ml) were removed from the incubation medium at 0, 15, 30 and 60min. Recoveries of radioactivity in the ethanol extract from samples removed at various times are shown in Table 5.

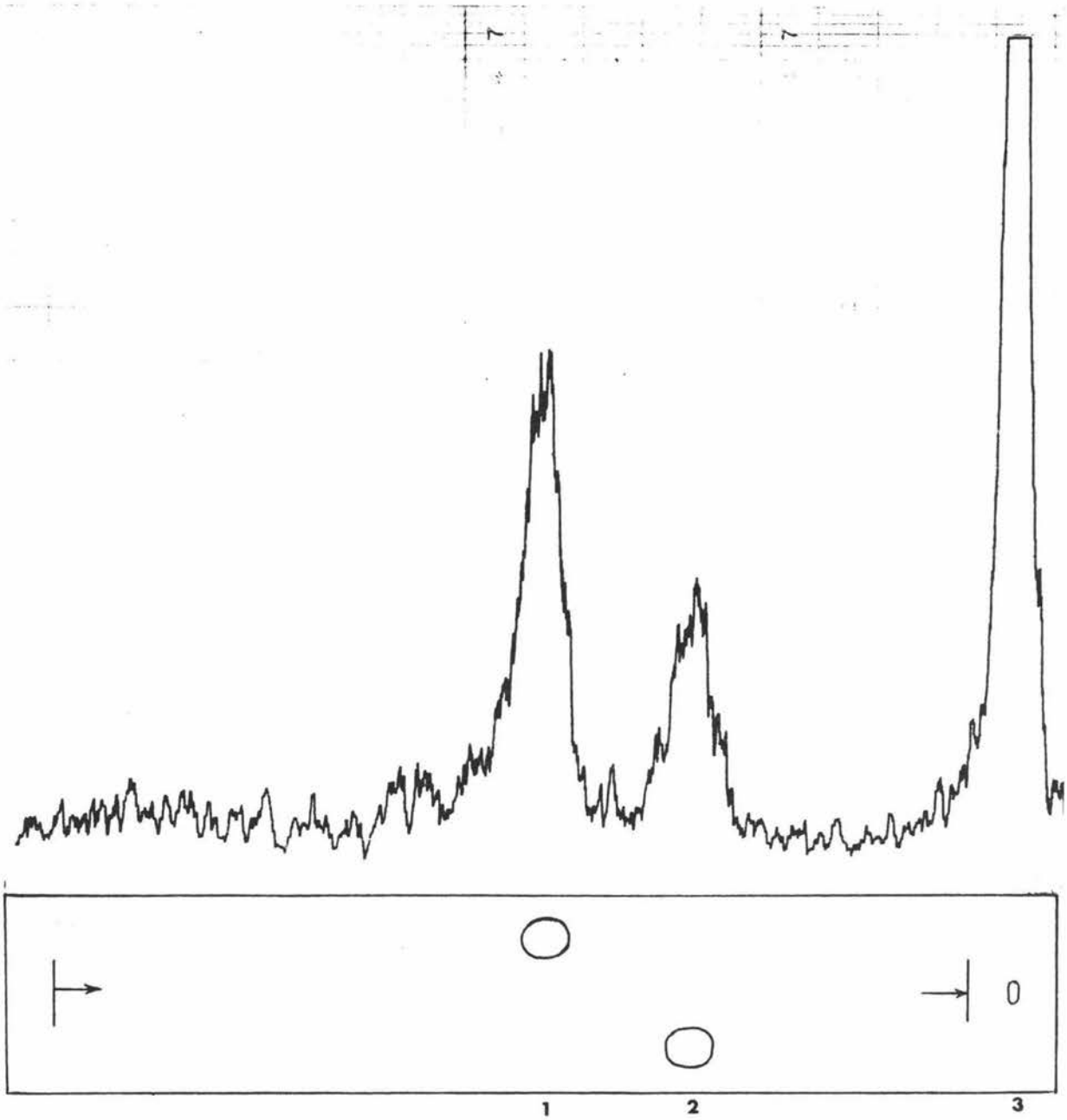


Figure 14A.

Radiochromatogram scan of ethanol extract obtained for the sample removed at 1hr, when uracil grown cells were incubated with thymine-methyl-C^{14}.

