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BARBITURIC ACID METABOLISM IN
NOCARDIA CORALLINA

A thesis presented in partial fulfilment of the
requirements for the degree of M.Sc. in Biochemistry
at Massey University.

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ABSTRACT

Barbituric acid utilisation in Nocardia corallina has been investigated. The enzyme barbiturase was induced using barbituric acid as the sole carbon and nitrogen source and a study of the optimum conditions for isolation of the enzyme was undertaken. Although it proved to be somewhat unstable, a partial purification was achieved.

Studies of barbituric acid utilisation were undertaken and it was shown that both cell-free extracts and the partially-purified enzyme were capable of the formation of an activated form of malonic acid from barbituric acid. Activation could not be demonstrated using malonic acid as substrate.

It is suggested that the barbiturase activity observed in vitro, i.e. malonic acid formation, is an artifact of isolation and that in vivo barbituric acid is utilised via malonyl-CoA.

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1.

INTRODUCTION

In living organisms there are both oxidative and reductive pathways for pyrimidine catabolism. Reductive catabolism of uracil and thymine involves an initial conversion to the corresponding dihydropyrimidine, while oxidative catabolism yields the respective barbituric acids as the initial products. Thymine may also be converted to uracil by the oxidative thymine-7-hydroxylase pathway in both animals and some micro-organisms.

I. Reductive Catabolism

The initial step in reductive catabolism is the formation of a dihydropyrimidine by an enzyme-catalysed reaction which requires either $\text{NADH} + \text{H}^+$ or $\text{NADPH} + \text{H}^+$, depending upon the source of the enzyme. This reaction is followed by a series of hydrolytic steps, shown in Figure 1, which ultimately lead to the formation of a β -amino acid, ammonia and carbon dioxide. Studies with ^{14}C -labelled uracil and thymine in both rat liver slices and intact rats, with supporting evidence from other mammalian species, reported by Fink et al. (1953 and 1956), Canellakis (1956), Fritzon (1957) and Fritzon and Pihl (1957) clearly demonstrated the existence of this pathway in animals.

Reductive catabolism has also been claimed to exist in bacteria. In 1952, DiCarlo et al. proposed a reductive pathway for cytosine catabolism, in Torula utilis, which

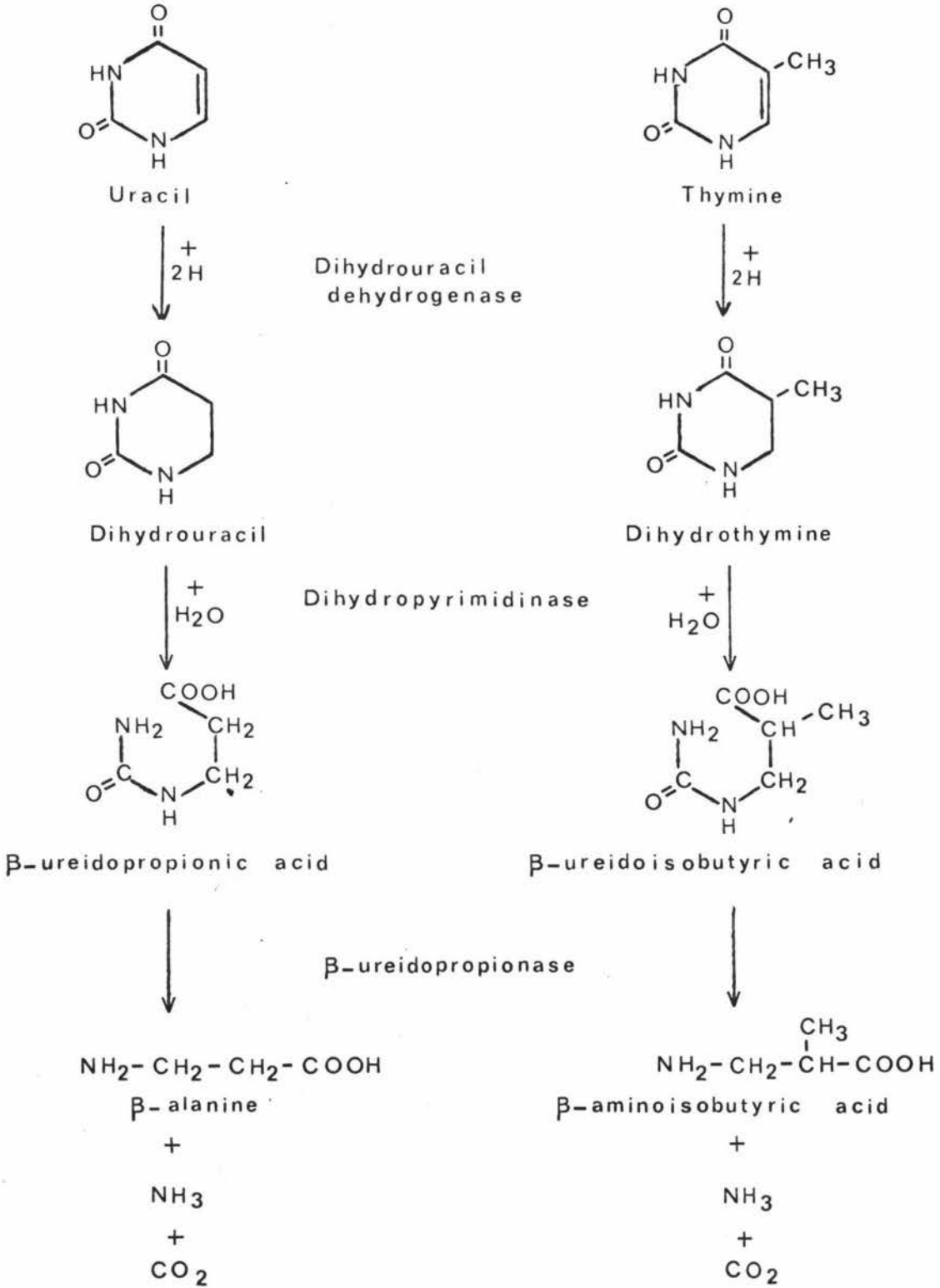


Figure 1. Pathway for the reductive catabolism of uracil and thymine.

involved dihydroorotic acid. However, Batt et al. (1953 and 1954) showed that this was unlikely since authentic dihydroorotic acid was not degraded by the organism. The formation of dihydropyrimidines and β -amino acids from uracil and thymine was demonstrated with Pseudomonas aeruginosa by Fink et al. in 1954, and an analogous reductive pathway for orotic acid degradation in Zymobacterium oroticum and some Corynebacterium species was similarly described by Lieberman and Kornberg (1953, 1954, 1955) and Reynolds et al. (1955). In 1957, Campbell (1957 a,b and c) showed that this pathway existed in Clostridium uracilium, and partially purified the enzyme uracil dehydrogenase.

In 1969, Kraemer and Kaltwasser (1969) showed that cell-free extracts of Hydrogenomonas facilis, grown on cytosine, converted cytosine to uracil and then to β -alanine, carbon dioxide and ammonia, with dihydrouracil and β -ureidopropionic acid as intermediates. Studies with mutants of this organism (1969b) indicated that the dehydrogenase and hydrase enzymes were non-specific with respect to uracil and thymine.

The existence of this pathway for uracil, thymine and orotic acid catabolism has also been demonstrated in plants, by Evans and Axelrod (1961) and Ross (1965), and in Chlorella fusca by Knutsen (1972).

II. Oxidative Catabolism Through Barbituric Acids

Investigations by Cerecedo (1927, 1930 and 1931) of pyrimidine catabolism in dogs suggested that uracil and

thymine were oxidatively catabolised and the pathway of uracil catabolism proposed at that time is shown in Figure 2. Cerecedo also claimed that thymine was degraded through thymine glycol. The work of Fink et al. (1953 and 1956), Fritzon (1957) and Fritzon and Pihl (1957) has, however, established that the main pathway for pyrimidine catabolism in animals is reductive.

The first reports on the current schemes for oxidative catabolism of pyrimidines in microorganisms appeared in 1951 (Batt and Woods; Wang and Lampen; Hayaishi and Kornberg). In 1952, these were followed by more detailed reports (Wang and Lampen; Hayaish; Lara; and Hayaishi and Kornberg), leading to the proposed pathways for oxidative catabolism through barbituric acids as shown in Figure 3.

Wang and Lampen (1951) reported the isolation of a soil bacterium which grew aerobically utilising uracil, thymine or cytosine as the sole source of nitrogen and carbon. Resting cells oxidised the pyrimidines to carbon dioxide and ammonia, while cell-free extracts oxidised uracil and thymine, in the presence of methylene blue and air, with an uptake of one atom of oxygen per mole of pyrimidine oxidised. Later, the organism was tentatively identified as a Bacterium species, (1952 a), and the only pyrimidines found which supported growth were cytosine, uracil, thymine and barbituric acid. The data suggested that the 6-position of the ring was initially oxidised. In a subsequent paper (1952 b), they showed that, in the

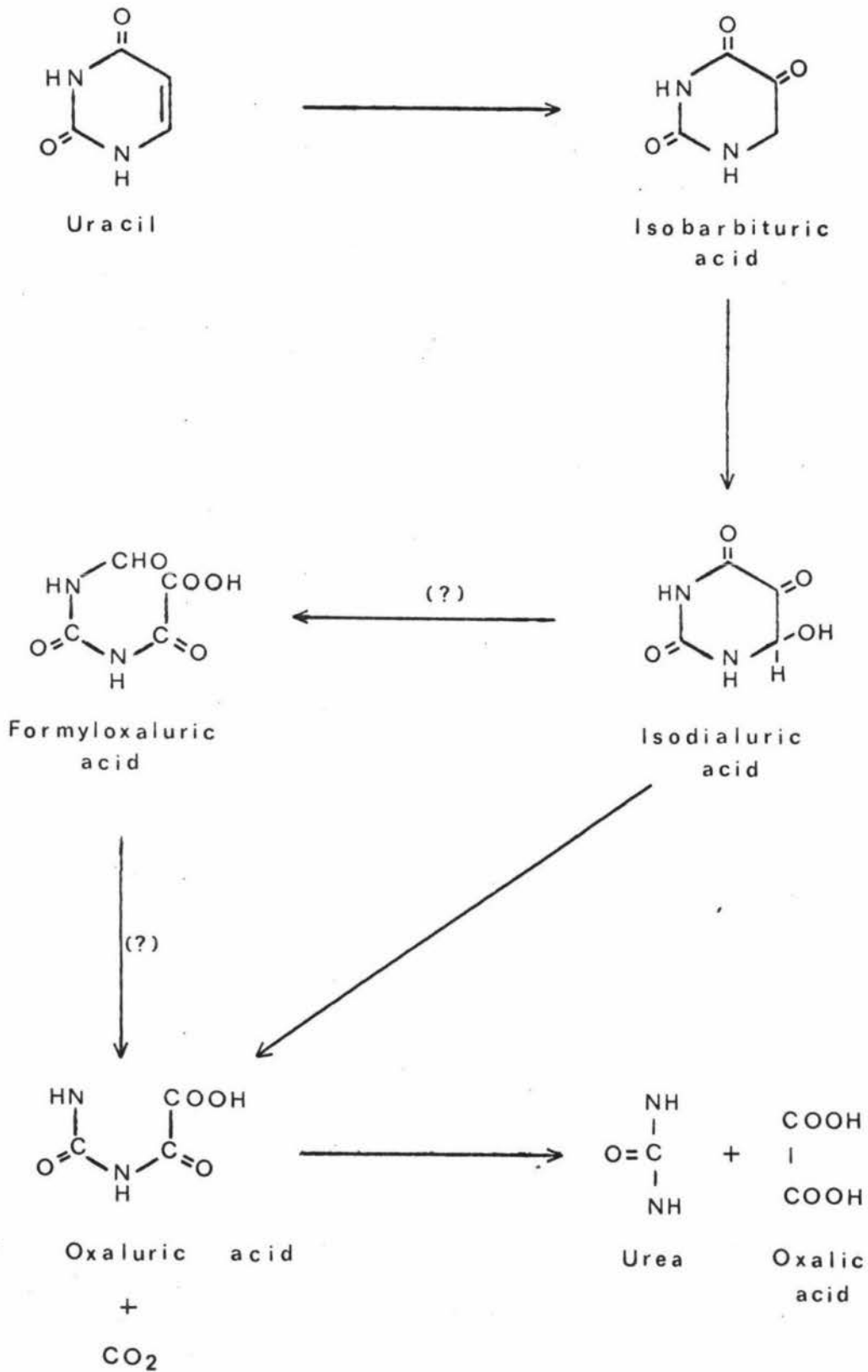


Figure 2. Pathway for the oxidative catabolism of uracil proposed by Cerecedo (1931).

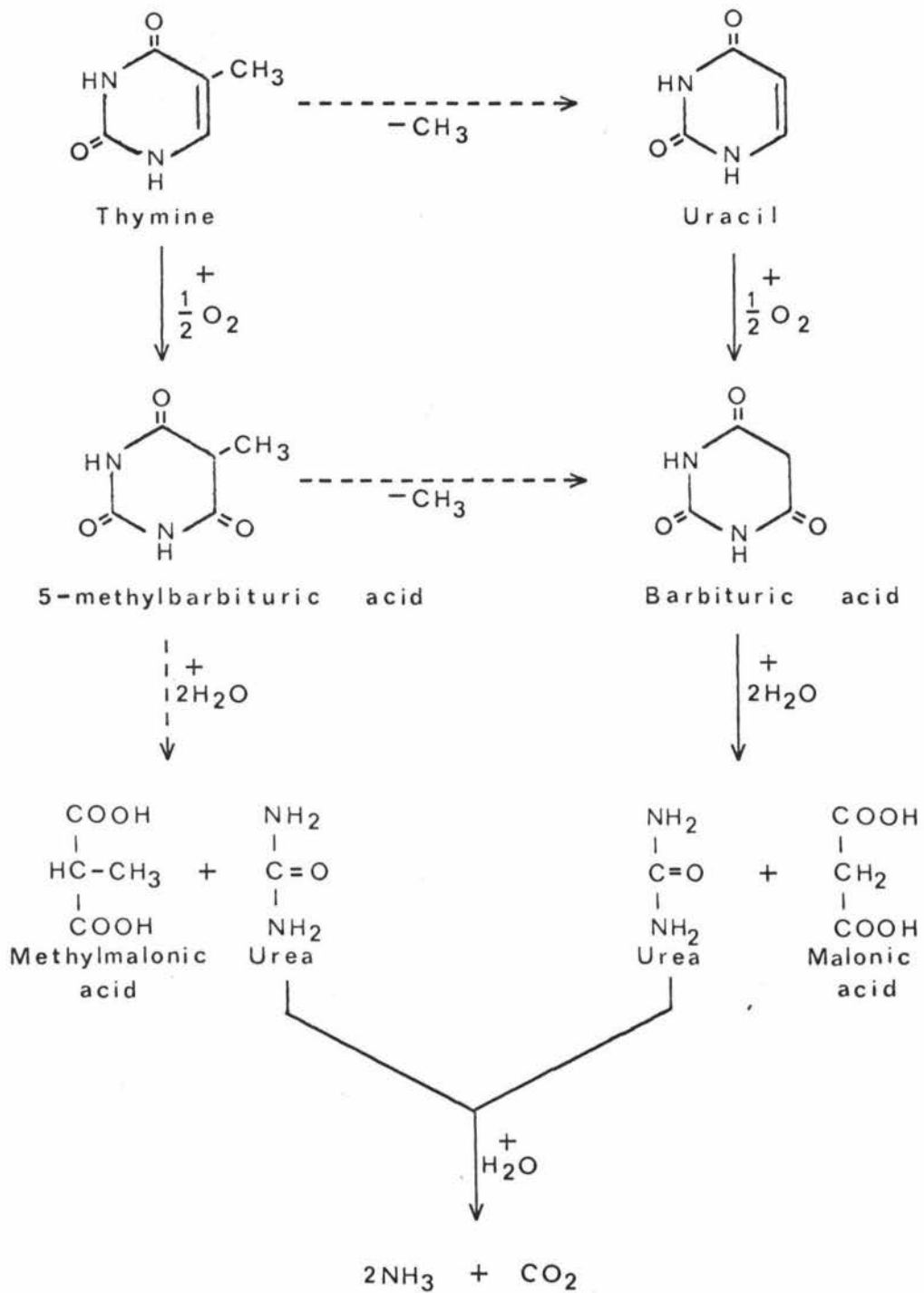


Figure 3. Pathways in bacteria for the oxidative catabolism of uracil and thymine through barbituric acids.

presence of oxygen and methylene blue, uracil was oxidised to barbituric acid and they proposed the name, uracil oxidase, for the enzyme catalysing this reaction.

In 1951, Batt and Woods also reported the isolation of soil organisms capable of oxidising pyrimidines. Suspensions of one isolate utilised thymine and uracil, but not cytosine, and cells grown on uracil oxidised thymine to a product with a λ_{max} of 268 nm, later shown to be 5-methylbarbituric acid. The organism was subsequently identified as a strain of Nocardia corallina.

Hayaishi and Kornberg (1951 and 1952) and Hayaishi (1952) isolated species of Mycobacterium and Corynebacterium capable of growing on uracil and thymine. Cells adapted to these compounds could utilise barbituric acid aerobically, but not cytosine, 5-methylcytosine, isobarbituric acid, 6-methyluracil, dihydrouracil, 2-thiouracil or 2-thio-5-methyluracil. The immediate product of uracil oxidation, in whole cells, was shown to be barbituric acid, and the so-called, uracil-thymine oxidase, partially purified from cell-free extracts, oxidised uracil and thymine to barbituric acid and 5-methylbarbituric acid respectively. The oxidase was reported to be inactive against barbituric acid, isobarbituric acid, dihydrothymine, dihydrouracil, 5-methylbarbituric acid, cytosine, 6-methyluracil, 2-thiouracil and 2-thio-5-methyluracil.

An enzyme active in hydrolysing barbituric acid, named barbiturase, was also partially purified from

pyrimidine-adapted cells, and the end products from its action were identified as urea and malonic acid. It was inactive against 5-methylbarbituric acid, orotic acid, barbital, pentobarbital, 2-thiobarbituric acid and isobarbituric acid. The optimum pH was found to be pH 8 to 9 and the Michaelis constant, $3.4 \times 10^{-3} \text{M}$.

Brennan (unpublished results, 1970), studying barbiturase from Nocardia corallina, found a pH optimum of 8.25 and a Michaelis constant of $7 \times 10^{-4} \text{M}$ for the enzyme.

Hayaishi and Kornberg (1952) did not establish the mechanism of barbituric acid hydrolysis but postulated an initial hydrolysis producing malonuric acid and a subsequent hydrolysis of this to give malonic acid and urea. No evidence was found for the accumulation of malonuric acid, but they suggested that the initial hydrolytic step may have been rate-limiting.