Key:
1. 5–Methylbarbituric acid
2. Thymine
3. Marker

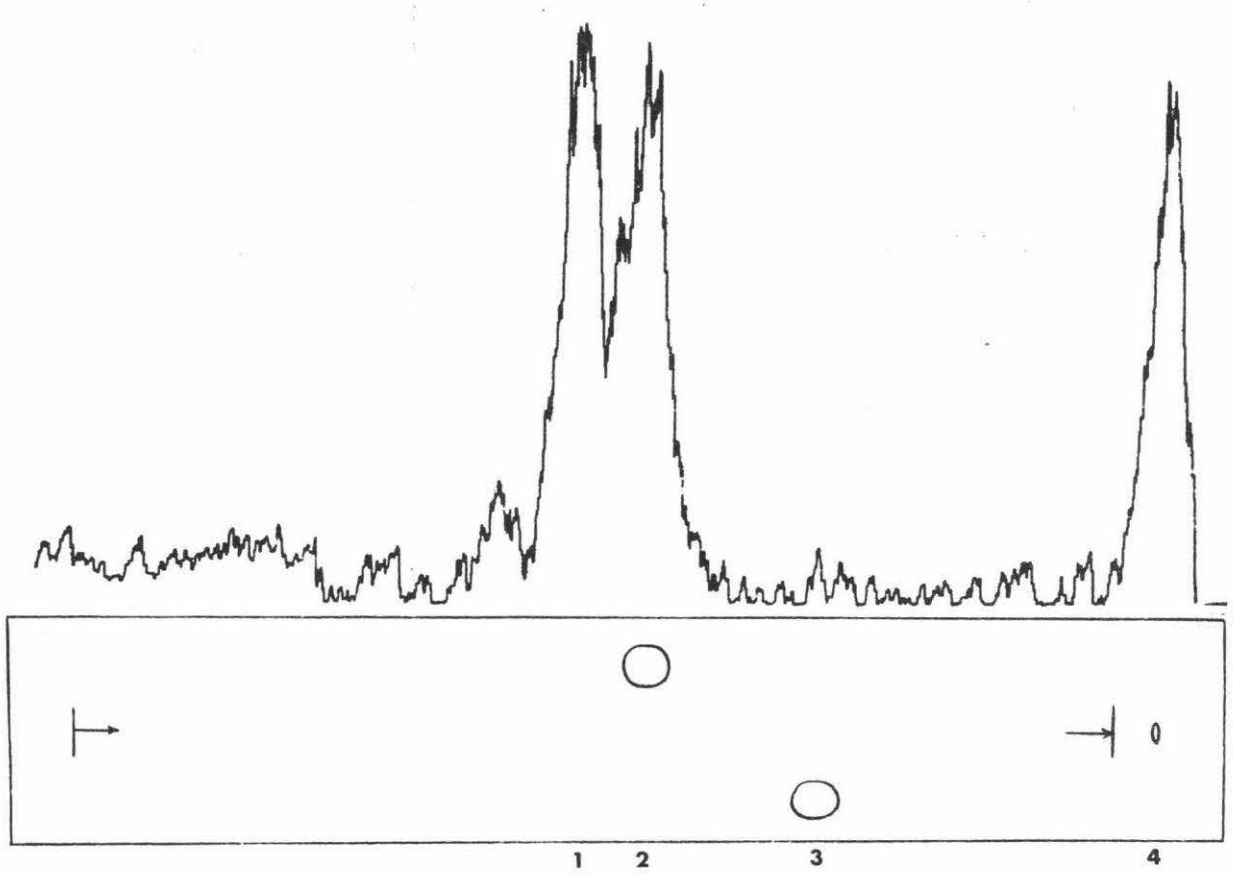


Figure 14B.

Radiochromatogram scan of ethanol extract obtained for the sample removed at 4hr, when uracil grown cells were incubated with thymine-methyl-C^{14}.

Key:
1. Unknown A (tentative)
2. 5–Methylbarbituric acid
3. Thymine
4. Marker

TABLE 5

Recoveries of radioactivity in ethanol extracts of samples removed when thymine grown organisms were incubated with thymine-methyl-C^{14} in the presence of diethyl malonate

Incubation time (min)	Dpm	% recovery
0	6,600	100
15	6,200	94
30	5,800	88
60	5,000	76

A. Radiochromatogram scanning of ethanol extracts

Radiochromatograms were developed using the following solvent systems.

1. N-butanol:acetic acid:H_2O (50:25:25 $^V/_V$)
11. Tert-butanol:methyl ethyl ketone:H_2O (40:30:25 $^V/_V$) in an ammonia atmosphere.

For the sample removed after 15 min incubation radiochromatogram scans showed apart from the major peak corresponding to the thymine marker, small peaks corresponding to the 5-methylbarbituric acid and methylmalonic acid markers respectively. This is exemplified in a scan obtained from a radiochromatogram developed with solvent 1. (Figure 15A). Scans also showed a small peak corresponding to unknown A.

For the sample removed after 30 min incubation radiochromatogram scans provided similar information. However the relative radioactivity

corresponding to the 5-methylbarbituric acid marker had increased, accounting for 18% of the total radioactivity of the material scanned. The band giving the peak was clearly discernable under ultraviolet light. Scans further showed an increase in relative radioactivity corresponding to the methylmalonic acid marker, however an accurate estimation was not obtainable.

The separation of radioactive compounds is shown in a scan (Figure 15B) of a radiochromatogram developed with solvent 1. Scans also showed a peak which appeared to be given by unknown A.

Radiochromatogram scans for the 60 min sample showed the peaks corresponding to 5-methylbarbituric acid and methylmalonic acid markers to be prominent. This is exemplified in the scan (Figure 15C) obtained from a radiochromatogram developed with solvent 1.

From scans, the peaks corresponding to methylmalonic acid and 5-methylbarbituric acid, together were estimated to account for almost 70% of the radioactivity of the material scanned. This contrasted with the results from the scan obtained for the sample removed at the same time for cells metabolising in the absence of diethylmalonate (Figure 13C) in which there was no radioactivity corresponding to the 5-methylbarbituric acid and methylmalonic acid markers.