Other alternatives proposed were (a) a second enzyme hydrolysing malonuric acid (b) that malonuric acid broke down spontaneously or (c) that, in vivo, barbiturase produced urea and a labile derivative of malonic acid.

The isolation of Nocardia and Corynebacterium species capable of utilising thymine as the sole source of carbon and nitrogen was reported by Lara in 1952.

Organisms adapted to thymine were also adapted to uracil and barbituric acid and, on this basis, a pathway for the conversion of thymine to uracil, and subsequently to barbituric acid, was postulated. Work with Nocardia cell-free extracts (Lara, 1952b), however, showed that, in

the presence of methylene blue and oxygen, thymine and uracil were oxidised to 5-methylbarbituric acid and barbituric acid respectively. In only one experiment was uracil isolated as an intermediate in thymine degradation, and Lara concluded that this was not the normal pathway of thymine catabolism. Cell-free extracts hydrolysed barbituric acid producing one mole malonic acid, one mole carbon dioxide and two moles ammonia per mole barbituric acid, which suggested that the products of hydrolysis were urea and malonic acid.

Batt (1957, 1958 and 1961) showed that uracil, thymine, barbituric acid, 5-methylbarbituric acid, 6-methyluracil and 5-hydroxymethyluracil all induced a uracil-thymine oxidase in N. corallina, although 6-methyluracil did not support growth of the organism. The oxidase was non-specific and catalysed the conversion of uracil, thymine, 2-thiouracil and 2-thiothymine to their corresponding barbituric acids.

In a study of pyrimidine utilisation by N. corallina, Batt and Woods (1961) measured oxygen uptakes during pyrimidine catabolism, and observed values much lower than the theoretical values for complete conversion to carbon dioxide and ammonia. Uracil oxidation by thymine-grown cells proceeded almost entirely through barbituric acid, and thymine oxidation by uracil-grown cells proceeded through 5-methylbarbituric acid. However, the discrepancies in the oxygen uptake values led them to suggest that uracil oxidation by uracil-grown

cells, and thymine oxidation by thymine-grown cells, may not proceed entirely through the corresponding barbituric acids.

Intact uracil-grown organisms utilised barbituric acid only under aerobic conditions and the only end products detected were urea, carbon dioxide and ammonia. Azide and isobarbituric acid inhibited this oxidation in whole cells. By contrast, cell-free extracts degraded barbituric acid anaerobically to malonic acid, carbon dioxide and ammonia, with azide and isobarbituric acid having no inhibitory effect on the enzyme action.

Dicarboxylic acids were tested as carbon sources for growth with N. corallina; succinic, malic, fumaric and methylmalonic acids supported growth well, but ketomalonic, tartronic and malonic acids did not. Hayaishi and Kornberg (1952) had also noted poor growth of uracil-adapted cells on malonic acid and had attributed this finding to permeability barriers. Batt and Woods, however, showed that the presence of malonic acid in the growth medium inhibited succinate oxidation in N. corallina, suggesting that malonic acid was, in fact, entering the cells.

Hayaishi (1955) showed that cell-free extracts from Pseudomonas fluorescens converted malonic acid to acetic acid through malonyl-CoA and acetyl-CoA. Batt and Woods, using N. corallina extracts, tested barbituric acid, malonic acid and other carboxylic acids for activation but only acetic and propionic acids were

activated. They suggested an "active derivative" of malonate, formed directly from barbituric acid, is immediately metabolised without being converted to the free acid. Hayaishi and Kornberg had suggested a labile intermediate, as one of several possibilities, in 1952.

There is good evidence that the products of barbituric acid degradation are urea and malonic acid, or a derivative of malonic acid, but rather less is known about 5-methylbarbituric acid catabolism. It is oxidised by intact organisms but cell-free extracts from pyrimidine-adapted organisms are inactive towards it, even with extracts which can degrade barbituric acid. Hayaishi and Kornberg (1952) and Batt and Woods (1961) noted that 5-methylbarbituric acid is spontaneously oxidised to 5-hydroxy-5-methylbarbituric acid, and Doumas and Biggs (1962), who confirmed this finding, showed that 5-hydroxy-5-methylbarbituric acid undergoes slow spontaneous hydrolysis to methyltartronylurea. It was thought possible that 5-methylbarbituric acid might undergo spontaneous oxidation in intact cells, and this possibility was tested by both Batt and Woods (1961), and Doumas and Biggs (1962). Batt and Woods observed that 5-hydroxy-5-methylbarbituric acid was not produced when 5-methylbarbituric acid was incubated with N. corallina, and that cell suspensions of the organism were incapable of oxidising 5-hydroxy-5-methylbarbituric acid. In agreement with these findings, Doumas and Biggs, who substituted 5-hydroxy-5-methylbarbituric acid or methyltartronylurea for thymine in cell suspensions of Corynebacterium, showed that neither

compound supported growth. These results indicated that 5-hydroxy-5-methylbarbituric acid and methyltartronylurea were not intermediates in the catabolism of 5-methylbarbituric acid.

Biggs and Doumas (1963) found that while urea plus malonic acid gave poor growth of Corynebacterium, urea plus methylmalonic acid supported growth well. Using (5^{14}C -) 5-methylbarbituric acid as the substrate with intact cells, labelled methylmalonic acid could be isolated from the growth medium supernatant and the specific activity of the acid was 96% of the labelled compound added. In a similar experiment, labelled urea, with the same specific activity as the starting material, was isolated using (2^{14}C -)5-methylbarbituric acid as substrate. Cell-free extracts from these organisms showed uracil-thymine oxidase and barbiturase activities but were inactive with 5-methylbarbituric acid. On the basis of these labelling and growth experiments they proposed that 5-methylbarbituric acid hydrolysis was analogous to barbituric acid hydrolysis.

Mountford, in 1971, further investigated thymine catabolism in N. corallina. By using metabolic inhibitors and (^{14}C -)labelled substrates it was hoped that labelled intermediates would accumulate and that identification of these would confirm or reject the proposal of Batt and Woods (1961) that thymine oxidation by thymine-grown cells might proceed by a pathway not involving barbituric acids. Preliminary experiments showed that uracil-grown cells oxidised thymine to 5-methylbarbituric acid, which was

subsequently utilised, but further intermediates could not be isolated. Experiments conducted with chloramphenicol in the growth medium confirmed the presence of an inducible enzyme system for 5-methylbarbituric acid utilisation.

In the absence of inhibitors thymine-grown cells, utilising unlabelled thymine, did not accumulate UV-absorbing compounds, and in only one experiment was methylmalonic acid detected, in small amounts. Arsenite and mercuric chloride were found to inhibit thymine utilisation, however, they did not cause an accumulation of intermediates. With diethylmalonate present and thymine as substrate, 5-methylbarbituric acid and three other compounds, which were tentatively identified by GLC of the methyl esters as methylmalonic acid, malonic acid and succinic acid, accumulated.

In inhibitor-free systems uracil-grown cells, utilising (^{14}C)-thymine, accumulated 5-methylbarbituric acid, methylmalonic acid and an unidentified compound, A, all of which were labelled, while thymine grown cells, utilising labelled thymine, accumulated three unidentified labelled compounds; A, B and C. With diethylmalonate present and (^{14}C)-thymine as substrate, thymine-grown cells accumulated 5-methylbarbituric acid, methylmalonic acid and the compounds A and B, all of which were labelled.

These results were interpreted as confirming the findings of Biggs and Doumas (1963) that methylmalonic acid and 5-methylbarbituric acid are intermediates of

thymine catabolism. Mountford was unable to establish whether this was the only pathway of thymine catabolism and it is still likely, from other in vivo studies, that at least one alternative pathway exists.

III. Oxidative Catabolism Through 5-hydroxymethyluracil

The first report of 5-hydroxymethyluracil as an oxidation product of thymine catabolism came from Fink et al. (1956) who found that 5-hydroxymethyluracil and uracil-5-carboxylic acid accumulated, along with products from reductive catabolism, in rat liver slices incubated with labelled thymine. Thymine hydroxylation has also been shown to occur in Neurospora crassa and Rhodotorula glutinus.

Abbott et al. (1964) showed that in the presence of oxygen, glutathione and $\text{NADPH} + \text{H}^+$, cell-free extracts of N. crassa mycelia converted thymine to 5-hydroxymethyluracil. The name thymine-7-hydroxylase was proposed for the enzyme catalysing this reaction, and, in 1967, Abbott et al. showed that Fe^{2+} , ascorbate and 2-oxoglutarate were essential cofactors for the enzyme. In 1968, Abbott et al. reported that cell-free extracts from N. crassa converted 5-hydroxymethyluracil to 5-formyluracil, and in 1970, Watanabe et al. demonstrated that 5-formyluracil was converted to uracil-5-carboxylic acid by cell-free extracts. In both cases oxygen, Fe^{2+} , ascorbate and 2-oxoglutarate were required. Palmatier et al., in 1970, partially purified an enzyme which catalysed the

decarboxylation of uracil-5-carboxylic acid without a requirement for cofactors. The steps for this series of reactions are shown in Figure 4.

Subsequent work carried out by Holme et al. (1970), McCroskey et al. (1971), Holme et al. (1971) and Liu et al. (1972) has shown that each oxidation step is coupled to the decarboxylation of 2-oxoglutarate, with molecular oxygen being incorporated into both the oxidation product and succinic acid. The oxidation product, succinic acid and carbon dioxide are produced in equimolar amounts. In 1973, Liu et al. reported the purification of a single enzyme from N. crassa which catalysed all three oxidative steps in the conversion of thymine to uracil-5-carboxylic acid.

Reports have appeared in Chemical Abstracts of work by various Russian authors which suggest that similar pathways may exist in micro-organisms. Vilks et al. (1972) (CA 78:133216) reported that Rhodotorula glutinis utilised thymine as a sole nitrogen source, and a pathway of thymine to uracil was suggested. In 1973, Vilks and Vitols demonstrated that 5-hydroxymethyluracil and uracil-5-carboxylic acid accumulated when the yeast was grown using thymine as the sole nitrogen source, and that cell suspensions were capable of converting uracil-5-carboxylic acid to uracil.

Zvyagintseva and Vitols (1968) (CA 70:45068) showed that a mixed culture of Pseudomonas and Bacillus species

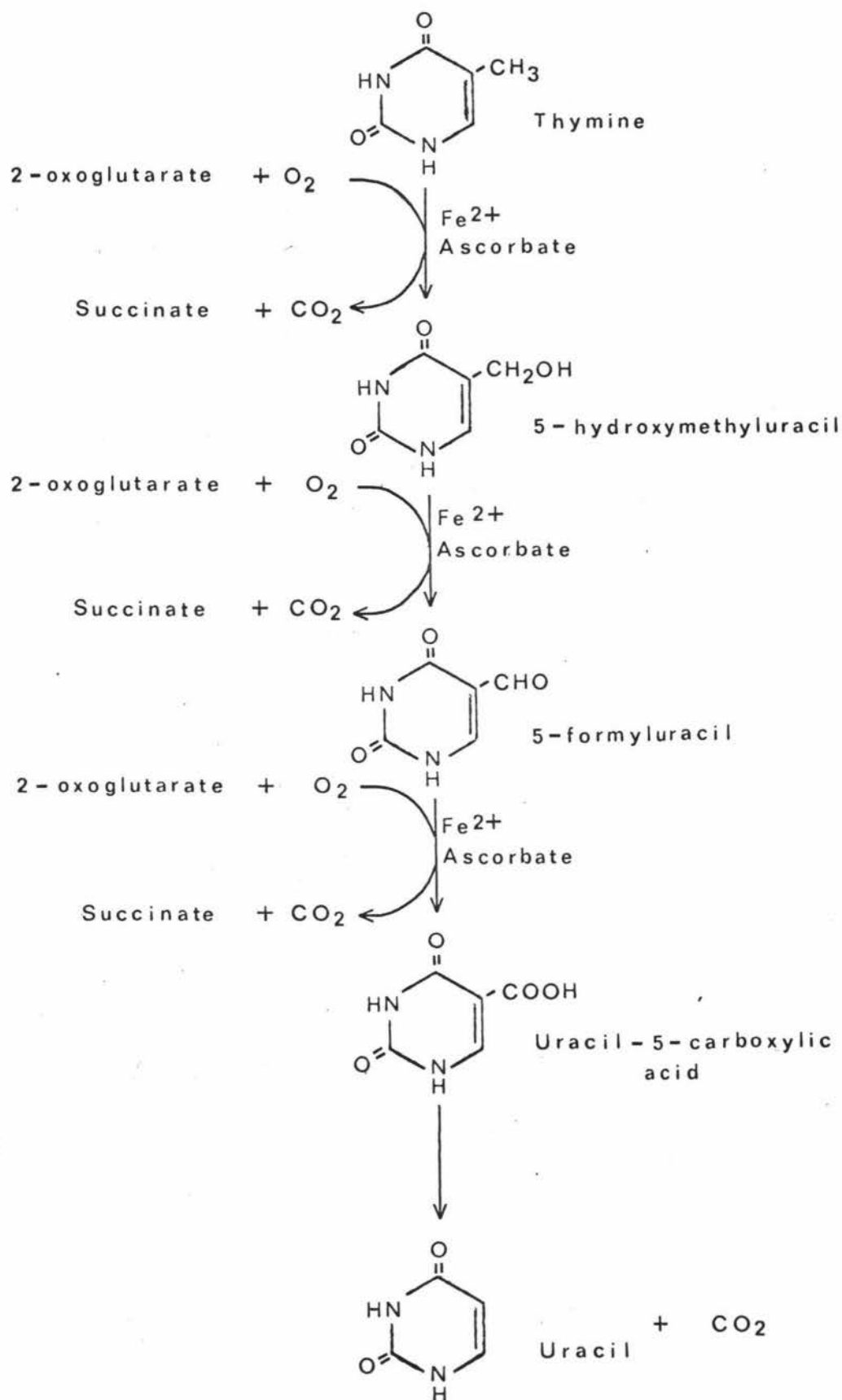


Figure 4. Pathway proposed for the oxidative demethylation of thymine (after Liu et al. 1973).

converted 6-methyluracil to 6-methyldihydrouracil and β -methyl- β -ureidopropionic acid, while Zvyagintseva and Mamulina (1969) (CA 72:39991) 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. Shvachkin et al. (1972) (CA 78:133221) showed that Mycobacterium species utilised L-isomers of β -(5-pyrimidyl)alanine derivatives as the sole source of nitrogen and that the alanine moiety was degraded first while the corresponding 5-hydroxypyrimidine accumulated in the culture medium.

IV. Aim Of The Investigation

A number of problems remain unsolved regarding oxidative pyrimidine catabolism in bacteria. These include

(a) The isolation and purification of the enzymes responsible for both the oxidation and hydrolytic steps.

(b) The existence of an enzyme for 5-methylbarbituric acid breakdown has not been conclusively demonstrated.

(c) There are anomalies regarding barbiturase activity in that, in vitro, malonic acid is produced although whole cells cannot utilise malonic acid for growth.

(d) The possibility that other oxidative pathways operate in vivo, e.g. the thymine hydroxylase pathway.

The objectives of the research were

(a) to isolate and purify the enzyme
barbiturase

(b) to investigate the anomalies regarding
barbiturase activity and the apparent non-utilisation of
malonic acid by pyrimidine-adapted bacteria.

2.

MATERIALS AND METHODSI. Materials

Materials used in this investigation were obtained from normal suppliers including BDH Ltd., (Poole, England), May and Baker, (Dagenham, England), Koch-Light Laboratories Ltd., (Colnbrook, England), Fluka AG, (Buchs, Switzerland), Riedel de Haen AG, (Hannover, Germany), and Ajax Chemicals Ltd., (Sydney, Australia). Some of the special compounds and materials were obtained as indicated below:

Coenzyme-A (Grade 1) and DEAE-Cellulose:
Sigma Chemical Corporation, (St. Louis, U.S.A.).

Sephadex G25 (Fine) and Sephadex G200 (Fine):
Pharmacia Fine Chemicals, (Uppsala, Sweden).

Microgranular DE52 DEAE-Cellulose and
Whatman No. 1 chromatography paper: W. and R. Balston,
(Maidstone, England).

Biogel P4 (100-200 mesh): Bio-rad
Laboratories, (Richmond, California, U.S.A.).

(^{14}C -)Malonic acid, sodium salt:
The Radiochemical Centre, (Amersham, England).

(^{14}C -)Malonyl-CoA: New England Nuclear,
(Boston, U.S.A.).

(5¹⁴C-)Barbituric acid, synthesised from (2¹⁴C-)malonic acid by the method of Vogel (1964) (Appendix 1).

Calcium phosphate gel, synthesised by the method of Kielin and Hartree (1938) (Appendix 2).

Streptomycin sulphate: Glaxo Laboratories (N.Z.) Ltd., (Palmerston North, New Zealand).

Dialysis tubing: Union Carbide Corporation, (Chicago, U.S.A.).

II. Methods

II.A. Analytical Techniques

II.A.1. Ultraviolet Spectrophotometry

Barbituric acid concentrations were estimated routinely by measuring the absorbance at 255 nm in a Hitachi 101 spectrophotometer. Samples were diluted with Tris/HCl buffer (0.05M, pH 8.0) to give an absorbance reading between 0.1 and 1.0.

II.A.2. Chromatography

II.A.2.a. Chromatography Paper

Whatman No. 1 chromatography paper was used in all chromatographic experiments.

II.A.2.b. Solvents

n-Butanol saturated with water (n-butanol: water, 84:16 v/v) was used for the separation of hydroxamic acids, (Stadtman and Barker, 1950), while n-butanol:acetic acid:water (5:2:3 v/v) was used to separate malonyl-CoA, barbituric acid and malonic acid.

II.A.2.c. Detection of Compounds on Chromatograms

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

Hydroxamic acids were detected after spraying with a solution containing 50 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ dissolved in 1 litre of 95% alcoholic 0.1M HCl (Stadtman and Barker, 1950).

Carboxylic acids were detected by spraying with 1% methyl red in 95% ethanol made alkaline by the addition of 0.1 M NaOH (Mountford, 1971).

II.A.3. Estimation of Radioactivity by Radioisotope Scanning

Developed chromatograms were cut into strips 2.5 cm wide and scanned for radioactivity using a Packard Radiochromatogram Scanner, Model 7200. The carrier gas was helium (98.7%) containing isobutane (1.3%) and the gas flow was $110 \text{ cm}^3/\text{min}$. Other operating conditions were voltage: 1.125 kV, time constant: 100 sec, speed: 50 cm/hr, maximum sensitivity: 1000 c.p.m. and slit width: 5 mm.

II.B. Bacteriological Methods

II.B.1. Organism

Nocardia corallina is an aerobic, gram-positive, rod-shaped bacterium, which is neither motile nor spore-forming. It exhibits considerable pleomorphism when grown on different media.

The strain of N. corallina used in this investigation was isolated by Batt and Wood (1951). It was maintained on glucose-yeast extract-agar slopes (Appendix 3) at 2°C, and subcultured monthly onto fresh slopes. These were incubated at 30°C for 24 hr and then stored at 2°C.

II.B.2. Growth in Liquid Media

Cells induced for pyrimidine utilisation were cultured in a medium containing barbituric acid, as the sole nitrogen and carbon source, together with inorganic salts and vitamin B1 (thiamine). The components of the medium in g/litre follow

KH_2PO_4	13.6
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1
Barbituric acid	2.0
Vitamin B1	0.025

Preparation of the medium is described in Appendix 4.

Inoculation was carried out using cells from a fresh agar slope which were transferred into 330 cm³ of liquid medium in a 1 litre conical flask. The inoculated flask was incubated at 30°C in a New Brunswick gyro-rotatory shaker (Model G-25) and growth was followed spectrophotometrically by absorbance measurements at 600 nm in a Spectronic 20 spectrophotometer.

Cells from the glucose-yeast extract-agar slopes required about 100 hr to reach the middle of the log-phase of growth in the pyrimidine liquid medium. At this stage 10 cm³ aliquots of cells were transferred, using a sterile pipette, to each of fifteen flasks containing 330 cm³ of liquid medium. These were then incubated in the shaker at 30°C for approximately 48 hr which corresponded nearly to the end of the log-phase.

II.B.3. Preparation of Cell Suspensions

Cells were harvested by centrifugation in 500 cm³ polypropylene bottles at 9000 g for 10 min at 0°C using a Sorvall RC-2B refrigerated centrifuge. The supernatant was discarded and the cells were resuspended in cold Tris/HCl buffer (0.05 M, pH 8.0) and recentrifuged for 10 min; this process was repeated a second time. The washed cells were then resuspended in a volume of the cold Tris/HCl buffer equal to 3-4 times the wet weight of the cells.

II.B.4. Preparation of Cell-free Extracts

A French press (Aminco, Maryland, U.S.A.) was used for cell disruption, two pressings at 7000 lb/sq.in. being required to give near complete cell disintegration (Mountford, 1971). The broken cells were centrifuged at 35,000 g for 10 min at 0°C (Sorvall RC-2B centrifuge) and the supernatant obtained by decanting was stored at -10°C. As a normal procedure cell-free extracts were prepared immediately after the cells had been harvested.

II.C. Experimental Methods

II.C.1. Enzyme Assay for Barbiturase

The following assay procedure, adapted from Hayaishi and Kornberg (1952), was used:- the assay system, which was incubated at 30°C, contained

0.1 cm ³	0.02 M barbituric acid
0.1 cm ³	2.5% bovine serum albumin
0.4 cm ³	0.05 M Tris/HCl buffer, pH 8.2
0.3 cm ³	distilled water
0.1 cm ³	cell-free extract

Usually 0.1 cm³ of cell-free extract was added but, with extracts of low activity, up to 0.4 cm³ was used with an appropriate change in the volume of water in the mixture.

Aliquots (0.05 cm³) were taken from the assay system, normally at 30 sec intervals, and diluted with 3.0 cm³ of Tris/HCl buffer (0.05 M, pH 8.0).

This dilution brought the absorbance at 255 nm into the range of 0.1 to 1.0 absorbance units and a Hitachi 101 spectrophotometer was used for measuring absorbance.

A linear relationship existed between the concentration of barbituric acid and absorbance in this range and a unit of enzyme activity was taken as that amount of enzyme which brought about an absorbance change of 0.01 absorbance units per minute.

II.C.2. Activation System Assays

II.C.2.a. Quantitative Assays

The method of Stadtman (1957), based on the Lipmann and Tuttle method (1945), was used to measure quantitatively the formation of activated compounds.

Tubes containing the following volumes of solution were incubated at 30°C for 0,1,2,5,10,20,30 or 60 min.

0.10 cm ³	substrate
0.10 cm ³	2.5% bovine serum albumin
0.10 cm ³	CoASH (10 mM)
0.05 cm ³	Na ₄ ATP (35 mM)
0.05 cm ³	Mg ²⁺ (40 mM)
0.40 cm ³	Tris/HCl buffer (0.05 M, pH 8.0)
0.10 cm ³	distilled water
0.10 cm ³	cell-free extract

After incubation 0.5 cm³ of fresh, neutral hydroxylamine (equal volumes of 4 M NH₂OH.HCl and 3.5 M NaOH) was added to the mixture which was then left to stand at room temperature for 10 min. FeCl₃/TCA/HCl solution (equal volumes of 5% FeCl₃, 12% trichloroacetic acid and 3 M HCl), (1.5 cm³), was then added, the precipitated protein removed by centrifugation at 10,000 g and the absorbance at 540 nm measured in a Hitachi 101 spectrophotometer.

This hydroxylamine assay system is specific for activated compounds of the thioester, anhydride and acyl phosphate type. Hydroxylamine reacts with normal esters and some other compounds when heated.

At a later stage in the investigation some quantitative assays were performed after the protein had been removed from the incubation mixture by the addition of 0.5 cm³ of 15% TCA. Fresh, neutral hydroxylamine (1.0 cm³) was added to the protein-free supernatant and, after 10 min at room temperature, 2.5 cm³ of the FeCl₃/TCA/HCl reagent was added to the mixture. The mixture was centrifuged and the absorbance read at 540 nm in a Hitachi spectrophotometer.

II.C.2.b. Qualitative Assays

Qualitative assays for activation reactions were carried out using (¹⁴C-)labelled barbituric acid or malonic acid as substrates in the assay system described above (II.C.2.a.). At the end of the incubation period the reaction was stopped by the addition of 0.5 cm³ of 15% TCA

to the reaction tube and the resultant solution was treated in one of two ways:-

(a) After centrifugation to remove the precipitated protein the supernatant was freeze-dried and the residue redissolved in 0.1 cm^3 of distilled water. Aliquots (0.02 cm^3) were spotted onto Whatman No. 1 chromatography paper and the chromatogram developed by descending chromatography using n-butanol:acetic acid:water (5:2:3 v/v) as the solvent.

(b) Fresh, neutral hydroxylamine (1.0 cm^3) was added to the solution and after standing at room temperature for 10 min it was poured into 25 cm^3 of acetone. This mixture was evaporated to dryness in a rotary-evaporator and the residue extracted with absolute ethanol (3.0 cm^3). The ethanol was evaporated off and the residue taken up in 0.1 cm^3 of 50% ethanol. An aliquot (0.02 cm^3) of this solution was spotted onto Whatman No. 1 chromatography paper and the chromatogram developed by descending chromatography using n-butanol:water (84:16 v/v) as the solvent. This qualitative hydroxamic acid assay is similar to that described by Stadtman and Barker (1951).

II.C.3. Estimation of Protein Concentration in Solutions

II.C.3.a. Quantitative Protein Estimations

Trichloroacetic acid was used to precipitate the proteins which were then estimated by the method of

Lowry et al. (1951) as follows:

An aliquot of 15% TCA, equal to the volume of the sample being tested, was added to the solution and the volume made up to 2.0 cm³ with 7% TCA. The solutions were mixed and after standing for 5 min were centrifuged for 10 min at 11,000 g (Sorvall RC-2B centrifuge). The supernatant was carefully decanted in a single movement and the precipitate resuspended in 7% TCA (2.0 cm³). After recentrifuging for 10 min at 11,000 g the supernatant was again carefully decanted and the protein pellet redissolved in 0.3 cm³ of 1.15 M NaOH. After standing at room temperature for 10 min, 3.0 cm³ of Reagent C (2% Na₂CO₃:1% CuSO₄:2% sodium potassium tartrate, 100:1:1 v/v) was added and after a further 10 min, 0.3 cm³ of Folin's reagent was added to the tubes. Thirty minutes later the absorbance at 750 nm was measured, using a Hitachi 101 spectrometer, and compared with a standard curve of 0-200 ug protein prepared using bovine serum albumin as the standard.

II.C.3.b. Qualitative Protein Estimations

Column eluants were monitored for the presence of protein by measuring the absorbance at 280 nm in a Hitachi 101 spectrophotometer.

3. PURIFICATION STUDIES ON THE
ENZYME BARBITURASE

I. DNA Precipitation Using Streptomycin Sulphate

Removal of DNA from the cell-free extract is an important step in the purification of bacterial enzymes. Although Hayaishi and Kornberg (1952) used protamine sulphate in their purification procedure, streptomycin sulphate can be just as effective and in this study 10% streptomycin sulphate (w/v) in Tris/HCl buffer (0.05 M, pH 8.0) was used. This, and all subsequent purification steps, were carried out at 0-2°C in either ice-buckets or cold-rooms. Precipitates were collected by centrifugation at 0°C in a Sorvall RC-2B refrigerated centrifuge.

Varying amounts of streptomycin sulphate solution were added to cell-free extracts (2.5 cm³) up to a concentration of 2 mg/mg protein and the precipitated DNA was removed by centrifugation. Enzyme assays on the supernatants showed that the addition of streptomycin sulphate up to a concentration of about 1 mg/mg protein gave a small increase in activity while at concentrations of 2 mg/mg protein, however, the activity was slightly lower than it was originally; this was partly due to the dilution effect of the larger volume of liquid. The results are summarised in Table 1 together with protein concentrations, estimated as outlined in Experimental Methods (II.C.3.a.).

TABLE 1.

BARBITURASE ACTIVITIES IN EXTRACTS TREATED WITH 10%
STREPTOMYCIN SULPHATE TO REMOVE DNA

Streptomycin-SO ₄ (cm ³)	0.00	0.15	0.25	0.40	0.50	0.65	0.80	1.0
<u>mg strep-SO₄ added</u> mg protein in CFE	0.0	0.3	0.5	0.8	1.0	1.3	1.6	2.0
Activity (units/cm ³)	40	40	41	43	45	44	40	38
Supernatant vol. (cm ³)	2.5	2.6	2.7	2.8	2.9	3.0	3.2	3.3
Total units	100	104	110	121	130	132	128	125
Protein (mg/cm ³)	10.0	9.7	9.2	8.0	7.0	6.8	6.6	6.5
Specific Activity (units/mg)	4.0	4.1	4.5	5.4	6.5	6.5	6.1	5.9

Step-wise addition of small volumes of streptomycin sulphate, followed by centrifugation after each addition, indicated that a streptomycin sulphate concentration of 1 mg/mg protein was sufficient to precipitate the DNA. Addition above a concentration of 1 mg/mg protein gave no further precipitation of DNA.

The addition of streptomycin sulphate at 1 mg/mg protein gave a 1.5 to 2 fold increase in the specific activity of the enzyme; the size of the increase fell off at concentrations greater than 1.3 mg/mg protein. In later experiments, streptomycin sulphate was used at a standard concentration of 1 mg/mg protein in the extract.

II. Ammonium Sulphate Precipitation

Brennan (1970) reported variable results when attempting to purify barbiturase using ammonium sulphate to precipitate proteins and he concluded that this was due to the presence of large quantities of DNA in the cell-free extract.

The results of an experiment in which amounts of powdered ammonium sulphate were slowly added to streptomycin sulphate-treated cell-free extract are shown in Table 2, where, in each case, 4.0 cm³ of treated extract was used. After centrifugation, the pellet of precipitated protein was taken up, in each case, in 1.0 cm³ of Tris/HCl buffer (0.05 M, pH 8.0) and both the supernatant and the resuspended precipitate were assayed for enzyme activity.

TABLE 2.

AMMONIUM SULPHATE FRACTIONATION OF STREPTOMYCIN
SULPHATE-TREATED CELL-FREE EXTRACTS

Supernatant Activities

	CFE*	CFE/SS ⁺	<u>%(NH₄)₂SO₄ Saturation</u>				
			40	45	50	55	60
Activity (units/cm ³)	38	46	41	37	37	29	13
Volume (cm ³)	4.0	4.0	4.2	4.5	4.6	4.8	4.8
Total units	152	176	168	166	170	140	63
Recovery (%)		100	97	97	98	82	40

Resuspended Precipitate Activities

	<u>%(NH₄)₂SO₄ Saturation</u>				
	40	45	50	55	60
Activity (units/cm ³)	6	3	6	8	27
Volume (cm ³)	1.2	1.1	1.1	1.1	1.1
Total units	7	3	7	9	30
Recovery (%)	4	2	4	5	17

* CFE = Cell-free extract.

+ CFE/SS = Streptomycin sulphate-treated cell-free extract.

At 50% saturation, all the barbiturase activity remained in the supernatant; however the enzyme began to precipitate out at saturations exceeding 50% with activity being recovered in the resuspended precipitate. Generally, the recovery of activity in the resuspended precipitate was low e.g. at 60% saturation about 60% of the enzyme was precipitated but only 17% of the original activity was recovered in the resuspended precipitate. In other experiments, a fraction from 50 to 75% ammonium sulphate saturation was obtained and the supernatant, under these conditions, retained 25 to 30% of the original activity while only 25% was recovered in the resuspended precipitate.

Estimations of the protein concentration in the supernatant of the 50% ammonium sulphate saturated extract indicated that a 4 to 5 fold increase in purity had been achieved by the combined streptomycin sulphate and ammonium sulphate treatments. Because of the poor recovery of enzyme activity at higher ammonium sulphate saturations, it was decided to work routinely with the 50% saturated supernatant.

III. Dialysis Studies

A number of experiments were performed to determine optimum conditions for using dialysis as a step in increasing enzyme specific activities and for the removal of salts from the extracts. Either cell-free extracts or supernatant from a 50% ammonium sulphate fractionation were used in these experiments.

III.A. Stability of Barbiturase During Dialysis at pH 8.0

Samples (2.0 cm³) of cell-free extracts were dialysed against 150 cm³ of 0.05 M Tris/HCl buffer, pH 8.0, at 2°C for times ranging from 0 to 8 hr and enzyme activities determined. Samples were also dialysed against the same buffer containing 10⁻³M EDTA or 10⁻³M β-mercaptoethanol and the results from these studies are shown in Table 3.

In a similar experiment samples (2.0 cm³) of the supernatant from the 50% ammonium sulphate fractionation were dialysed against 200 cm³ of the Tris/HCl buffer for times ranging from 0 to 4.5 hr. The activities and recoveries after dialysis are also shown in the table.

With both the cell-free extracts and the ammonium sulphate-treated extracts there was a rapid loss of activity during dialysis. Dialysis of cell-free extracts for 8 hr led to the loss of nearly 90% of the activity and the presence of EDTA or β-mercaptoethanol in the buffer did not effect an appreciable reduction in the loss. After dialysis of the ammonium sulphate-treated extracts for 4.5 hr less than 50% of the activity remained and after 16 hr all activity had disappeared.

III.B. Effect of Alteration of pH and Ionic Strength on Barbiturase Stability During Dialysis

Ammonium sulphate-treated extracts (2.0 cm³, activity 16 units/cm³) were dialysed against 0.05 M

TABLE 3.

EFFECT OF DIALYSIS ON ENZYME RECOVERY

<u>Dialysis of Crude Extracts</u>	<u>Duration of Dialysis (hr)</u>				
	0	2	3	4	8
Crude extracts dialysed against buffer	100*	42	25	20	8
Crude extracts dialysed against buffer + EDTA	100	47		30	10
Crude extracts dialysed against buffer + CH ₃ CH ₂ SH	100	37		27	10

*Figure is percentage recovery of enzyme activity.

Dialysis of (NH₄)₂SO₄-treated extract

	<u>Duration of Dialysis (hr)</u>							
	0	1.5	2	2.5	3	3.5	4	4.5
Recovery %	100	99	99	99	60	57	55	48

Tris/HCl buffer of varying pH's for 2 or 5 hr at 2°C and the activities of the extracts after dialysis are shown in Table 4.

After 2 hr dialysis recoveries ranged from 37% (pH 7.2) to 94% (pH 8.2) while after 5 hr dialysis recoveries ranged from 10% (pH 7.2) to 29% (pH 8.0 - 8.2). From the results obtained, it would seem that the optimum pH for dialysis is pH 8.0 - 8.2.

Ammonium sulphate-treated extracts were also dialysed against pH 8.0 Tris/HCl buffers of molarity up to 0.25 M and against 0.05 M Tris/HCl buffers, pH 8.0, which were 0.1 and 0.2 M in NaCl. Dialysis against the higher molarity Tris buffers resulted in losses of enzyme activities similar to those observed earlier. After dialysis for 2 hr against the 0.1 M and 0.2 M NaCl buffers the recovery of activity was about 85% while after 5 hr dialysis the recovery was down to only 25% with 0.1 M NaCl and 28% with 0.2 M NaCl buffers.

III.C. The Effect of Different Buffer Solutions on Barbiturase Stability During Dialysis

The following buffers were prepared:

- (a) 0.05 M triethanolamine hydrochloride/NaOH
- (b) 0.02 M sodium 5:5-diethylbarbituric acid/HCl
- (c) 0.05 M 2-amino-2-methyl-1:3-propanediol/HCl
- (d) 0.05 M phosphate/NaOH and
- (e) 0.05 M 2:4:6-collidine/HCl

as described by Dawson et al. (1969). The pH of each

TABLE 4.

EFFECT OF pH ON ENZYME RECOVERIES DURING DIALYSIS
OF AMMONIUM SULPHATE-TREATED EXTRACTS

2 hr Dialysis

	pH						
	7.2	7.4	7.6	7.8	8.0	8.2	8.4
Volume (cm ³)	3.4	3.4	3.3	3.4	3.4	3.6	3.4
Activity (units/cm ³)	3.5	4.0	5.5	6.5	7.5	9.0	7.0
Total units	12	13	18	22	26	30	24
Recovery (%)*	38	41	57	69	80	95	75

5 hr Dialysis

	pH						
	7.2	7.4	7.6	7.8	8.0	8.2	8.4
Volume (cm ³)	3.4	3.5	3.6	3.6	3.7	3.7	3.6
Activity (units/cm ³)	1.0	1.5	1.5	2.0	2.5	2.5	2.0
Total units	3.4	5.2	5.4	7.2	9.2	9.2	7.2
Recovery (%)*	10	16	17	22	29	29	22

* In each case the ammonium sulphate-treated extract (2.0 cm³) had an initial activity of 16 units/cm³, i.e. 32 units were taken for each dialysis.

buffer was adjusted to pH 8.0 at 0°C and small volumes (usually 2.0 cm³) of cell-free extracts with an activity of 33 units/cm³ were used in dialysis experiments.

After dialysis for 6 hr against the triethanolamine, aminomethylpropanediol or diethylbarbituric acid buffers, the activity of the extracts was less than 5 units/cm³ and similar results were obtained after dialysis against the phosphate buffer for the shorter period of 5 hr. By contrast, after dialysis for 6 hr against the collidine buffer, the activity of the extract was 10 units/cm³.