B. Further evidence for the presence of 5-methylbarbituric acid and methylmalonic acid

Approximately 0.5ml of radioactive ethanol extract from a 60min sample, was streaked on a 10 x 20 cm thin layer plate (cellulose: thick-

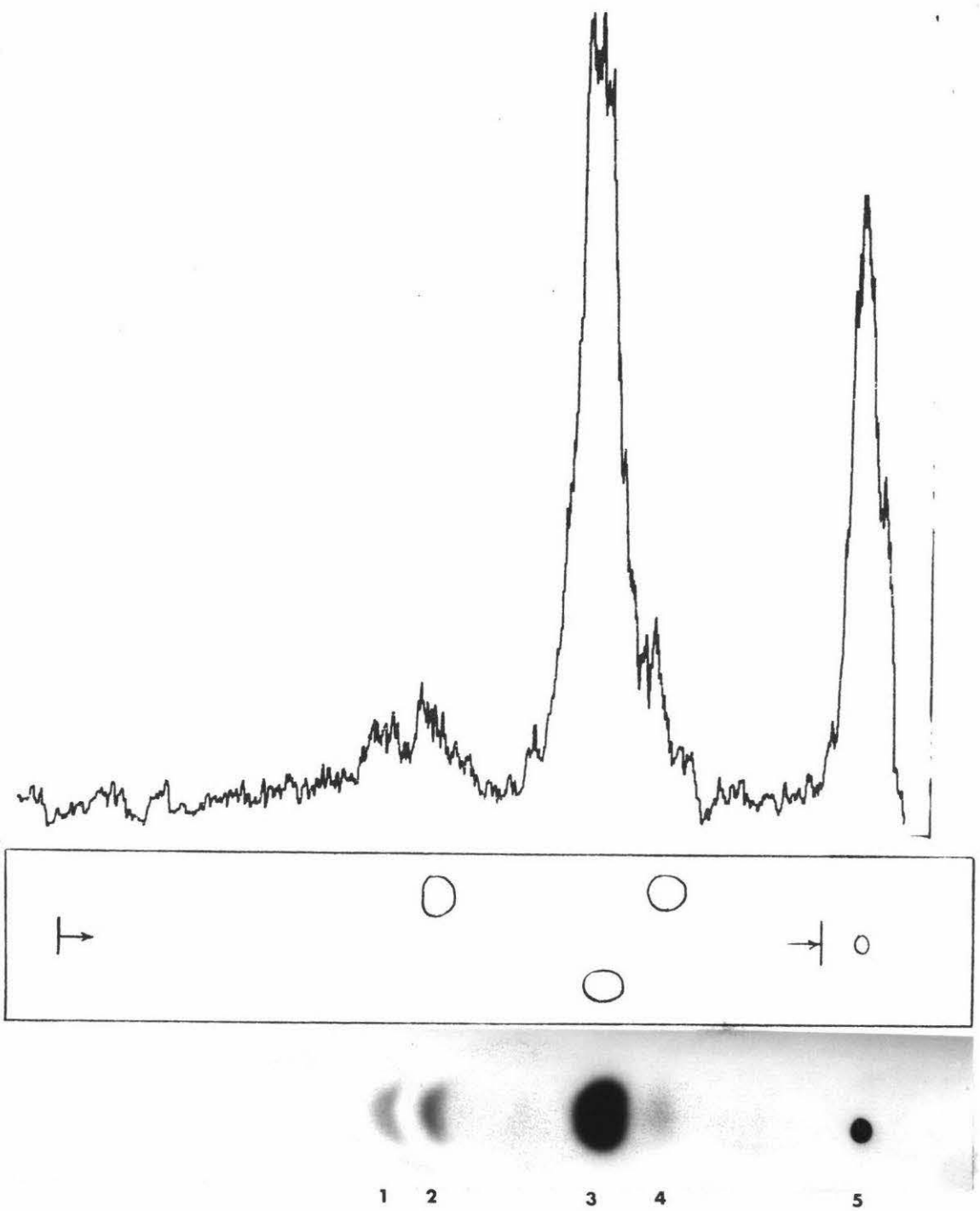


Figure 15A.

Radiochromatogram scan of ethanol extract obtained for the sample removed at 15 min, when thymine grown cells were incubated with thymine-methyl-C^{14} in the presence of diethylmalonate.

Key:
1. Unknown A (tentative)
2. 5–Methylbarbituric acid
3. Thymine
4. Methylmalonic acid
5. Marker

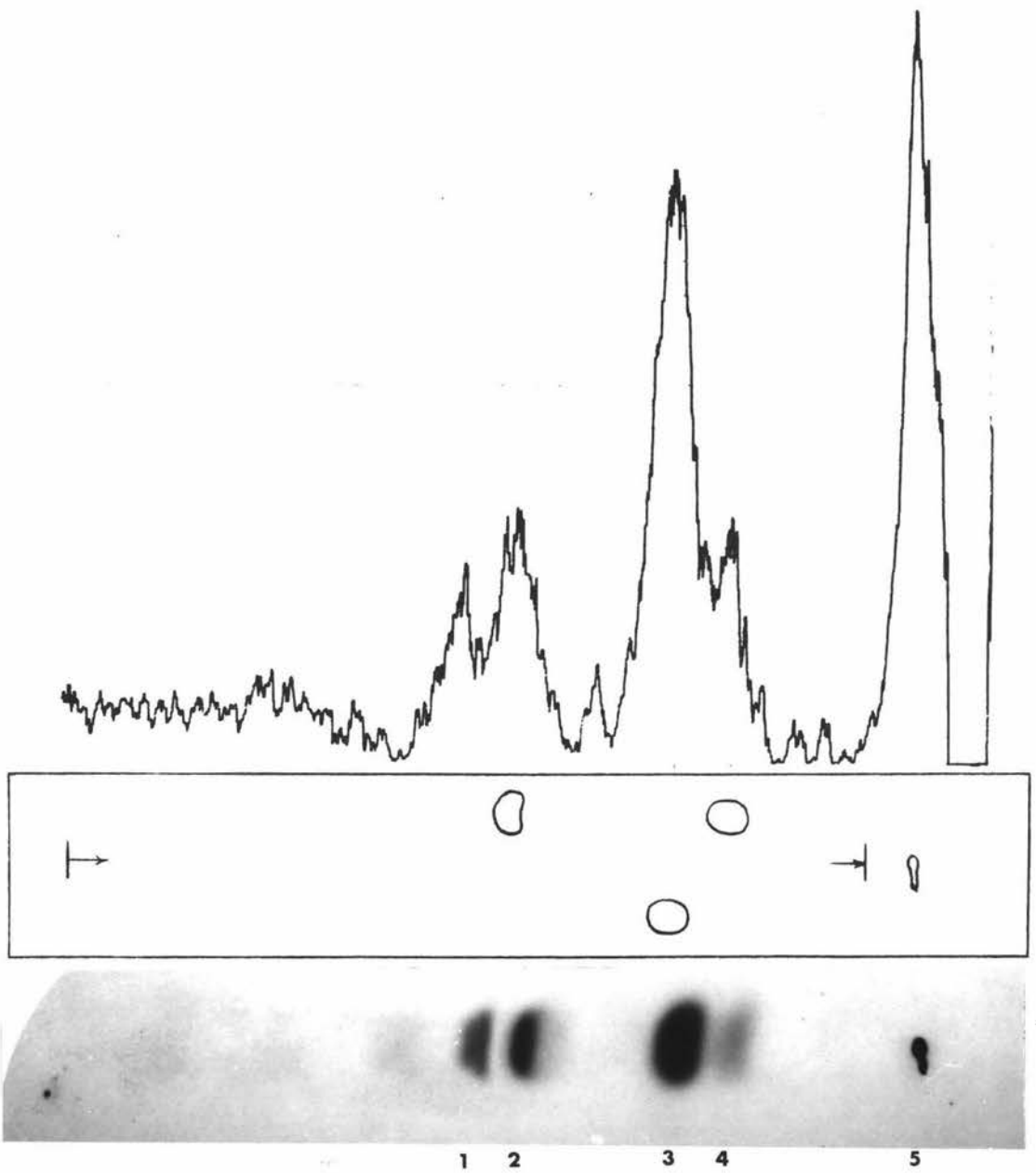


Figure 15B.

Radiochromatogram scan of ethanol extract obtained for the sample removed at 30 min, when thymine grown cells were incubated with thymine-methyl-C^{14} in the presence of diethylmalonate.

Key:
1. Unknown A and B (tentative)
2. 5–Methylbarbituric acid
3. Thymine
4. Methylmalonic acid
5. Marker