A range of collidine buffers of pH's between 7.0 and 8.0 was prepared and cell-free extracts were dialysed against these, in each case for 6 hr. The optimum pH appeared to be pH 7.8. In a further series of experiments, ammonium sulphate-treated extracts (2.0 cm³) were dialysed against 0.05 M collidine/HCl buffer (pH 7.8) for 2, 4 or 8 hr. Before dialysis the activity of the extract was 46 units/cm³, after dialysis for 2 hr it was 15 units/cm³ (3.6 cm³, 65% recovery), after 4 hr, 9 units/cm³ (4.0 cm³, 40% recovery) and after 8 hr, 5 units/cm³ (4.0 cm³, 22% recovery). These results are similar to those obtained using Tris/HCl buffers.

Enzyme assays carried out with these buffers added to the assay solutions showed that the components of the buffers were not inhibitors of barbiturase.

III.D. Dialysis of Cell-free Extracts Against Buffers Containing Metal Ions

A range of Tris/HCl buffers (0.05 M, pH 8.0) containing 10^{-3} M Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} , Na^+ , K^+ or NH_4^+ was prepared and cell-free extracts were dialysed against each of these buffers and against Tris/HCl containing no metal ions, for 4 hr at 2°C .

The activity of the dialysed extracts, in all cases, was between 8 and 10 units/cm³ compared with an activity of 40 units/cm³ before dialysis.

III.E. Dialysis Against Buffers Containing Substrate or Substrate Analogues

Tris/HCl buffers (0.05 M, pH 8.0) containing barbituric acid (0.005 M), uracil (0.01 M), diethylbarbituric acid (0.01 M) or 2-hydroxypyridine (0.01 M) were prepared and either cell-free extracts or ammonium sulphate-treated extracts were dialysed against the barbituric acid-containing buffer for 2 to 4.5 hr. The results of assays performed after the dialysis of the ammonium sulphate-treated extract are shown in Table 5 and similar results were obtained from the dialysis of cell-free extracts against the barbituric acid-containing buffer.

In other experiments, cell-free extracts were dialysed against the buffers containing either uracil, diethylbarbituric acid or 2-hydroxypyridine for 6 hr at 2°C . In each case, the activity of the extract before

TABLE 5.

EFFECT OF SUBSTRATE ON THE RECOVERY OF ENZYME ACTIVITY
DURING DIALYSIS

Without Barbituric Acid

	Duration of Dialysis (hr)					
	0	2.0	2.5	3.0	3.5	4.0
Volume (cm ³)	2.0	3.2	3.2	3.3	3.4	3.4
Activity (units/cm ³)	30	17	15	12	11	10
Total units	60	51	47	44	37	34
Recovery (%)	100	90	79	73	62	57

With Barbituric Acid (0.005M)

	Duration of Dialysis (hr)						
	0	2.0	2.5	3.0	3.5	4.0	4.5
Volume (cm ³)	2.0	3.3	3.4	3.3	3.4	3.3	3.2
Activity (units/cm ³)	30	17	16	12	11	10	9
Total units	60	57	54	44	37	33	29
Recovery (%)	100	95	90	73	62	55	49

dialysis was 30 units/cm³ and less than 5 units/cm³ after dialysis.

III.F. Dialysis Against Buffers Containing Glycerol

Tris/HCl buffers (0.05 M, pH 8.0) containing 5, 10 or 15% v/v glycerol were prepared and ammonium sulphate-treated extracts were dialysed against these buffers for 2.5 or 5 hr.

The recovery of activity after 2.5 hr dialysis was 55%, 65% and 62% for the 5%, 10% and 15% glycerol buffers respectively, and after 5 hr dialysis, the corresponding recoveries were 50%, 60% and 55%.

III.G. Dialysis Against Buffers Containing Urea, Ammonium Bicarbonate or Ammonium Sulphate

Tris/HCl buffers (0.05 M) were prepared which were 0.2 or 1 M with respect to urea, 0.1 M with respect to ammonium bicarbonate or 50% saturated with ammonium sulphate, and the pHs were adjusted to pH 8.0 at 0°C. Ammonium sulphate-treated extracts (3.0 cm³) were then dialysed against these buffers for 2, 4 or 8 hr.

After 8 hr dialysis against the buffers containing urea or ammonium bicarbonate the activity of the extracts was 25 to 30% of that in the original extract while, after 8 hr dialysis against the 50% saturated buffer, full activity was retained in the extract.

IV. Desalting By Gel Filtration and UltrafiltrationIV.A. Gel Filtration

A column (1.4 x 10 cm) was packed with degassed Sephadex G 25 (Fine) which had been allowed to swell for 6 hr in Tris/HCl buffer at room temperature. After equilibration with 0.05 M Tris/HCl buffer (pH 8.0), 2.0 cm³ of the ammonium sulphate-treated extract was applied to the column. When the sample had been washed in, the column was eluted with 0.05 M Tris/HCl buffer, pH 8.0, at a flow rate of approximately 30 cm³/hr. Ten 2 cm³ fractions were collected and assayed for protein, which began to appear in fraction 3; ammonium sulphate was eluted in fractions 6 to 10.

All fractions were assayed for barbiturase activity and the results are shown in Table 6. A total of 74 units of barbiturase were applied to the column and a total of 28 units was recovered; a recovery of 38%.

This experiment was repeated using buffers of pH 7.5 and pH 7.8, and with buffers containing 10⁻³ M EDTA or 10⁻³ M β-mercaptoethanol. The recovery using the pH 7.5 buffer was 25% and with the pH 7.8 buffer, about 30%. The addition of EDTA or β-mercaptoethanol to the pH 8.0 buffer did not improve the recoveries.

Similar results were obtained when Biogel P4 (100-200 mesh) was substituted for Sephadex G25.

TABLE 6.

ENZYME ACTIVITIES IN FRACTIONS ELUTED FROM A
SEPHADEX G25 COLUMN

	Fraction Number									
	1	2	3	4	5	6	7	8	9	10
Volume (cm ³)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Activity (units/cm ³)	0	0	1	5	6	2	0	0	0	0
Number of units	0	0	2	10	12	4	0	0	0	0

Of 74 units applied to the column, 28 units were found in the eluate; a recovery of 38%.

IV.B. Ultrafiltration

A C50 ultrafiltration cell (Chemlab Instruments Ltd. Ilford, England), fitted with an Amicon XM50 membrane (Amicon Corporation, Massachusetts, U.S.A.) was filled with ammonium sulphate-treated extract (35 cm^3) and ultrafiltration was carried out at 0°C under a nitrogen pressure of 50 lb/sq.in.; the solution was stirred continuously. When the volume of liquid remaining in the cell was about 5 cm^3 , 25 cm^3 of Tris/HCl buffer was added and the solution concentrated again to about 5 cm^3 ; this process was repeated a second time.

The final volume of solution was 4.8 cm^3 with an activity of 88 units/ cm^3 ; the activity of the treated extract was originally 40 units/ cm^3 (in 35 cm^3) so the recovery of activity was approximately 30%.

V. Adsorption By Calcium Phosphate Gels

Streptomycin sulphate-treated cell-free extracts (5.0 cm^3) were added to 5.0 g of calcium phosphate gel which had been pre-equilibrated with 0.05 M Tris/HCl buffer, pH 8.0, and after stirring for 20 min, the slurry was centrifuged (5000 g, 5 min) giving a clear supernatant. Tris/HCl buffer (2.0 cm^3), 0.01 M in phosphate was added to the pellet, and, after stirring for 20 min, the mixture was recentrifuged (5000 g, 5 min) giving a second supernatant fraction. This process was repeated successively with 2.0 cm^3 volumes of Tris/HCl buffers 0.02 to 0.10 M and 0.50 M in phosphate, pH 8.0.

The supernatant from each of these elutions was assayed for barbiturase activity and the results, given in Table 7, showed that most of the activity was recovered with phosphate concentrations between 0.025 and 0.065 M.

The experiment was repeated with a streptomycin sulphate-treated extract (4.0 cm^3) to which 4.0 g of gel was added and Tris/HCl buffers 0.025 M, 0.065 M (twice) and 0.5 M in phosphate, pH 8.0, were used successively to elute the enzyme. The supernatants obtained after the first centrifugation and from the 0.025 M buffer elution had activities of 0 and 5 units/ cm^3 respectively. The supernatants from the two 0.065 M elutions had activities of 70 and 50 units/ cm^3 while the supernatant from the 0.5 M elution had an activity of 10 units/ cm^3 . The volume of buffer used for each elution was 2.0 cm^3 and the total recovery of enzyme was 270 units compared with 340 units in the original extract i.e. an overall recovery of 80%.

After determining the protein concentration in the pooled 0.065 M elutions it was estimated that this step had given a three-fold increase in purification. However, when the experiment was repeated later, using different batches of gel, considerably lower recoveries, 55% and 40%, were obtained.

VI. DEAE-Cellulose Ion Exchange Chromatography

In these experiments, both batch-wise techniques and column chromatography were used.

TABLE 7.

RECOVERY OF ENZYME ACTIVITY AFTER ADSORPTION ON TO
CALCIUM PHOSPHATE GEL

Eluant	Volume (cm ³)	Activity (units/cm ³)
0.01 M PO ₄ ³⁻	2.0	0
0.02 M "	"	0
0.03 M "	"	5
0.04 M "	"	14
0.05 M "	"	13
0.06 M "	"	10
0.07 M "	"	2
0.08 M "	"	0
0.09 M "	"	0
0.10 M "	"	0
0.50 M "	"	0

Calcium phosphate gel was added to streptomycin sulphate-treated cell-free extracts (5 cm³, 40 units/cm³) and, after centrifuging, the supernatant (5.5 cm³, 2 units/cm³) obtained. The gel was then successively eluted using the buffers listed above (see text for details).

VI.A. Gradient Elution Column Chromatography

A column, 0.9 x 22 cm, was packed with Sigma DEAE-Cellulose which had been pre-cycled prior to equilibration with 0.05 M Tris/HCl buffer, pH 8.0. Streptomycin sulphate-treated extract (5.0 cm³) was applied to the column followed by 30 cm³ of pH 8.0, 0.05 M Tris/HCl buffer. A linear buffer gradient from 0 to 0.5 M NaCl in 0.05 M Tris/HCl buffer was used to elute the column into 5 cm³ fractions. This was followed by elution with 50 cm³ of buffer 1 M in NaCl.

All fractions were assayed for protein concentration (280 nm absorbance method) and for barbiturase activity. Several bands of protein were eluted from the column but enzyme activity could not be detected in any fraction.

Similar results were obtained when a column, 1.4 x 9 cm, packed with pre-swollen Whatman DE52 Microgranular DEAE-Cellulose was used. The ion-exchanger was washed with buffer 1.5 M in NaCl and equilibrated with 0.05 M Tris/HCl, pH 8.0 before the column was packed. Streptomycin sulphate-treated cell-free extract (7.0 cm³, activity 60 units/cm³) was applied to the resin and washed in with a small quantity of equilibration buffer. Subsequently, this was followed successively by equilibration buffer (20 cm³); equilibration buffer 0.1 M in NaCl (20 cm³); a linear buffer gradient from 0.1 M to 0.5 M NaCl (150 cm³) and finally, equilibration buffer 1.5 M in NaCl (50 cm³).

Fractions (2.0 cm^3) were collected and assayed for protein and barbiturase activity. As before, several bands of proteins had been eluted from the column but enzyme activity could not be detected.

VI.B. Batchwise Elution

Streptomycin sulphate-treated cell-free extract (5.0 cm^3) was added to 5.0 g pre-cycled, equilibrated DEAE-Cellulose (Sigma) and after gentle stirring for 10 min the slurry was centrifuged (5000 g , 5 min). The supernatant was assayed for barbiturase activity and found to be completely inactive.

Cold 0.05 M Tris/HCl buffer, pH 8.0, (4.0 cm^3) which was 1.5 M with respect to NaCl, was added to the DEAE-Cellulose pellet and stirred for 10 min. After centrifugation, the supernatant was decanted and assayed and the pellet was eluted again using the same buffer. The supernatant from this second elution was also assayed for enzyme activity.

The supernatant obtained from the first elution (3.8 cm^3) had an activity of 17 units/cm^3 while the supernatant from the second elution showed 6 units/cm^3 . A total of 92 units were recovered from the 275 units of enzyme which had been added to DEAE-Cellulose; a recovery of 33% which suggests that the enzyme was being deactivated on the DEAE-Cellulose. With nearly 70% of the activity lost within the 40 min that this experiment lasted, it is not surprising that activity could not be detected in the fractions from the gradient elutions, as nearly three hours

was required for the column procedure.

VII. Gel Filtration

A column, 4.0 x 30 cm, was packed with degassed Sephadex G 200 which had been allowed to swell for 72 hr at room temperature in 0.05 M Tris/HCl buffer. The column was equilibrated by elution with two bed volumes of 0.05 M Tris/HCl buffer, pH 8.0, at a flow rate of 25-30 cm³/hr.

Streptomycin sulphate-treated cell-free extract (10 cm³) was applied to the column and washed in with equilibration buffer (5.0 cm³). The column was then eluted with 500 cm³ of equilibration buffer and 10 cm³ fractions collected and assayed for protein and barbiturase activity. Several fractions had relatively high protein concentrations but enzyme activity could not be detected in any fraction.

In a second experiment the same column was repacked with Sephadex G200 which had been washed in buffer (0.05 M Tris/HCl, pH 8.0) 50% saturated with ammonium sulphate. The column was equilibrated with two bed volumes of 50% saturated buffer, and ammonium sulphate-treated extract (10 cm³) was applied to the column. After the sample had been washed into the gel, the column was eluted with 50% saturated buffer. Fractions (10 cm³) were collected and assayed for protein and barbiturase and the results are shown in Figure 5. The recovery of activity from fractions 15 to 22 was greater than 85% and

the procedure was found to be reproducible with recoveries varying from 85 to 95%.

The active fractions were assayed for protein using the TCA precipitation method outlined in Experimental Methods II.C.3.a., and the results are shown in Table 8. Fractions 16 to 21 were pooled and concentrated by ultrafiltration to a final volume of about 8 cm³. Attempts to remove the ammonium sulphate from this concentrate, using the methods described earlier, were unsuccessful and only resulted in a rapid loss of activity; usually about 90% of the activity was lost after 4 hr dialysis.

VIII. Preparation And Use Of Partially-Purified Barbiturase

Using the purification scheme outlined in Table 9, a 34 fold purification of barbiturase was achieved in the pooled active fractions obtained from the gel filtration step. It was not possible to determine the degree of purification by methods such as analytical gel electrophoresis, since the presence of ammonium sulphate interferes with such techniques and the removal of ammonium sulphate leads to enzyme inactivation.

Cell-free extracts and the partially-purified enzyme preparations were assayed for barbiturase activity, with samples taken over a one hour period, and the results are shown in Figure 6. With cell-free extracts, the disappearance of barbituric acid followed a hyperbolic

TABLE 8.

ENZYME ACTIVITY IN ACTIVE FRACTIONS OBTAINED AFTER
GEL FILTRATION ON SEPHADEX G200

Fraction Number	Volume (cm ³)	Activity (units/cm ³)	Protein (mg/cm ³)	Specific Activity (units/mg)
15	10.5	0	0.03	0
16	10.4	8	0.13	61
17	10.4	17	0.15	113
18	10.4	19	0.18	106
19	10.4	17	0.21	83
20	10.5	15	0.22	69
21	10.6	14	0.17	83
22	10.5	7	0.14	50
23	10.6	2	0.10	20

Buffers 50% saturated with ammonium sulphate were used for equilibration and elution (see text for details).

TABLE 9.

PURIFICATION SCHEME FOR THE PARTIAL PURIFICATION
OF BARBITURASE

Step Number	Fraction	Volume (cm ³)	Protein (mg/cm ³)	Activity (units/cm ³)
1	Cell-free extract	15.0	20	50
2	Streptomycin sulphate	17.6	12.1	55
3	Ammonium sulphate	18.0	4.0	51
4	Sephadex G200	58.0*	0.18	15

Step Number	Specific Activity (units/mg)	Yield (%)	Purification (fold)
1	2.5		
2	4.5	100	1.8
3	12.7	95	5.1
4	85	90	34

* The six most active fractions from the column were pooled.

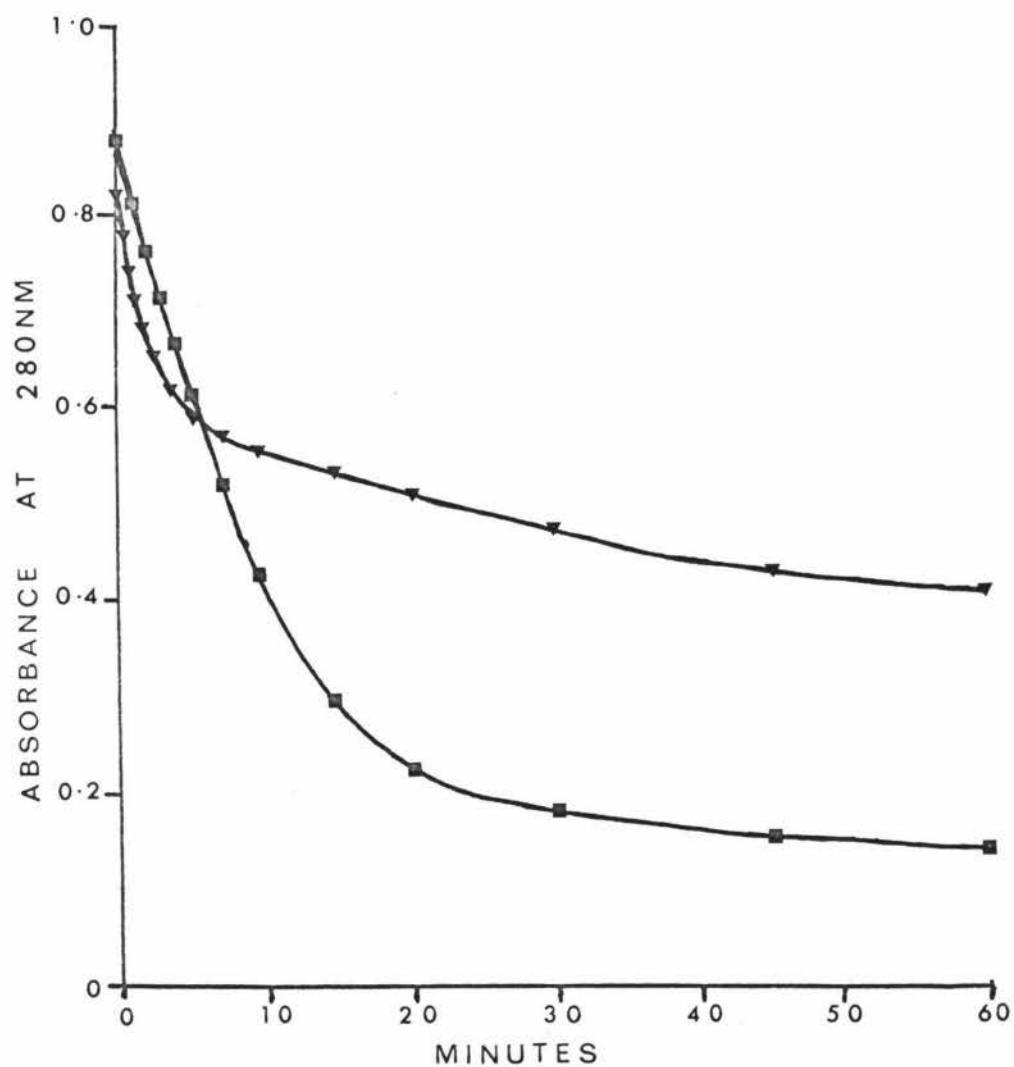


Figure 6. Enzyme assays for barbiturase activity in cell-free extracts and in partially-purified barbiturase.