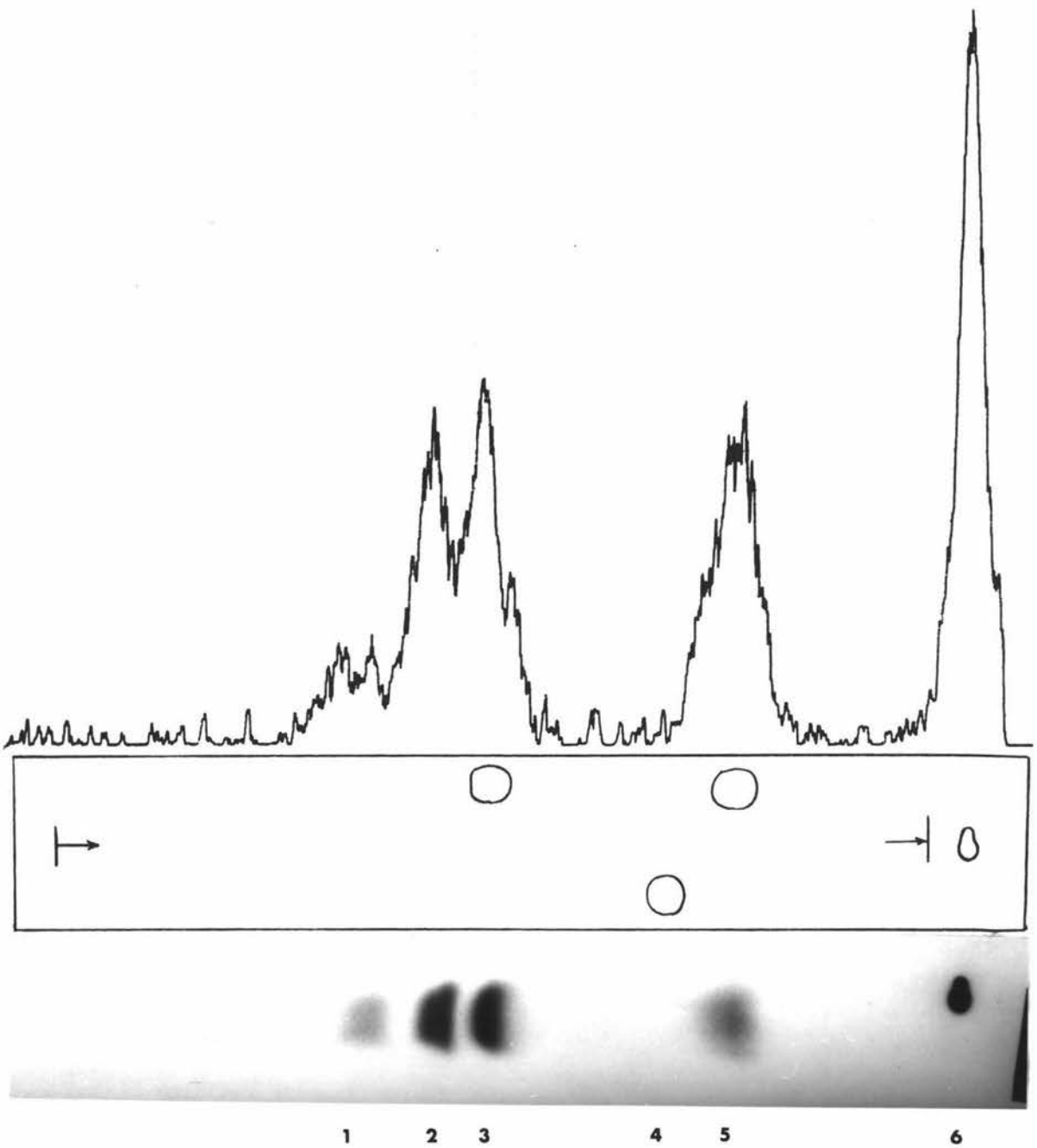


Figure 15C.

Radiochromatogram scan of ethanol extract obtained for the sample removed at 60 min, after thymine grown cells had been incubated with thymine–methyl–C^{14} in the presence of diethylmalonate.

Key:
1. Unknown
2. Unknown A and B (tentative)
3. 5-Methylbarbituric acid
4. Thymine
5. Methylmalonic acid
6. Marker

ness 0.5mm) using a Shandon sample streaker (London, England). Methylmalonic and 5-methylbarbituric acid were also spotted at positions corresponding to the streak on either side of the plate. The chromatogram was developed using a solvent of N-butanol:acetic acid:H_2O (50:25:25 $^{V}/_{V}$).

After the chromatogram had been dried, cellulose was immediately scraped off in the bands corresponding to 5-methylbarbituric acid and methylmalonic acid, and the radioactive material from each band separately eluted with H_2O. Successive elutions and centrifugations were carried out until only a small amount of radioactivity remained associated with the cellulose from each band.

Ultraviolet spectrophotometry of the material from the ultraviolet absorbing band, revealed the presence of a compound having spectral characteristics identical to 5-methylbarbituric acid with λ max values in 0.1M HCl and 0.1M NaOH at 262nm and 269nm respectively.

Material from the band corresponding to methylmalonic acid was concentrated in the presence of ethanol under reduced pressure with heating at 80°C. Concentrated radioactive material was spotted on a 20 x 20 cm thin layer plate, at an amount equivalent to 3,500 dpm. Malic, malonic, methylmalonic, succinic and isocitric acid standards were also spotted over the same point.

The radiochromatogram was developed two dimensionally using amyl alcohol:formic acid:H_2O (40:40:2 $^{V}/_{V}$) and N-butanol:acetic acid:H_2O (50:25:25 $^{V}/_{V}$) as the first and second solvent systems respectively.

To help identify components separated in the radiochromatogram on the basis of R_f values, standards were also chromatographed in one dimension. One plate was run in amyl alcohol:formic acid:H_2O and the other in N-butanol:acetic acid:H_2O simultaneously with the development of the radiochromatogram.

By this method all acids were identified on the developed radiochromatogram except for one which was found to be an impurity in the citric acid sample.

Autoradiography of the radiochromatogram showed virtually all of the radioactivity corresponding to methylmalonic acid (Figure 16). Traces of radioactivity were also found in succinic acid.

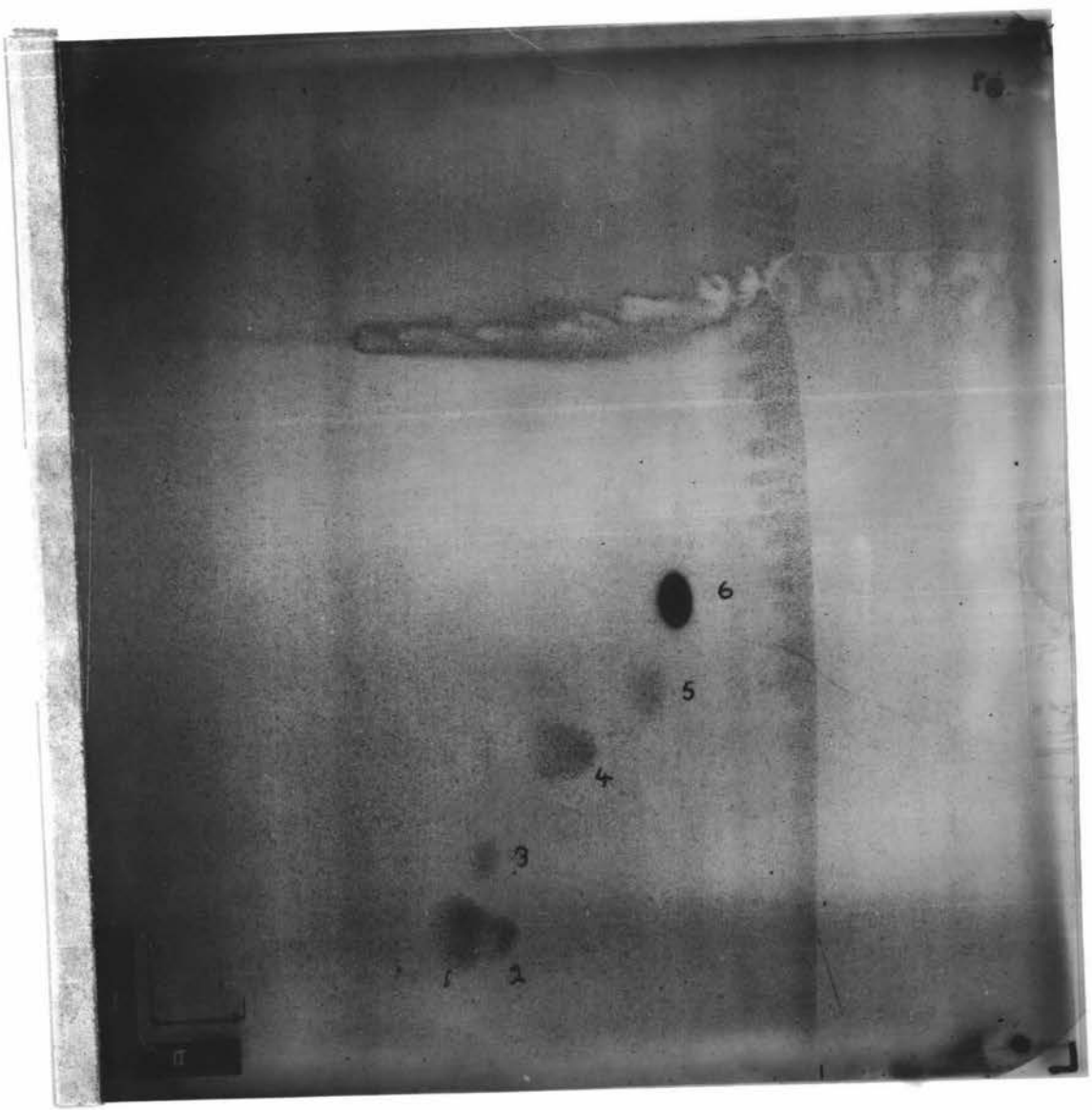


Figure 16.

Autoradiograph of radiochromatogram for radioactive material removed by preparative thin layer chromatography in band corresponding to methylmalonic acid.

Key:
1. Citric acid
2. Impurity in citric acid
3. Malic acid
4. Malonic acid
5. Succinic acid
6. Methylmalonic acid

IV Summary

a. When thymine grown organisms degraded thymine-methyl-C^{14} in the absence of diethyl malonate.

i. Radioactive 5-methylbarbituric acid and methylmalonic acid could not be detected by radio-isotope scanning or autoradiography.

ii. At least three unidentified radioactive compounds (non-ultraviolet absorbing) were detected. These were tentatively referred to as unknowns A, B, and C.

b. When uracil grown organisms catabolised thymine-methyl-C^{14} in the absence of diethyl malonate.

i. A radioactive compound was identified as 5-methylbarbituric acid, which absorbed under ultraviolet light.

ii. Another radioactive compound (non-ultraviolet absorbing) was detected and appeared to be that of unknown A.

c. When thymine grown organisms metabolised thymine-methyl-C^{14} in the presence of diethyl malonate.

i. Two radioactive compounds accumulated which were identified by radioisotope scanning and autoradiography as 5-methylbarbituric acid and methylmalonic acid.

ii. Unidentified radioactive compounds which appeared to be those of unknown A and perhaps unknown B were also detected.

SECTION III

DISCUSSION

From the evidence presented in this investigation there is little doubt that 5-methylbarbituric acid is a primary intermediate in the oxidative catabolism of thymine in N. corallina. Studies with uracil grown organisms had shown that 5-methylbarbituric acid accumulated almost quantitatively over the first phase of thymine oxidation. Such findings were consistent with those of Batt and Woods (1961).

With thymine grown organisms 5-methylbarbituric acid accumulated only when cells metabolised thymine in the presence of diethyl malonate. The extent of the accumulation amounted to 30% of the original thymine concentration.

Results from this investigation did not support the claim by Batt (1961) that 5-hydroxymethyluracil is oxidised by thymine grown organisms and thus the role of this compound as a possible intermediate of thymine catabolism remains doubtful.