KEY: ▼▼▼▼▼ = Partially-purified barbiturase

■ ■ ■ ■ ■ = Cell-free extracts

curve, but with the partially-purified enzyme, a rapid decrease in barbituric acid concentration was observed for the first 3-5 min, followed by a slower, steady decrease in concentration which continued for the rest of the assay period. The addition of (a) cofactors (ATP with Mg^{2+} and CoASH) or (b) heat-treated cell-free extracts to the incubation mixture did not significantly alter the shape of the curve obtained using the partially-purified enzyme, suggesting that cofactors or metal ions are not responsible for the marked change in the rate of barbituric acid removal observed after about five minutes. Assays were carried out at pH 7.0 and at 25°C and 37°C and the effect was still observed.

One possibility is that the enzyme is undergoing denaturation during the first five minutes, forming a denatured enzyme which has only limited barbiturase activity. Alternatively, it may be that, in vitro, barbiturase hydrolyses barbituric acid only as far as an intermediate product e.g. malonuric acid, and that, in the absence of a second enzyme to hydrolyse the intermediate, the increased levels of this substance inhibit the barbiturase. The slow disappearance of barbituric acid observed after five minutes incubation could be explained by postulating a slow spontaneous breakdown of the intermediate.

Attempts were made to synthesis malonuric acid ($COOH-CH_2-CO-NH-CO-NH_2$) as a possible intermediate of barbituric acid degradation, but these were unsuccessful. The main problem was the unstable nature of the product

which very readily undergoes decarboxylation forming
1-N-acetylurea.

4. STUDIES OF THE UTILISATION OF BARBITURIC
 ACID AND MALONIC ACID

In Chapter 4, the partial purification of the enzyme barbiturase was described.

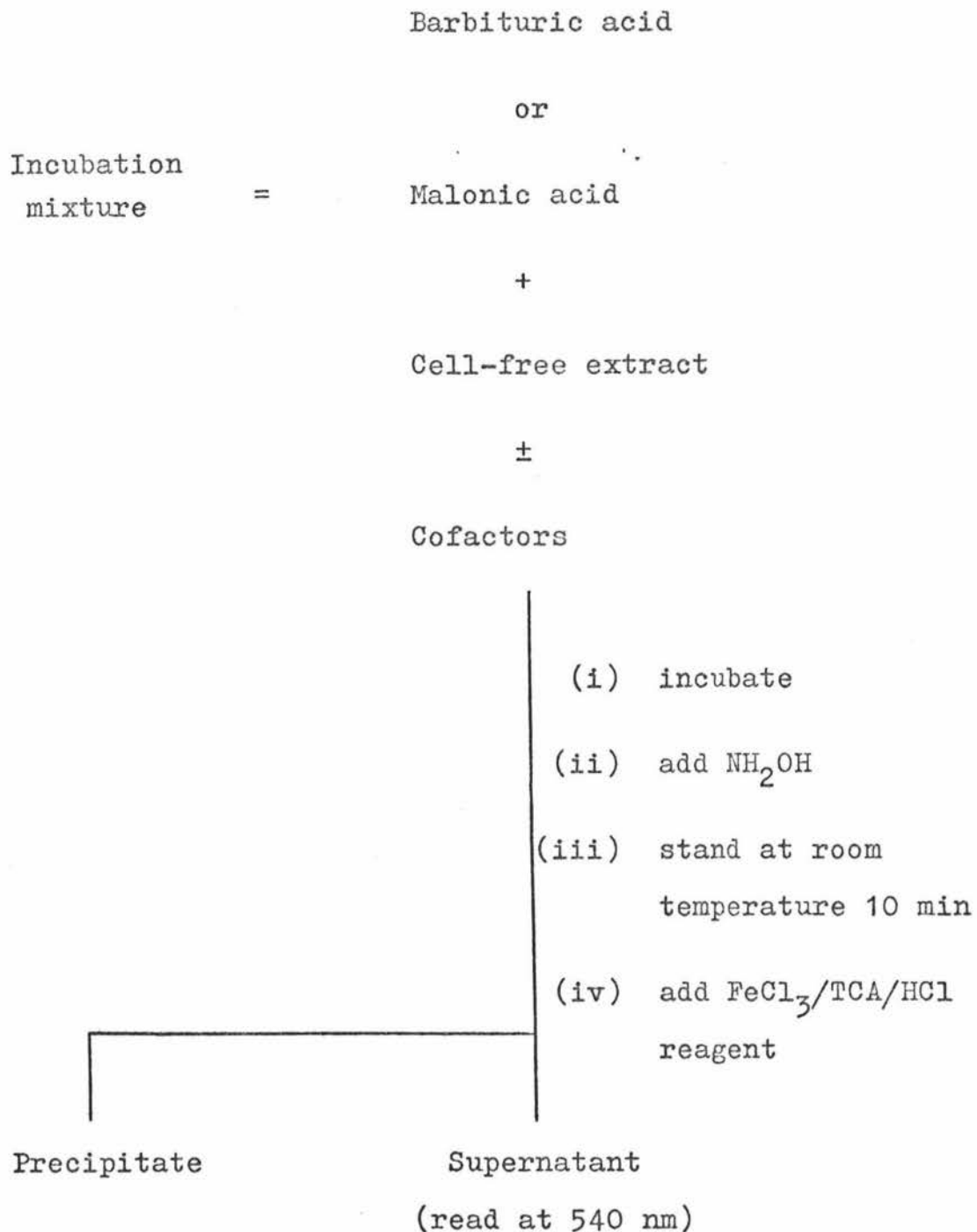
Hayaishi and Kornberg (1952) and Batt and Woods (1961) suggested that malonic acid may not be a product of barbituric acid catabolism in intact cells, and it was considered possible that, *in vivo*, a labile derivative of malonic acid might be an intermediate. A likely derivative was malonyl-CoA and experiments to test this possibility were undertaken using cell-free extracts and the partially-purified enzyme preparation of barbiturase.

I. Preliminary Studies

The formation of hydroxamic acids when hydroxylamine is added to thioesters, acyl phosphates or anhydrides is the basis of the Lipmann and Tuttle (1945) method to assay for the presence of activated compounds. To determine if either barbituric acid or malonic acid were converted to activated compounds, activation assays were performed using the quantitative, hydroxamic acid activation assay described in Experimental Methods II.C.2.a. This procedure is also outlined in Scheme 1.

I.A. Activation Assay Using Barbituric Acid As
 Substrate

Tubes containing barbituric acid plus the



Scheme 1: Scheme for the quantitative activation assay for the formation of malonyl-CoA from malonic acid or barbituric acid. (Chapter 5, I.A. and I.B.).

cofactors listed in Table 10, were incubated with cell-free extracts for the times indicated in the table before hydroxylamine was added. The absorbance readings, measured after treatment with the $\text{FeCl}_3/\text{TCA}/\text{HCl}$ reagent, are also given in the table.

The absorbance change in the tubes containing barbituric acid and all three cofactors was 0.150 units in ten minutes while the absorbance change in the tubes containing barbituric acid, but no cofactors, was 0.135 units in ten minutes. The omission of one cofactor from the assay system had little effect on the absorbance readings measured after ten minutes incubation. This suggests that a hydroxamic acid is being formed by a process which does not require cofactors.

I.B. Activation Assay Using Malonic Acid As
 Substrate

An experiment, similar to that described above, was performed by incubating tubes containing malonic acid, plus the cofactors listed in Table 11, for the times indicated in the table. The absorbance readings, measured after treatment with the $\text{FeCl}_3/\text{TCA}/\text{HCl}$ reagent, are also shown in the table.

In contrast to the results obtained above, (I.A.), where an absorbance change of about 0.15 units in ten minutes was observed, absorbance readings did not change significantly during the incubation period, even though the tubes were incubated for up to sixty minutes.

TABLE 10.

FORMATION OF HYDROXAMIC ACIDS IN DIFFERENT
BARBITURIC ACID-CONTAINING INCUBATION SYSTEMS

Incubation Mixture							Incubation Time	Absorbance
Buffer*	BSA	BA	ATP	Mg	CoASH	CFE	(min)	(540 nm)
+	+	-	-	-	-	+	0	0.125
+	+	-	-	-	-	+	10	0.130
+	+	+	-	-	-	+	0	0.165
+	+	+	-	-	-	+	10	0.300
+	+	-	+	+	+	+	0	0.150
+	+	-	+	+	+	+	10	0.162
+	+	+	+	+	+	+	0	0.185
+	+	+	+	+	+	+	10	0.335
+	+	+	-	+	+	+	10	0.315
+	+	+	+	-	+	+	10	0.325
+	+	+	+	+	-	+	10	0.315

* Buffer	= Tris/HCl buffer (0.05 M, pH 8.0)	- 0.4 cm ³
BSA	= 2.5% bovine serum albumin	- 0.1 cm ³
BA	= Barbituric acid, 2 μ moles	- 0.1 cm ³
ATP	= Adenosine triphosphate, 1.75 μ moles	- 0.05 cm ³
CoASH	= Coenzyme-A, 1 μ mole	- 0.1 cm ³
Mg	= Mg ²⁺ as MgCl ₂ , 2 μ moles	- 0.05 cm ³
CFE	= Cell-free extract	- 0.1 cm ³

The final volume was made up to 1.0 cm³ with distilled water.

TABLE 11.

FORMATION OF HYDROXAMIC ACIDS IN DIFFERENT
MALONIC ACID-CONTAINING INCUBATION SYSTEMS

Incubation Mixture							Incubation Time	Absorbance
Buffer*	BSA	MA ^o	ATP	Mg	CoASH	CFE	(min)	(540 nm)
+	+	-	-	-	-	+	0	0.115
+	+	-	-	-	-	+	10	0.120
+	+	+	-	-	-	+	0	0.365
+	+	+	-	-	-	+	2	0.375
+	+	+	-	-	-	+	10	0.345
+	+	+	-	-	-	+	30	0.370
+	+	+	-	-	-	+	60	0.350
+	+	-	+	+	+	+	0	0.165
+	+	-	+	+	+	+	10	0.185
+	+	+	+	+	+	+	0	0.415
+	+	+	+	+	+	+	10	0.430
+	+	+	+	+	+	+	60	0.425

* See Table 10 for abbreviations and explanations.

^o MA = Malonic acid, 2 μ moles - 0.1 cm³.

However, a comparison of absorbance readings from tubes containing cofactors only, and incubated with cell-free extracts, with tubes containing malonic acid plus cofactors, and incubated with cell-free extracts, shows the formation of a hydroxamic acid in the tubes to which malonic acid had been added. Similarly, the absorbance readings from tubes containing malonic acid but no cofactors are markedly higher than those from which the malonic acid had been omitted, indicating hydroxamic acid formation. The presence of cofactors in the incubation mixture had little effect on the amount of hydroxamic acid formed and it appears that the hydroxamic acid formation observed in these preliminary studies may not be a true measure of activation compound formation.

II. Enzymatic Formation Of Hydroxamic Acids

A possible explanation for the results obtained in the preliminary studies is that there is an enzyme present in the cell-free extracts capable of catalysing the formation of hydroxamic acids from hydroxylamine and carboxylic acids. Meister et al. (1955) reported rat liver ω -amidase catalysed the synthesis of succinylmonohydroxamic acid and glutarylmonohydroxamic acid, in the presence of hydroxylamine, from succinic and glutaric acids respectively. Oxalic, malonic, aspartic and glutamic acids were not substrates for this enzyme. They also reported that asparaginase and glutaminase from Escherichia coli catalysed the formation of L-aspartylmonohydroxamic acid and L-glutamylmonohydroxamic acids from their respective L-amino acids.

The presence of a similar enzyme in Nocardia corallina cell-free extracts, acting on malonic acid, would explain the following results which were obtained in the preliminary studies.

(a) Low hydroxamic acid levels after treatment of barbituric acid incubated with cell-free extracts for 0 min (low malonic acid).

(b) High hydroxamic acid levels after treatment of barbituric acid incubated with cell-free extract for 10 min (high malonic acid).

(c) High hydroxamic acid levels after treatment of malonic acid incubated for either 0 or 10 min with cell-free extracts (high malonic acid).

(d) The apparent independence of the hydroxamic acid level on the cofactor level.

To test for the presence of a hydroxamic acid synthesising enzyme in the cell-free extracts, the following experiments were performed:-

(a) The effect of hydroxylamine on the hydrolytic activity of barbiturase was tested.

(b) Substrate plus hydroxylamine was incubated with either normal or heat-treated cell-free extracts as a test for enzymatic hydroxamate formation.

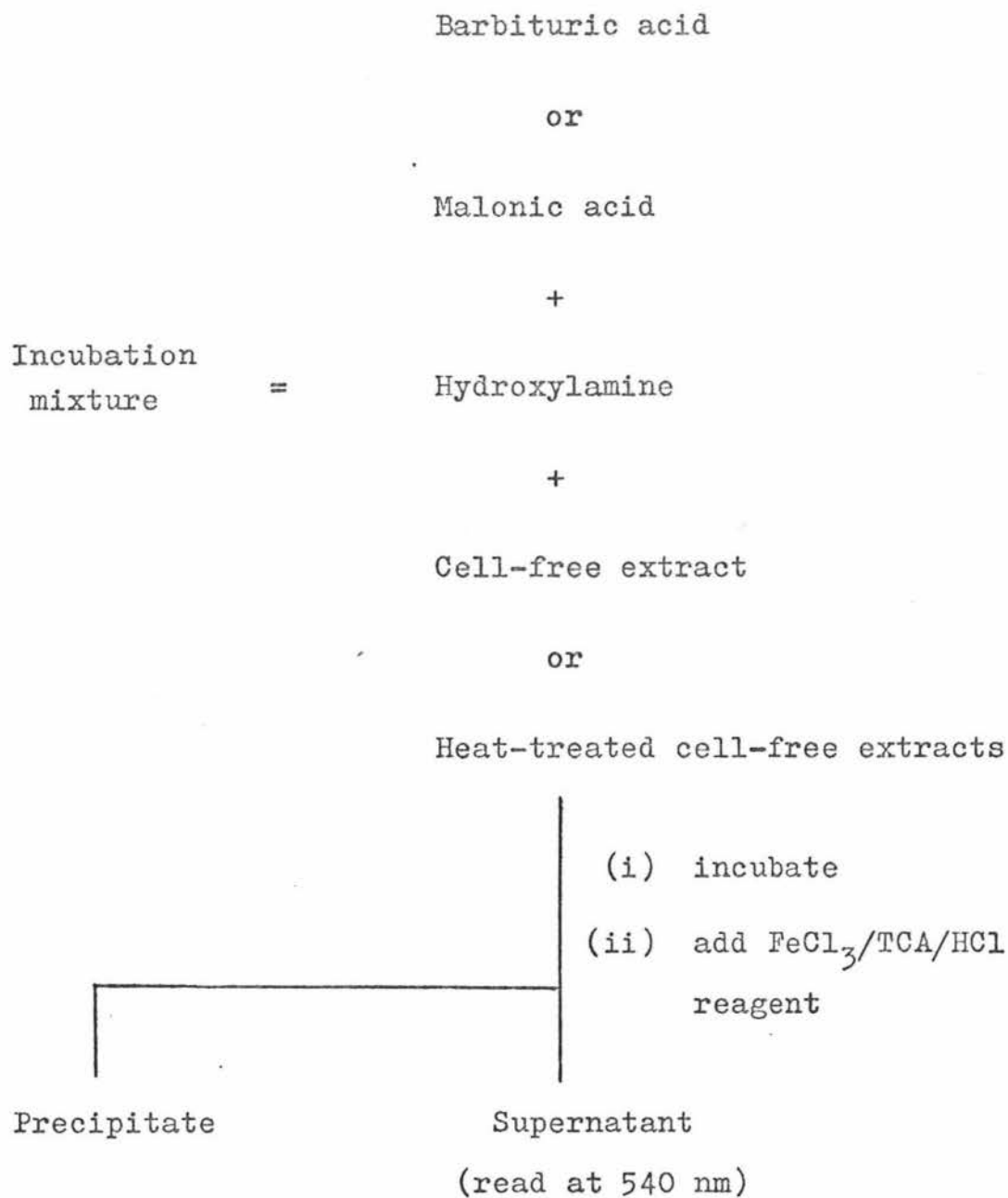
(c) Substrate, with and without added cofactors, was treated with TCA after incubation with cell-free extracts but before hydroxylamine treatment as a further test for enzymatic hydroxamic acid formation, and as a test for activation systems.

II.A. Effect Of Hydroxylamine On Barbiturase Activity

To determine the effect of hydroxylamine on barbiturase, the distilled water (0.3 cm^3) used in the normal assay procedure (Experimental Methods II.C.1.) was replaced with fresh, neutral hydroxylamine. The replacement caused almost complete inhibition of barbiturase activity; this inhibition is probably due to the lowering of the pH to pH 6.5 on the addition of hydroxylamine. (The pH optimum for barbiturase is about pH 8.2).

II.B. Incubation Of Substrate Plus Hydroxylamine With Either Normal Or Heat-treated Cell-free Extracts

In this experiment, outlined in Scheme 2, either normal or heat-treated (10 min, 100°C) cell-free extracts were added to tubes containing substrate (barbituric acid or malonic acid) plus hydroxylamine but no added cofactors. Details of the tube contents and incubation times are given in Table 12 together with absorbance readings measured following incubation and treatment with $\text{Fe Cl}_3/\text{TCA}/\text{HCl}$ reagent.



Scheme 2: Scheme for assaying the enzyme-catalysed formation of hydroxamic acids from malonic acid or barbituric acid. (Chapter 5, II.B.).

TABLE 12.