The initial step in the breakdown of 5-methylbarbituric acid was suggested to be enzymatic from studies with chloramphenicol, an inhibitor of protein synthesis. The inhibitor was used to prevent the synthesis of the inducible enzyme or enzymes thought to act upon 5-methylbarbituric acid. In the presence of chloramphenicol, the utilisation of 5-methylbarbituric acid by uracil grown organisms was markedly suppressed. This indicated that the synthesis of the enzyme system acting on 5-methylbarbituric acid was inhibited.

The enzymatic nature of 5-methylbarbituric acid breakdown was also suggested from the observation that thymine grown organisms utilised 5-methylbarbituric acid more rapidly than uracil-grown organisms.

Although studies with chloramphenicol suggested that the initial reaction in the degradation of 5-methylbarbituric acid was enzymatic, cell free extracts of thymine grown organisms were found to be inactive towards the compound, even in the presence of ATP, NAD^+ and $NADP^+$. The same extracts were active towards barbituric acid, in containing barbiturase.

The failure to demonstrate activity of bacterial cell free extracts towards 5-methylbarbituric acid has been evident in studies by other workers.

Lara (1952) found that cell free extracts from a pyrimidine adapted strain of N. corallina were active towards thymine, uracil, and barbituric acid, but not 5-methylbarbituric acid. Similar results were obtained by Hayaishi and Kornberg (1952) working with cell free extracts of Mycobacterium and Corynebacterium.

Biggs and Doumas (1963) found that although cell free extracts of a Corynebacterium sp. were active towards barbituric acid, they were inactive towards 5-methylbarbituric acid.

The inactivity of bacterial cell free extracts towards 5-methyl-barbituric acid suggests that the enzyme or enzymes active upon the compound are either very labile or a complex set of factors are required

for activity.

On one occasion it was claimed that 5-methylbarbituric acid was degraded via barbituric acid. Lara (1952) showed that thymine-grown organisms of N. corallina oxidised barbituric acid more rapidly than yeast extract grown organisms. Batt and Woods (1961) demonstrated that uracil adapted N. corallina oxidised barbituric acid more rapidly than thymine grown organisms.

The results implied that the level of enzyme, active upon barbituric acid was higher in uracil grown organisms than those grown on thymine which in turn had a higher level of activity than glucose-grown organisms. Studies by Brennan (unpublished work) confirmed this finding in demonstrating that the specific activity of barbiturase from thymine grown organisms was 10% of that for the enzyme from uracil grown organisms. Barbiturase from glucose grown organisms was found to have a specific activity of less than 0.01% of that for the enzyme from thymine grown organisms.

The results from studies on barbiturase levels would be consistent with the possibility that in thymine grown organisms the decomposition of 5-methylbarbituric acid could occur at least in part via barbituric acid. However, the high level of barbiturase in uracil grown organisms does not necessarily imply that 5-methylbarbituric acid breakdown is initiated by a demethylation.

The results from this investigation provide little evidence to either support or reject the possible existence of a pathway for thymine

catabolism, in which 5-methylbarbituric is demethylated to give barbituric acid.

In studies on the catabolism of thymine-methyl-C^{14} by thymine grown organisms several radioactive compounds were detected but not identified (Figure 13A, B and C). The possibility that these compounds could have been produced as a result of demethylation of 5-methyl-C^{14} barbituric acid has not been examined experimentally.

The accumulation of 5-methylbarbituric acid in the presence of diethyl malonate would seem to be due to a mechanism analogous to negative feedback inhibition. Malonate produced by intracellular hydrolyses of diethyl malonate could be directly inhibiting the enzyme which acts on 5-methylbarbituric acid.

Diethyl malonate was shown to have a further effect during thymine catabolism in thymine grown organisms by evoking the accumulation of a compound identified as methylmalonic acid. The mechanism causing this accumulation has not been established, but it is reasonable to suggest that the de-esterified malonate acts as a competitive inhibitor on the enzyme acting upon methylmalonic acid.

The observation that in the presence of diethyl malonate, methylmalonic acid accumulated together with 5-methylbarbituric acid, suggests that the compound is an intermediate of thymine catabolism. This would be consistent with the conclusion of Biggs and Doumas (1963) from a study on the oxidative catabolism of thymine in Corynebacterium. These workers provided evidence that methylmalonic acid was derived

directly from 5-methylbarbituric acid.

Further work is required before methylmalonic acid can be definitely established as an intermediate on the only pathway for thymine catabolism in this organism. Such studies should also include a more detailed investigation of thymine catabolism by uracil grown organisms.

The possibility that a pathway exists for thymine catabolism via barbituric acid certainly requires further investigation. Although the unidentified radioactive compounds formed as a result of thymine-methyl-C^{14} catabolism, would appear to have occurred at later stages on the metabolic sequence or sequences, the possibility that any of these, were formed as a result of 5-methylbarbituric acid demethylation cannot be rejected. It would not only seem important to identify these compounds, but also study in more detail the catabolism of thymine labelled in the pyrimidine ring.

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APPENDIX I

A. Glucose culture medium

KH_2PO_4	20.41 gm.
$(NH_4)_2SO_4$	4.50 gm.
Thiamine HCl (Vit B_1)	0.037 gm.

were dissolved in approximately 1350 ml of distilled water. After neutralisation with 5M NaOH (pH 7.0)

Mg SO_4:$7H_2O$	0.15 gm

was added. The total solution was adjusted to 1425 ml. and dispensed evenly into 5 conical flasks (1 L.) fitted with muslin coated cotton wool plugs.

Glucose	13.5 gm

was dissolved in 90 ml of glass distilled water in a 250 ml conical flask which was plugged with muslin coated cotton wool. All solutions were sterilised by autoclaving (15 min: 121°C). After cooling, 15 ml of glucose solution was pipetted aseptically into each conical flask giving a final volume of 300 ml of phosphate buffered medium/flask.

B. Pyrimidine culture medium

KH_2PO_4	24.5 gm
$MgSO_4$:$7H_2O$	0.18 gm
Uracil or thymine	3.6 gm
Thiamine HCl (Vit B_1)	0.045 gm

were dissolved in approximately 1,500 ml of distilled water. The medium was then neutralised (pH 7.0) with 5N NaOH and the volume was then made up to 1.8 litres. This was dispensed evenly into 6 conical flasks (1 L). which were each plugged with muslin coated cotton wool.

Flasks were autoclaved (15 min at 121°C) allowed to cool, and were ready for inoculation.

C. Agar medium for slope cultures

KH_2PO_4	1.70 gm
$(NH_4)_2SO_4$	1.50 gm
Oxoid Yeast Ext	0.25 gm
Thiamine HCl (Vit B_1)	0.025 gm
Glucose	3.75 gm

were dissolved in approximately 450 ml of distilled water and neutralised with 5M NaOH

$MgSO_4:7H_2O$	0.05 gm

was dissolved in several ml of distilled water and added. The final volume was adjusted to 0.5L and 10 gm of Agar (Davis) was added. The preparation was heated and prior to autoclaving, was poured into McCartney bottles for slopes.

APPENDIX II

A. The preparation of diazomethane

(Based on the method of Schlenk and Gellerman 1960)

35 ml of methyl cellulose (2-methoxyethanol), 50 ml of ether, and 10 ml of 60% KOH were placed in a 250 ml distilling flask, which was cooled for 10 min in an ice-bucket. Approximately 1g of N-methyl-N-nitrosotoluene-p-sulphonamide was added to the mixture which was allowed to stand 30 minutes. The flask was then attached to a splash head and receiving condenser and the contents were warmed to about $70^{\circ}C$. The distillate was collected in a 50 ml receiving tube placed in ice.

The preparation was carried out in a fume cupboard.

B. The preparation of 5-hydroxymethyluracil

(Based on the method of Fink, Fink and Cline, 1959)

32 ml of 0.5N KOH, 2.24g of uracil, and 2 ml of 37% HCHO were mixed and allowed to stand for two weeks at room temperature. Dowex 50 W-X (100 - 200 mesh H^{+}) was added to neutralise the solution the cation exchange resin was then filtered off, and the solution concentrated in vacuo, then refrigerated. Crystals were obtained by filtration, and recrystallisation was carried out with ethanol:H_2O (1:1 V/V) the product which had a melting point of 310° was obtained in a yield of 70% (tentative).

Tests for homogenuity using thin layer chromatography and N.M.R. Spectroscopy revealed that the product was impure containing a signi-

ficant quantity of uracil.

Preparative thin layer chromatography, with ethyl acetate:H_2O: HCl conc (60:35:5 $^{V}/_{V}$) as the solvent, was used to separate the uracil impurity. From elution with H_2O and recrystallisation with Ethanol: H_2O (1:1 $^{V}/_{V}$) 5-hydroxymethyluracil was obtained at a purity of greater than 90%, as established by N.M.R. Spectroscopic data.

Ultraviolet spectrophotometry was used to estimate the extinction coefficients of the compound in 0.1M NaOH, which were as follows

a. $E^{1cm}_{286\ gm} = 8.1 \times 10^3$

b. $E^{1cm}_{245\ nm} = 2.45 \times 10^3$

C. <u>The preparation of 5-methylbarbituric acid</u>

<u>(Based on the method of Holmberg, 1945)</u>

Sodium (2.5g) was dissolved in absolute alcohol (40ml) 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 a reflux for 4 hr at 115° - 120°C. Absolute alcohol (50ml) 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. 5-methylbarbituric 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 (3g) obtained after washing with absolute ethanol and drying over $CaCl_2$ in a dessicator, had a melting point of

195°C and the following extinction coefficients

i. In 0.1M HCl $E^{1.0cm}_{262nm} = 520$

ii. In 0.1M NaOH $E^{1.0cm}_{269nm} = 1.67 \times 10^4$

D. The preparation of 5-hydroxy-5-methylbarbituric acid

(Based on the method of Biggs and Doumas, 1962)

5-methylbarbituric acid (0.5g) was shaken in 30ml of 3% H_2O_2 until the absorption at 269nm disappeared. The solution was then evaporated to dryness at 60° under reduced pressure, and the residue was dissolved in water and taken again to dryness under reduced pressure. After being dried over $CaCl_2$ the compound was recovered in almost quantitative yield, and had a melting point of 226°C.