FORMATION OF HYDROXAMIC ACIDS IN INCUBATION SYSTEMS
CONTAINING HYDROXYLAMINE AND EITHER BARBITURIC ACID
OR MALONIC ACID

Incubation Mixture							Incubation Time	Absorbance
Buffer*	BSA	MA	BA	NH ₂ OH ^o	CFE	HTE ⁺	(min)	(540 nm)
+	+	-	-	+	-	+	0	0.025
+	+	-	-	+	-	+	10	0.125
+	+	-	+	+	+	-	0	0.045
+	+	-	+	+	+	-	10	0.165
+	+	+	-	+	+	-	0	0.030
+	+	+	-	+	+	-	1	0.155
+	+	+	-	+	+	-	2	0.235
+	+	+	-	+	+	-	5	0.325
+	+	+	-	+	+	-	10	0.365
+	+	+	-	+	+	-	30	0.375
+	+	+	-	+	-	+	0	0.025
+	+	+	-	+	-	+	10	0.095
+	+	+	-	+	-	-	0	0.020
+	+	+	-	+	-	-	10	0.025

* See Table 10 for abbreviations and explanations.

^o NH₂OH = Neutral 2.0 M hydroxylamine solution - 0.5 cm³

+ HTE = Heat-treated cell-free extract - 0.1 cm³

The final volume was made up to 1.5 cm³ with distilled water.

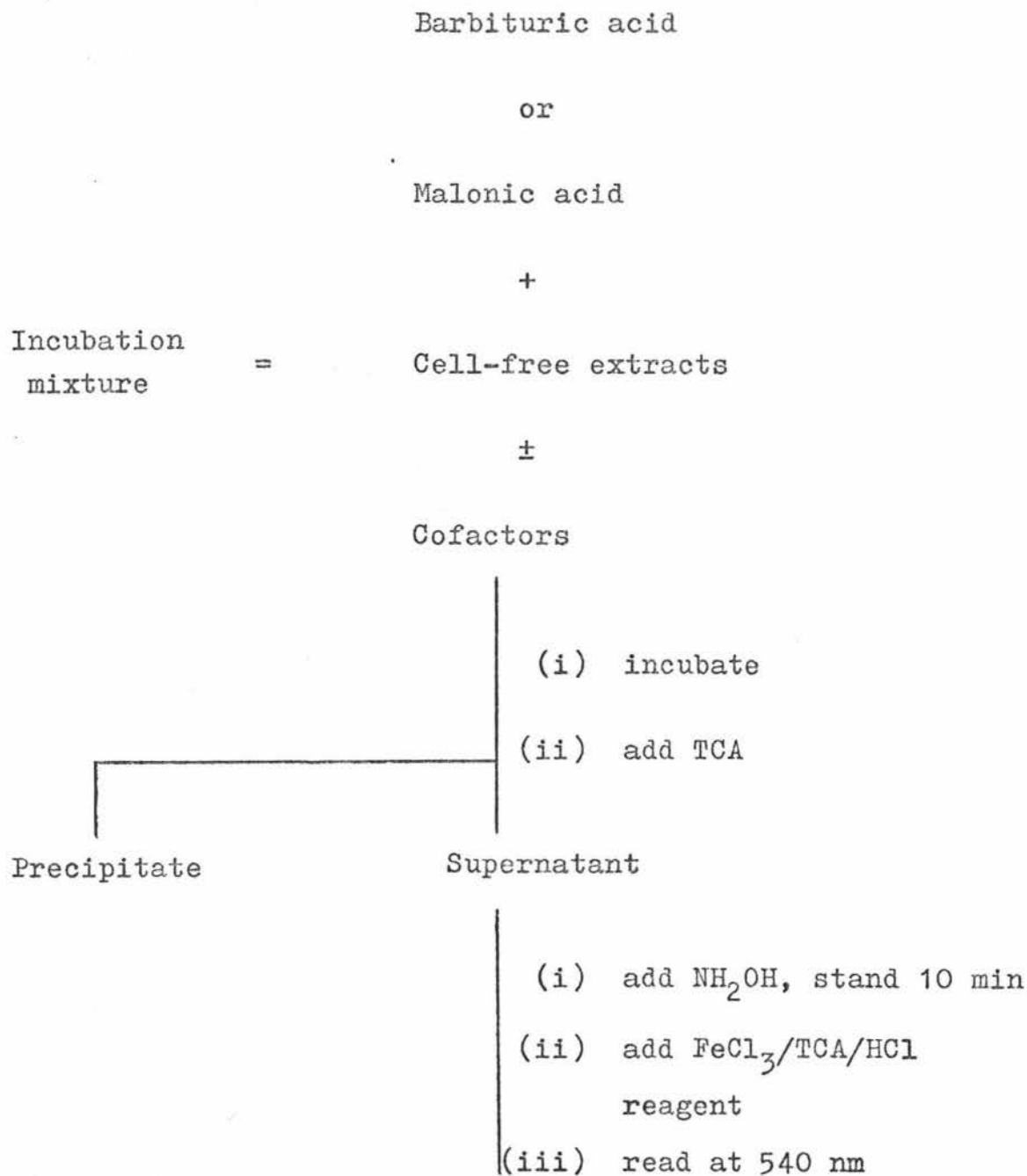
With barbituric acid plus hydroxylamine, incubated with normal cell-free extracts, the absorbance change in ten minutes was 0.120 units, while the comparable absorbance change in tubes containing hydroxylamine, but no substrate, and incubated with heat-treated cell-free extracts was 0.100 units. This, together with the finding that the presence of hydroxylamine inhibits barbiturase activity, suggests that most of the absorbance change observed with barbituric acid present in the incubation system is due to the formation of a hydroxamic acid from endogenous components in the cell-free extracts.

Absorbance readings from tubes containing malonic acid plus hydroxylamine, incubated with normal cell-free extracts, increased rapidly during the first five minutes of incubation, reaching a maximum by about ten minutes with an absorbance change of 0.335 units in ten minutes. The absorbance change when malonic acid plus hydroxylamine was incubated with heat-treated cell-free extracts was 0.07 in ten minutes.

The large, rapid absorbance changes observed during the first ten minutes of incubation, which are markedly reduced if heat-treated cell-free extracts are used, suggest that most of the absorbance change could be due to the enzyme-catalysed formation of a hydroxamic acid from the added malonic acid.

II.C. Effect Of TCA Addition After Incubation But Before Hydroxylamine Treatment

The following experiment, outlined in Scheme 3,



Scheme 3: Scheme for the modified quantitative activation assay for the formation of malonyl-CoA from malonic acid or barbituric acid. (Chapter 5, II.C.).

was performed as a check that the hydroxamic acid formation observed earlier was enzyme-catalysed, and not a measure of activated compound formation, and also to determine whether barbituric or malonic acids were activated by cell-free extracts.

Trichloroacetic acid was added to tubes containing substrate, either barbituric acid or malonic acid, with and without cofactors after they had been incubated with cell-free extracts for the times listed in Table 13. The protein-free supernatant was treated with hydroxylamine and, ten minutes later, with $\text{FeCl}_3/\text{TCA}/\text{HCl}$ reagent. Details of this procedure are given in Experimental Method II.C.2.a.

No changes in the absorbance readings, given in the table, were observed when cofactors were omitted from the incubation mixture and very little hydroxamic acid was formed. This contrasts with the results obtained in Experiment I.B. (this chapter), in which the TCA step was omitted where no absorbance change was observed but large amounts of hydroxamic acid were formed. When cofactors were added to the incubation mixture a small, slow increase in absorbance readings was observed, the total absorbance changes being about 0.06 and 0.09 units for barbituric and malonic acids respectively.

As well as showing that much of the hydroxamic acid formation observed earlier was enzyme-catalysed, these results show that, in the presence of cofactors, activation may occur, although the compound which was

TABLE 13.

HYDROXAMIC ACID FORMATION IN ASSAY SYSTEMS TO WHICH TCA
WAS ADDED AFTER INCUBATION BUT BEFORE
HYDROXYLAMINE TREATMENT

Incubation Time (min)	Absorbance readings (540 nm) obtained following incubation and treatment of tubes containing			
	MA	MA + cofactors	BA	BA + cofactors
0	0.032	0.037	0.035	0.043
2		0.046		0.050
5	0.033	0.052	0.036	0.055
10	0.032	0.062	0.036	0.062
25		0.084		0.085
30	0.035		0.034	
50		0.110		0.105
75		0.123		0.108
100		0.125		0.106

The tubes contained either malonic acid or barbituric acid, buffer, BSA and cell-free extracts, with or without cofactors, as described in Table 10.

activated may not have been the added substrate.

III. Identification Of The Enzyme-Synthesised Hydroxamic Acid

Tubes containing (2^{14}C -)malonic acid or (5^{14}C -)barbituric acid (synthesis described in Appendix 1) were incubated with cell-free extracts for 0, 5 or 30 minutes. Hydroxylamine was then added to the tubes and the solution treated with alcohol and acetone as described in Experimental Methods II.C.2.b.

Radiochromatograms of the alcohol extracts were developed using n-butanol:water (84:16 v/v) as solvent. The dried radiochromatograms were examined under UV light before spraying with the alcoholic FeCl_3/HCl reagent to detect hydroxamic acids.

Scans of radiochromatograms of (5^{14}C -)barbituric acid incubated for 0 min showed one peak which corresponded to a UV-absorbing spot on the radiochromatogram; this was identified as barbituric acid. After 5 min incubation two peaks were found in the scans; one was barbituric acid and the second, which gave a positive (purple/brown) colour reaction with the spray reagent, had an R_F of 0.36, very similar to that quoted by Stadtman and Barker (1950) for malonylmonohydroxamic acid (0.37). The scans of (5^{14}C -)barbituric acid after 30 min incubation showed only one peak corresponding to a spot giving a positive hydroxamic acid colour reaction and having the same R_F as malonylmonohydroxamic acid.

With (2^{14}C -)malonic acid the radiochromatogram scans of the samples incubated for 0, 5 and 30 min each showed one peak corresponding to a spot which gave a positive colour reaction with the spray and had an R_F similar to that of malonylmonohydroxamic acid. These results suggest that the hydroxamic acid formed is malonylmonohydroxamic acid.

IV. Activation Assays Using Substrates Other Than Malonic Acid And Barbituric Acid

Batt and Woods (1961) used the method of Rose (1955) to test for activation systems. A number of substrates were incubated with cofactors and cell-free extracts in the presence of hydroxylamine but only acetic and propionic acids were activated.

The results of the experiment described in II.C. (this chapter) appear to confirm Batt and Woods' result that barbituric and malonic acids are not activated. In the following experiment tubes containing the solutions listed in Table 14 were incubated for the times shown in the table and hydroxylamine was then added. Absorbance readings were measured after treatment with $\text{FeCl}_3/\text{TCA}/\text{HCl}$ reagent and are also given in the table.

The changes in absorbance readings confirm the finding of Batt and Woods (1961) that acetic acid is activated, and they also show a possible activation system for succinic acid. The other compounds tested do not appear to be activated.

TABLE 14.

ACTIVATION ASSAYS USING SUBSTRATES OTHER THAN
BARBITURIC ACID AND MALONIC ACID

Substrate	Cofactors	Incubation Time (min)	Absorbance (540 nm)
Acetate	-	0	0.145
"	-	10	0.147
"	+	0	0.195
"	+	10	0.310
Succinate	-	0	0.115
"	-	5	0.113
"	+	0	0.158
"	+	5	0.173
Malate	-	0	0.117
"	-	5	0.115
"	+	0	0.159
"	+	5	0.162

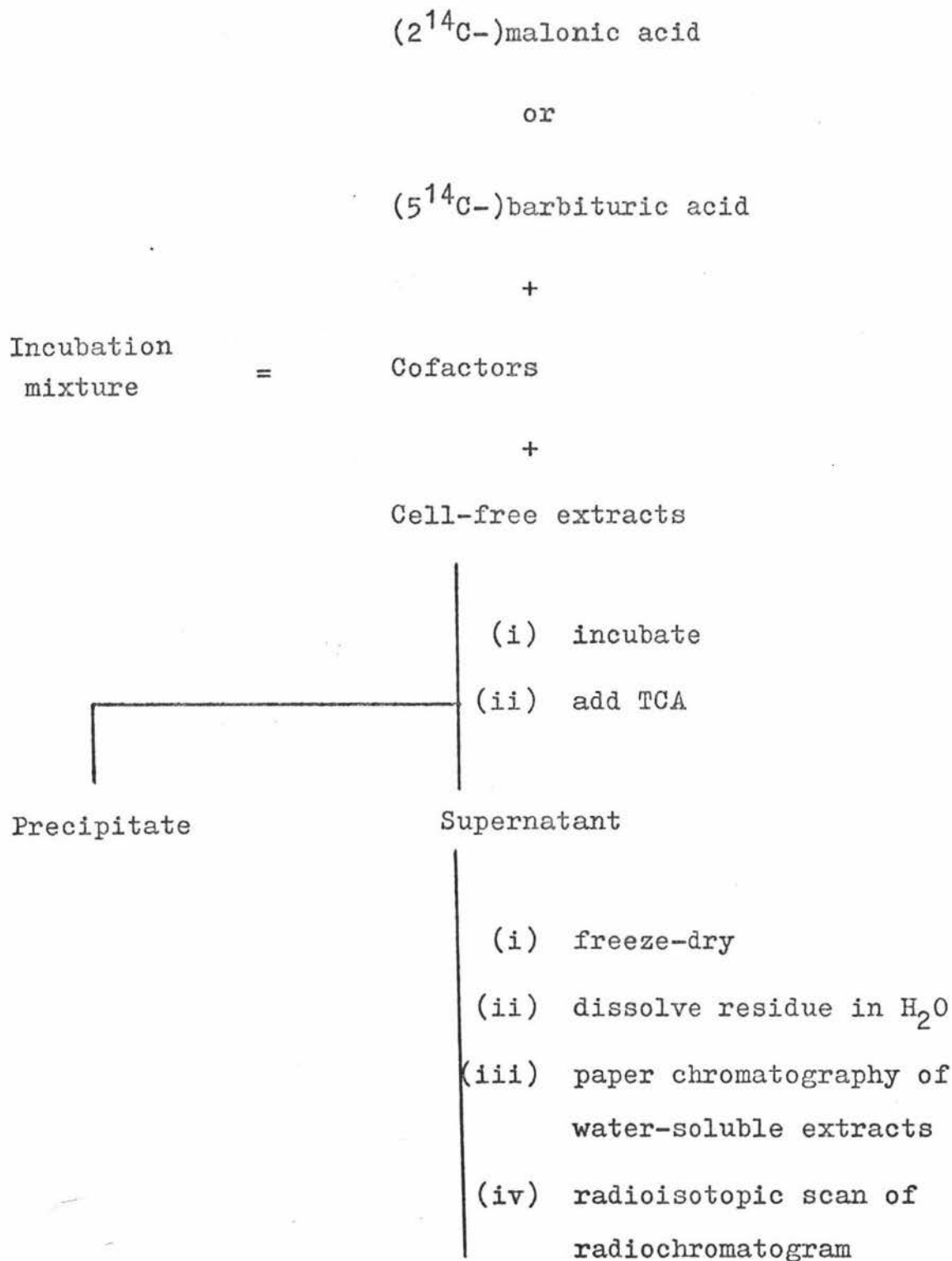
The tubes contained substrate (6.6 μ moles) and cofactors, as indicated above, together with buffer, BSA and cell-free extract as described in Table 10.

V. Qualitative Experiments Using (2^{14}C -)Malonic Acid Or (5^{14}C -)Barbituric Acid And Cell-free Extracts

The results of earlier experiments suggested that malonic and barbituric acids were not activated by cell-free extracts. To confirm this, experiments using (14C -)labelled substrates were performed. In one type of experiment, outlined in Scheme 4, substrate and cofactors were incubated with cell-free extract for varying times, after which the reaction was stopped by the addition of TCA, and following treatment, the solution was assayed directly, using paper chromatography, for the presence of labelled malonyl-CoA. In a second type of experiment, outlined in Scheme 5, substrate, cofactors and cell-free extracts were incubated. The reaction was stopped by adding TCA; hydroxylamine was added to the protein-free supernatant and, after further treatment, the solution was assayed indirectly, using paper chromatography, for labelled malonyl-CoA. Details of these direct and indirect assays are given in Experimental Methods, II.C.2.b.

V.A. Direct Assay For Malonyl-CoA Formation From Malonic Acid

Trichloroacetic acid was added to tubes containing (2^{14}C -)malonic acid, cofactors and cell-free extracts after 0, 5, 15 or 30 min incubation and the protein-free supernatant freeze-dried (Scheme 4). Radiochromatograms of the water-soluble freeze-dried extracts were developed by descending chromatography using n-butanol:acetic acid:water (5:2:3 v/v) as solvent.



Scheme 4: Scheme for the direct qualitative assay for malonyl-CoA formation from either malonic acid or barbituric acid. (Chapter 5, V.A. and V.C.).

(2¹⁴C-)malonic acid

or

(5¹⁴C-)barbituric acid

±

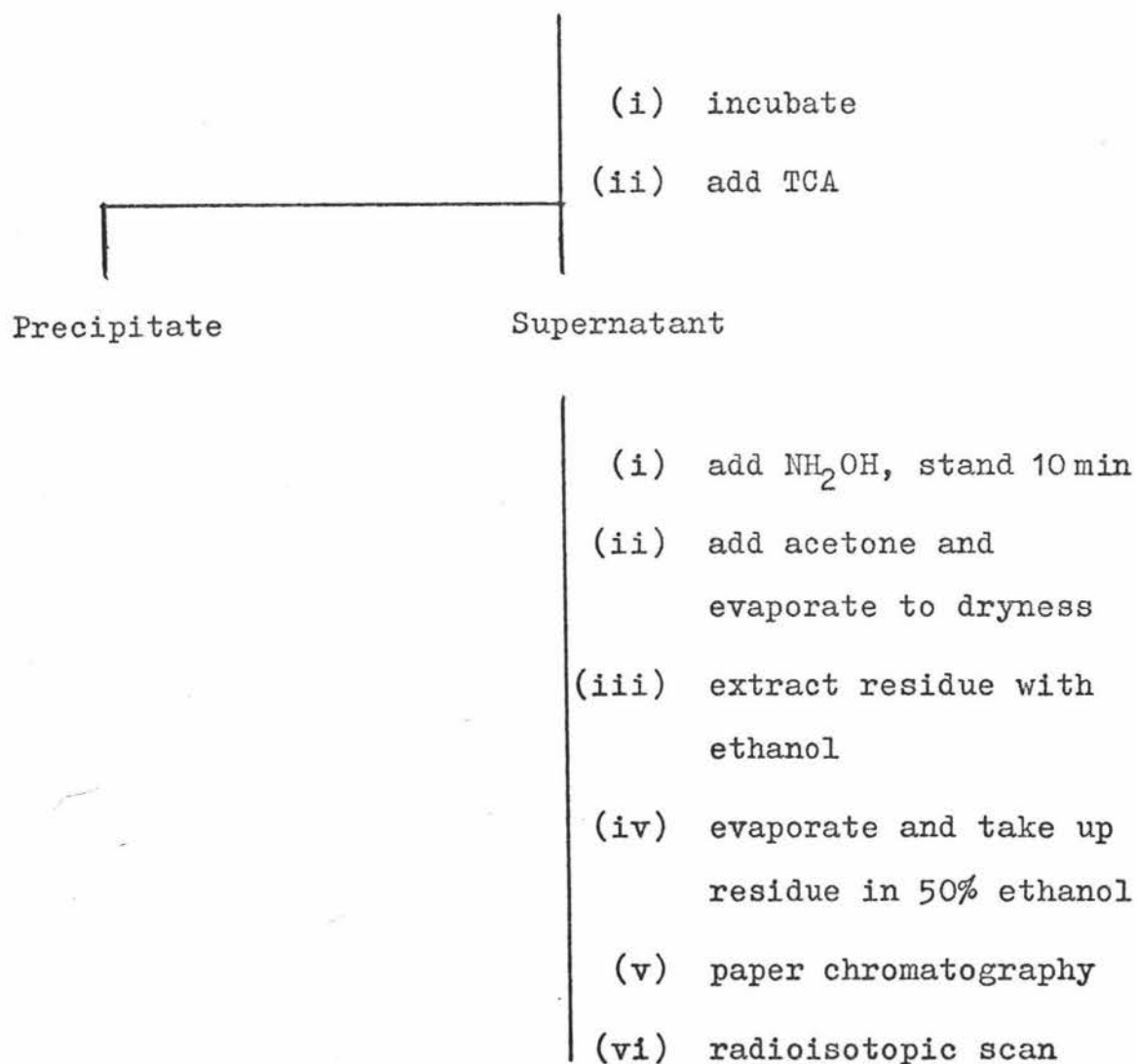
Incubation
mixture

=

Cofactors

+

Cell-free extracts



Scheme 5: Scheme for the indirect qualitative assay for malonyl-CoA formation from either malonic acid or barbituric acid (Chapter 5, V.B. and V.D.).

Scans of the radiochromatograms revealed one peak in each scan which corresponded to the peak obtained from the radiochromatogram of standard (2^{14}C -)malonic acid. The radiochromatogram scans of the 0 and 30 min, and the standard malonic acid are shown in Figure 7.

The results suggest that malonyl-CoA is not formed from malonic acid.

V.B. Indirect Assay For Malonyl-CoA Formation
 From Malonic Acid

The previous experiment was repeated with hydroxylamine being added to the protein-free supernatant. After treatment with acetone and alcohol (Scheme 5), radiochromatograms of the alcohol-soluble extracts were developed using n-butanol:water (84:16 v/v) as solvent. A radiochromatogram of (2^{14}C -)malonic acid which had undergone the same treatment as the samples was also developed.

Peaks were absent in the radiochromatogram scans of the incubated samples and the standard (2^{14}C -) malonic acid. The absence of a peak corresponding to (2^{14}C -)malonic acid is probably due to the conversion of the malonic acid to the sodium salt following the addition of the neutral hydroxylamine to the solution and subsequent evaporation to dryness. The sodium salt would be insoluble in the absolute alcohol extraction which follows the evaporation step.

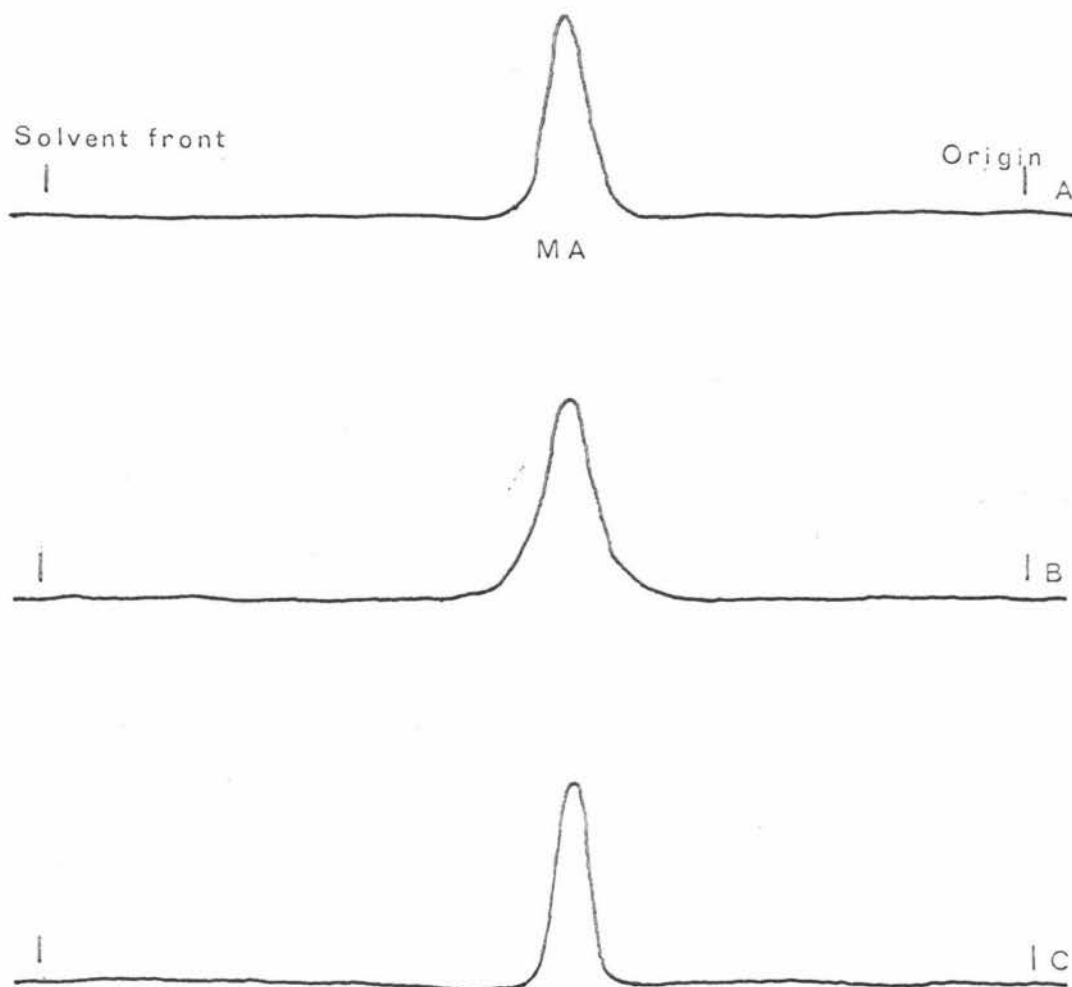


Figure 7. Radiochromatogram scans of water extracts of $[2^{14}\text{C}]$ malonic acid after incubation with cofactors & cell-free extracts, TCA treatment & freeze-drying.

KEY: A = 0 min

B = 30 min

C = $[2^{14}\text{C}]$ malonic acid standard

MA = malonic acid

The absence of peaks corresponding to malonyl-monohydroxamic acid confirms the previous findings that malonic acid is not converted to malonyl-CoA.

V.C. Direct Assay For Malonyl-CoA Formation From
Barbituric Acid

Trichloroacetic acid was added to tubes containing (5^{14}C -)barbituric acid, with and without added cofactors, and cell-free extracts, after 0, 5, 15 or 30 min incubation, and the protein-free supernatant freeze-dried (Scheme 4). Radiochromatograms of the water-soluble freeze-dried extracts were developed using n-butanol:acetic acid:water (5:2:3 v/v). Radiochromatogram scans of the extracts from incubations performed in the absence and presence of cofactors are shown in Figures 8 and 9 respectively.

The scans of the 0 min incubations showed one peak, corresponding to a UV-absorbing spot on the radiochromatogram, which was identified as barbituric acid. The 5 min incubation scan in Figure 8 shows two peaks; one was identified as barbituric acid and, after spraying with alkaline methyl red, the second peak was identified as malonic acid. The 30 min incubation scan shown in Figure 8 showed one peak corresponding to malonic acid.

The scan of the 5 min incubation with cofactors present (Figure 9) showed three peaks; two of these corresponded to barbituric acid and malonic acid while the third had an R_F the same as standard (2^{14}C -)malonyl-CoA.

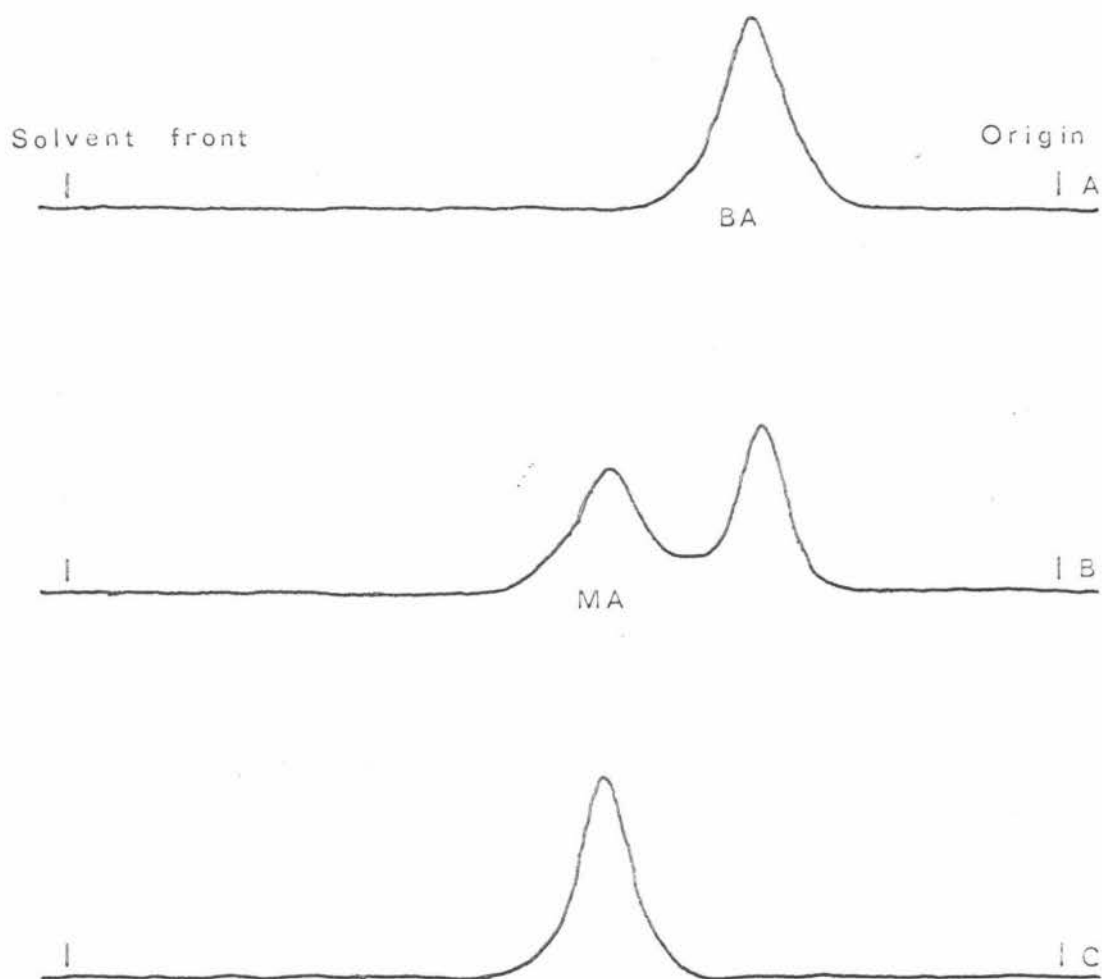


Figure 8. Radiochromatogram scans of water extracts of $[5^{14}\text{C}]$ barbituric acid after incubation with cell-free extracts, TCA treatment and freeze-drying.

KEY: A = 0 min

B = .5 min

C = 30 min

BA = barbituric acid

MA = malonic acid

(The radiochromatogram scan of the (2^{14}C -)malonyl-CoA is also shown in Figure 9). The scan of the 30 min incubation showed one major peak, malonic acid, and several minor peaks, one of which had the same R_F as the (2^{14}C -)malonyl-CoA standard.

These results suggest that, in the presence of cofactors, barbituric acid may be converted to malonyl-CoA, as well as malonic acid, by an enzyme(s) in the cell-free extract. In this qualitative experiment it is difficult to estimate the amount of malonyl-CoA formed but, using peak sizes as a basis for estimation, probably less than 5% of the barbituric acid is converted to malonyl-CoA under the conditions used in this experiment.

V.D. Indirect Assay of Malonyl-CoA Formation From
Barbituric Acid

The previous experiment was repeated with hydroxylamine being added to the protein-free supernatant obtained after TCA precipitation. After treatment with acetone and alcohol (Scheme 5), radiochromatograms of the alcohol-soluble extracts and of samples of (5^{14}C -)barbituric acid and (2^{14}C -)malonyl-CoA which had been similarly treated were developed using n-butanol:water (84:16 v/v) as solvent.

The radiochromatogram scans of the extracts from the 0 min incubations in the presence and absence of added cofactors are shown in Figures 10 and 11 respectively. The scans each showed one peak which matched the peak in the scan of the treated, labelled barbituric acid standard.

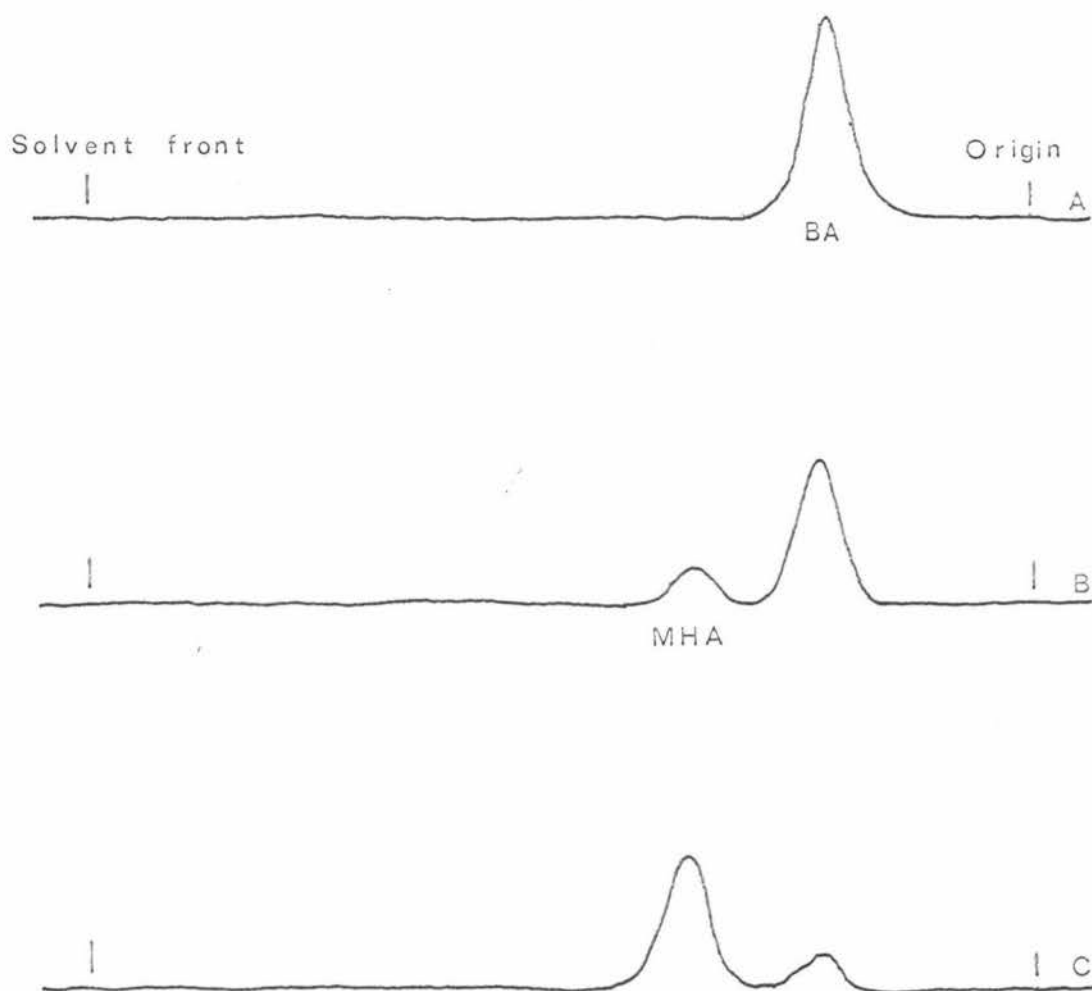


Figure 11. Radiochromatogram scans of alcohol extracts of $[5^{14}\text{C}]$ barbituric acid after incubation with cell-free extracts & treatment with hydroxylamine.

KEY: A = 0 min

B = 5 min

C = 30 min

BA = barbituric acid

MHA = monomalonylhydroxamic acid

Two peaks were observed in the scans of radiochromatograms of the samples incubated for 5 min and 30 min in the presence of cofactors; these are also shown in Figure 10 together with scans of the treated (5^{14}C -)barbituric acid and (2^{14}C -)malonyl-CoA standards. One peak was identified as barbituric acid and the other matched the peak obtained from the treated, labelled malonyl-CoA. The radiochromatogram scan of the sample incubated for 30 min without added cofactors (Figure 11) also showed two peaks which corresponded to the peaks obtained from the treated standards.

The results appear to confirm the findings of the previous experiment that, in the presence of cofactors, barbituric acid may be converted to malonyl-CoA as well as malonic acid. However, using this indirect assay system, it appears as if an activated derivative of malonic acid is produced from barbituric acid if cofactors are omitted from the incubation mixture. The malonylmonohydroxamic acid peak was larger after 30 min incubation if cofactors had been added to the assay system, suggesting that their presence enhances malonic acid activation.

VI. Qualitative Activation Assays Using Partially-Purified Barbiturase And (5^{14}C -)Barbituric Acid

Barbiturase, partially purified as described in Chapter 4, was used in this experiment to establish if the enzyme preparation also catalysed malonyl-CoA formation. The indirect (hydroxamic acid) assay system (Scheme 5) was used except that 0.20 cm^3 of enzyme preparation was used

instead of 0.10 cm³ of cell-free extracts plus 0.10 cm³ distilled water.

Trichloroacetic acid was added to tubes containing substrate, with and without added cofactors, after 0 or 30 min incubation with the partially-purified enzyme. Radiochromatograms of the alcohol extracts of the hydroxylamine-treated samples and a hydroxylamine-treated (2¹⁴C-)malonyl-CoA standard were developed using n-butanol:water (84:16 v/v) as the solvent. Scans of the radiochromatograms of extracts from samples incubated for 0 min and for 30 min with and without added cofactors are shown in Figure 12 together with the scan of the treated, labelled malonyl-CoA.

The scans showed one peak after 0 min (barbituric acid) and two peaks after 30 min incubation. One peak corresponded to barbituric acid while the second corresponded to the peak obtained in scans of the treated malonyl-CoA standard (malonylmonohydroxamic acid). The malonylmonohydroxamic acid peak was larger in the scans of the samples which had been incubated in the presence of cofactors.

The results indicate that the partially-purified barbiturase is capable of forming an activated derivative of malonic acid from barbituric acid. The formation of an activated form of malonic acid in the absence of added cofactors and the enhancement of its formation when cofactors are added suggest that the enzyme may be complex having cofactors associated with it.

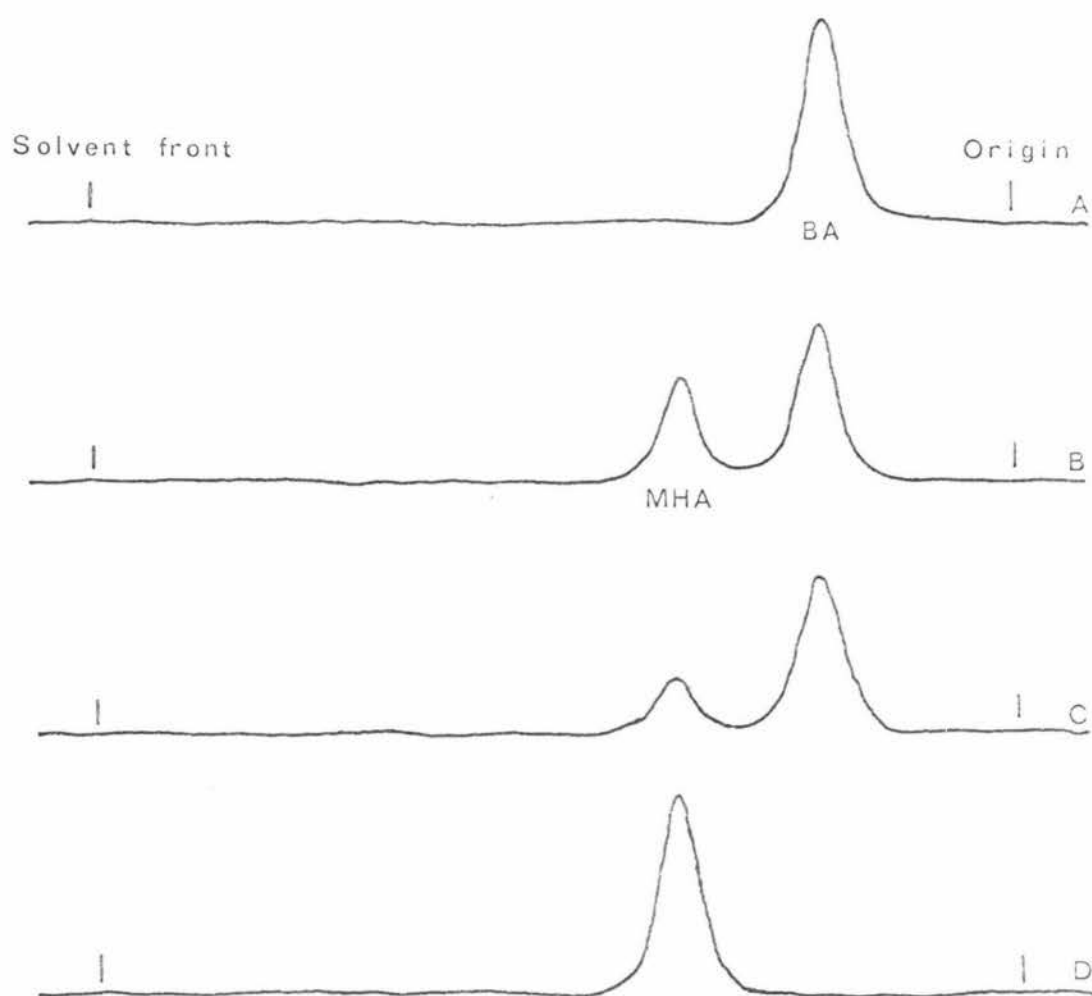


Figure 12. Radiochromatogram scans of alcohol extracts of $[5^{14}\text{C}]$ barbituric acid after incubation with partially purified barbiturase and cofactors (scans A & B) or partially purified barbiturase only (scan C) & treatment with hydroxylamine.

KEY. A = 0 min BA = barbituric acid
 B = 30 min MHA = monomalonylhydroxamic acid
 C = 30 min
 D = hydroxylamine-treated $[2^{14}\text{C}]$ malonyl-CoA

5.

DISCUSSION

Barbiturase is an induced enzyme found only in microbial cells which have been grown on, or incubated with pyrimidines — especially uracil or barbituric acid.

It was first described in the early 1950's but since then few attempts have been made to purify it or to study its kinetics.

I. Enzyme Purification

Although hydrolytic enzymes are usually relatively amenable to standard purification procedures, when attempts were made to purify barbiturase from cell-free extracts it was found to be surprisingly unstable. This instability, together with the low yields of bacteria obtained from the relatively small volumes of culture media which could be readily handled proved to present major difficulties during the investigation.

Losses in activity were observed during ammonium sulphate fractionation, dialysis, ion-exchange chromatography and gel-filtration steps, although the enzyme was stable in crude, cell-free extracts. The enzyme was also stable (a) in the presence of 50% ammonium sulphate (b) when dialysed against buffers which were 50% saturated with ammonium sulphate and (c) when gel-filtration was carried out using buffers saturated to 50% with ammonium sulphate.

These observations suggested that the loss of cofactors and/or metal ions was not responsible for the

loss of activity unless perhaps the high salt concentrations enhanced the binding of such substances to the enzyme.

The most likely cofactor would appear to be Coenzyme-A, but the testing of this in dialysis solutions would clearly have been exceedingly expensive and probably unjustified while other experimental approaches could still be tested.

(In some later studies it may be necessary, however, to use buffers containing this cofactor.) Dialysis against solutions containing various metal ions did not improve enzyme stabilities or recoveries.

Attempts were continued to purify the enzyme, primarily because anomalous findings had been reported in the literature by various workers related to barbituric acid metabolism in micro-organisms shown to be capable of oxidising pyrimidines. A partial purification of barbiturase was eventually achieved.

In these purification studies enzyme activity was measured by following the disappearance of barbituric acid. However, in the light of later results it would seem to be more appropriate to use malonyl-CoA formation as the basis for an enzyme assay.

II. Barbituric Acid Metabolism

Although there is good evidence that barbituric acid is converted to urea and malonic acid by cell-free extracts obtained from various micro-organisms capable of oxidising uracil, evidence has not been obtained for this conversion taking place in intact cells. In fact, there is

some positive evidence which suggests that free malonic acid is not an intermediate in vivo. In summary

(a) Cells adapted for pyrimidine catabolism cannot utilise malonic acid plus urea for growth (Batt and Woods (1961), Hayaishi and Kornberg (1952), and Biggs and Doumas (1963)).

(b) Free malonic acid does not accumulate in the medium when cells are grown on a pyrimidine (Batt and Woods (1961)).

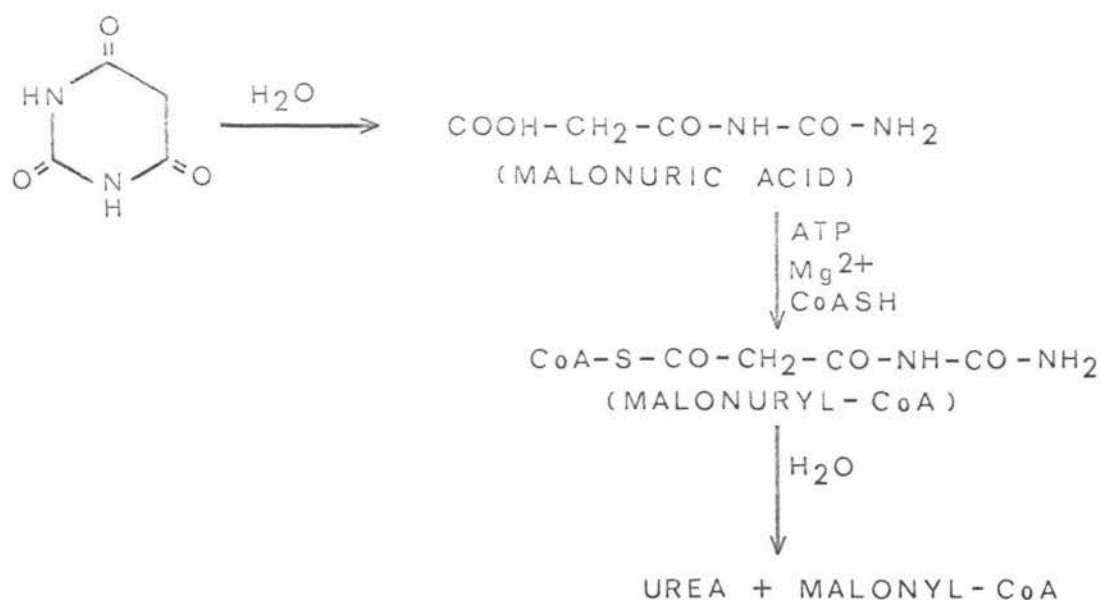
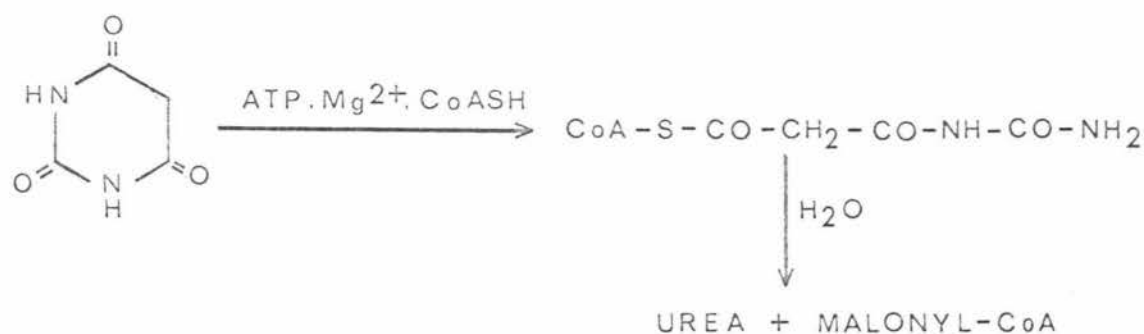
(c) Activation systems for malonic acid have not been demonstrated in cell-free extracts of N. corallina (Batt and Woods (1961)).

(d) Succinate dehydrogenase inhibition was observed when whole cells were incubated in the presence of malonic acid which suggested that malonic acid could enter intact cells, even although it was not oxidised (Batt (1952) Ph.D. thesis, Oxford University). (There is also evidence that oxidative assimilation occurs, suggesting that the 3-carbon portion of barbituric acid is utilised).

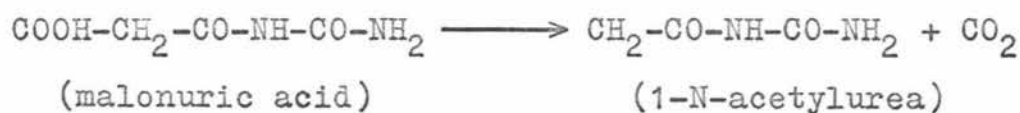
If malonic acid is not an in vivo product of barbituric acid metabolism, the most likely intermediate would be malonyl-CoA. The results of the experiments described in Chapter 5 have, in fact, suggested that both cell-free extracts and the partially purified barbiturase are capable of the formation of activated malonic acid from barbituric acid. However, neither the cell-free extracts nor the partially purified enzyme were capable of converting

malonic acid to malonyl-CoA. With barbituric acid, it was shown that the activation was enhanced by the addition of cofactors to the incubation mixture.

There are several hypothetical pathways by which barbituric acid might be converted to malonyl-CoA including:



Of these, the second would seem to be the more likely but attempts to synthesise malonuric acid for testing as an intermediate were unsuccessful; malonuric acid is unstable and readily undergoes the following reaction:



III. Malonic Acid Formation In Vitro

As already noted, malonic acid is formed from barbituric acid, in vitro, and it is possible that this barbiturase activity is due to the action of the same protein induced for barbituric acid breakdown, in vivo. It is possible that during isolation the protein is modified in some way and that this perhaps occurs when the cells are disintegrated. Since barbiturase activity is not observed in non-induced cells, the formation of barbiturase activity from an induced protein may reasonably be assumed.

If this hypothesis is correct the modification could be associated with a conformational change of the induced protein, producing the barbiturase protein, or alternatively, there could be an unmasking of a barbiturase site on the induced protein. In whole cells it is unlikely that two active enzymes are involved in barbituric acid breakdown, or that an induced protein has two enzyme activities, since these possibilities would lead to some malonic acid being produced in vivo.

IV. Enzyme Induction By Pyrimidines

The results of in vivo studies by Batt and Woods (1961) suggested that separate enzymes were responsible for the breakdown of barbituric acid and 5-methylbarbituric acid in N. corallina. Thymine-grown cells and uracil-grown cells utilised thymine and uracil respectively with little accumulation of their respective barbituric acids. However, thymine-grown cells converted uracil and uracil-grown cells converted thymine almost

quantitatively to their respective barbituric acids. Conversion was complete after about $1\frac{1}{2}$ hr when the barbituric acids then began to be utilised.

This suggested that the oxidase responsible for uracil and thymine oxidation is non-specific, and experimental evidence supporting this was first reported by Hayaishi and Kornberg in 1952. By contrast, it appears that the enzymes responsible for barbituric acid and 5-methylbarbituric acid utilisation are different and substrate-specific. The experimental results also suggested that thymine and uracil are poor inducers of barbiturase and "5-methylbarbiturase" activities respectively. In this context, Brennan (1970) showed, for example, that the specific activity of barbiturase in thymine-grown cells was only $\frac{1}{10}$ of that found in uracil-grown cells.

If it is correct that separate enzymes exist for barbituric acid and 5-methylbarbituric acid utilisation, and that barbituric acid is converted to malonyl-CoA in vivo, studies using thymine-grown cells and 5-methylbarbituric acid should be undertaken to determine whether 5-methylbarbituric acid is converted to methylmalonyl-CoA.

V. Other Oxidative Pathways

There is some evidence for the existence of an oxidative pathway for the conversion of thymine to uracil in N. corallina. Batt (1957, 1958 and unpublished results) showed that 6-methyluracil, 5-hydroxymethyluracil and uracil-5-carboxylic acid induced a non-specific uracil-

thymine oxidase in N. corallina, although 6-methyluracil did not support growth and uracil-5-carboxylic acid gave only a poor growth response. Barbituric acid appeared to be produced from 6-methyluracil in cells in which this compound had been used for oxidase induction. There was also some evidence that in thymine grown cells, 5-hydroxymethyluracil may be produced from thymine.

This raises the possibility that a pathway of thymine conversion through 5-hydroxymethyluracil, 5-formyluracil and uracil-5-carboxylic acid to uracil may exist in pyrimidine-adapted N. corallina.

At this stage, therefore, it would seem that barbiturase is an artifact formed from an induced protein during isolation and that, in intact cells, barbituric acid is oxidised - and the carbon atoms assimilated in part - via an activated form of malonic acid, probably malonyl Coenzyme-A.

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APPENDIX 1.

(5¹⁴C-)barbituric acid was synthesised from (2¹⁴C-) malonic acid by esterification and subsequent condensation with urea. The esterification and condensation methods were based on those of Vogel (1964).

Synthesis of (2¹⁴C-)diethylmalonate

Malonic acid (0.52 g), (2¹⁴C-)malonic acid in 50% EtOH (0.1 cm³ \equiv 25 μ Cl) and EtOH (1.0 cm³) were added to a flask (10 cm³) containing dry benzene (4.5 cm³). Concentrated H₂SO₄ (0.15 cm³) was slowly added to the flask and, after refluxing for 6 hr, the mixture was poured into a separating funnel together with water (15 cm³). The benzene layer was retained and the water layer was washed several times with ether. The benzene layer and the pooled ether extracts were combined and washed with saturated NaHCO₃. After washing with water and drying with CaSO₄ the solvents were removed by distillation at atmospheric pressure. The product (0.72 g) had a refractive index and an infra red absorption spectrum consistent with diethylmalonate.

Synthesis of (5¹⁴C-)barbituric acid

Urea (0.27 g) was added to a round bottom flask containing EtOH (4.0 cm³) in which sodium metal (0.10 g) had been dissolved. (2¹⁴C-)diethylmalonate (0.70 g) was added and the mixture refluxed for 4 hr.

After cooling, the EtOH was drained and the residue washed twice with cold EtOH (5.0 cm³). The residue was then dissolved in 3.0 M HCl (2.0 cm³) and left to crystallise in a refrigerator overnight. The crystals were washed twice with cold water (1.15 cm³) and dried in vacuum dessicator. The product (0.5 g) had a melting point and a UV spectrum consistent with barbituric acid.

APPENDIX 2.Synthesis of calcium phosphate gel

The following method, based on the Keilin and Hartree method (1938), was used. Calcium chloride solution (132 g $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ /litre, 150 cm^3) was diluted to 1600 cm^3 with tap water to which trisodium phosphate solution (152 g $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ /litre, 150 cm^3) was added with vigorous stirring. The pH of the solution was adjusted to pH 7.4 using dilute acetic acid and the precipitate was washed four times by decantation with about 15 litres of water. The precipitate was finally washed with distilled water and, after centrifugation, the gel was suspended in a small volume of distilled water. The gel was kept in a refrigerator for 3-4 weeks before using.

APPENDIX 3.Glucose-Yeast Extract-Agar Medium For Slope Cultures

Glucose	0.75 g
$(\text{NH}_4)_2\text{SO}_4$	0.30 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.01 g
Yeast extract	0.05 g
KH_2PO_4	0.34 g
Thiamine HCl (Vitamin B1)	0.003 g

were dissolved in approximately 90 cm³ distilled water. After neutralisation with 5 M NaOH, the final volume was made up to 100 cm³ and Davis agar (2.0 g) was added to the solution. The preparation was heated and poured into McCartney bottles prior to autoclaving at 121°C for 15 min.

APPENDIX 4.Pyrimidine Culture Medium

KH_2PO_4	68.0 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
Barbituric acid	10.0 g
Thiamine HCl (Vitamin B1)	0.25 g

were dissolved in approximately 4.8 litres of distilled water. After neutralisation with 5 M NaOH, the final volume was made up to 5.0 litres with distilled water. The solution was dispensed equally into 15 one litre conical flasks which were plugged with muslin-coated cotton wool and autoclaved for 15 min at 121°C.