PURIFICATION AND PROPERTIES OF URACIL DEHYDROGENASE FROM *Nocardia corallina*

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ABSTRACT

Uracil dehydrogenase (EC 1.2.99.1), an enzyme which catalysed the oxidation of uracil or thymine to the corresponding barbituric acids, has been purified in a typical preparation, 85-fold with an 18% recovery of the initial activity present in a cell-free extract from Nocardia corallina. The enzyme was considered to be 95-98% pure by gel electrophoresis. It has a molecular weight of approximately 298 000, determined by both gel filtration and sedimentation equilibrium centrifugation and consists of two each of three dissimilar subunits, with molecular weights of 91 000, 36 000 and 21 000 as determined by sodium dodecyl sulphate — polyacrylamide gel electrophoresis. The spectrum of the enzyme is similar to that of milk xanthine oxidase and rabbit liver aldehyde oxidase (Rajagopalan & Handler, 1964), and is typical of nonheme iron flavoproteins. The flavin prosthetic group was identified as flavin adenine dinucleotide. It was demonstrated that the enzyme contains, per mole of protein, 1 mole of FAD, 4 atoms of nonheme iron and 4 moles of labile sulphide. The amino acid composition of uracil dehydrogenase has been determined. Other properties reported for the enzyme include substrate and electron acceptor specificity, K_m for uracil and thymine, pH optimum, and the effect of various inhibitors on enzyme activity. In addition, the purified enzyme has been shown to exhibit 'aerobic dehydrogenase' activity.

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CHAPTER 1

INTRODUCTION

1.1 General introduction

1.1.1 Pyrimidine catabolism

The present study of pyrimidine catabolism in Nocardia corallina was undertaken as an extension of previous investigations by Batt & Woods (1951, 1961), which had shown that, in this organism, uracil and thymine were oxidised to the corresponding barbituric acids. The enzyme, "uracil dehydrogenase (EC 1.2.99.1)" which catalyses the initial step in oxidative catabolism of uracil or thymine, is the subject of this thesis.

Pyrimidine bases are degraded in humans, higher animals, parasitic worms, plant seedlings and microorganisms. Two principal pathways for pyrimidine catabolism occur in nature: a reductive pathway and an oxidative pathway. Thymine and uracil can be catabolised by either pathway, while cytosine and 5-methylcytosine are first deaminated to uracil or thymine, respectively. In addition, thymine may be oxidised to uracil and CO_2. The above pathways are discussed below, in order to give some appreciation of the role of uracil dehydrogenase in pyrimidine catabolism.

Deamination of pyrimidines

Cytosine and methylcytosine are initially deaminated to yield uracil and thymine, respectively, before further degradation of the pyrimidine ring system occurs.

It has been reported that cytosine and 5-methylcytosine were converted to uracil and thymine, respectively, by a yeast extract (Hahn & Haarman, 1926) and bacteria (Iwatsura

& Chikano, 1923; Friedman & Gots, 1953). Yeast cells as well as cell-free extracts and intact cells of Escherichia coli contained cytosine deaminase which catabolised the conversion of cytosine to uracil (Chargaff & Kream, 1948). Studies with Bacterium sp. (Wang & Lampen, 1952a) and Corynebacterium (Hayaishi & Kornberg, 1952) indicated that deamination of cytosine to uracil was probably the first step and the uracil was then oxidised. For example, Wang & Lampen (1952a) showed that when cytosine-adapted Bacterium sp. was incubated with cytosine anaerobically, the spectrum of uracil was observed in the incubation mixture. Suspensions of Rhodospirillum rubrum rapidly deaminated cytosine and 5-methylcytosine to uracil and thymine, respectively (Goodwin & Passorn,1961). Uracil was rapidly absorbed and metabolised by germinating rape seeds but part of the uracil was converted into cytosine (Evans & Axelrod, 1961). Recently, Vilks & Vitols (1973) showed that the yeast Rhodotorula, grown on 5-methylcytosine as sole nitrogen source deaminated this compound to thymine.

Oxidative conversion of thymine to uracil

A pathway for the conversion of thymine to uracil, suggested by the results of Fink et al. (1956) using rat liver slices, was later established in Neurospora crassa (Williams & Mitchell, 1969). The sequential, enzymic oxygenation of thymine resulted in the formation of 5-hydroxymethyluracil, 5-formyluracil and 5-carboxyuracil, respectively. The latter was then converted to uracil. This system has not been reported in bacteria.

Reductive pathway

The steps involved in the reduction of uracil and thymine have been established as shown in Figure 1.1 (Fink et al., 1956). The initial reaction is the reduction of uracil or thymine to the corresponding dihydropyrimidine. Subsequent hydrolysis in two steps leads to β-alanine or β-aminoisobutyrate, CO_2 and ammonia.

It appears that this pathway occurs widely in nature. Evidence for a reductive pathway was initially obtained by various investigators from studies with animals (Fink et al., 1953, 1956; Batt & Exton, 1956; Fritzson & Nakken, 1956; Fritzson, 1957; Fritzson & Pihl, 1957), Clostridium uracilicum (Campbell, 1957a, b) and N. crassa (Woodward et al., 1957). It has also been described in man (Gartler, 1959), normal and leukemic leukocytes (March & Perry, 1964), rat tape worm, Hymenoleptis diminuta (Campbell, 1960), Hydrogenomonas facilis (Kraemer & Kaltwasser, 1969), germinating rape seeds (Evans & Axelrod, 1961; Tsai & Axelrod, 1965), and Jack pine seedling (Pitel & Durzan, 1975).

The individual enzymes involved in the reductive pathway have been purified and studied by Grisolia & Cardoso (1957), Wallach & Grisolia (1957), Caravaca & Grisolia (1958), Campbell (1957, 1958), Fritzson (1960) and Goedde et al. (1970).

Oxidative pathway

The results obtained by various authors with bacteria, as described below, have led to the scheme for the oxidative degradation of uracil and thymine as shown in Figure 1.2. The initial reaction is the oxidation of uracil or thymine

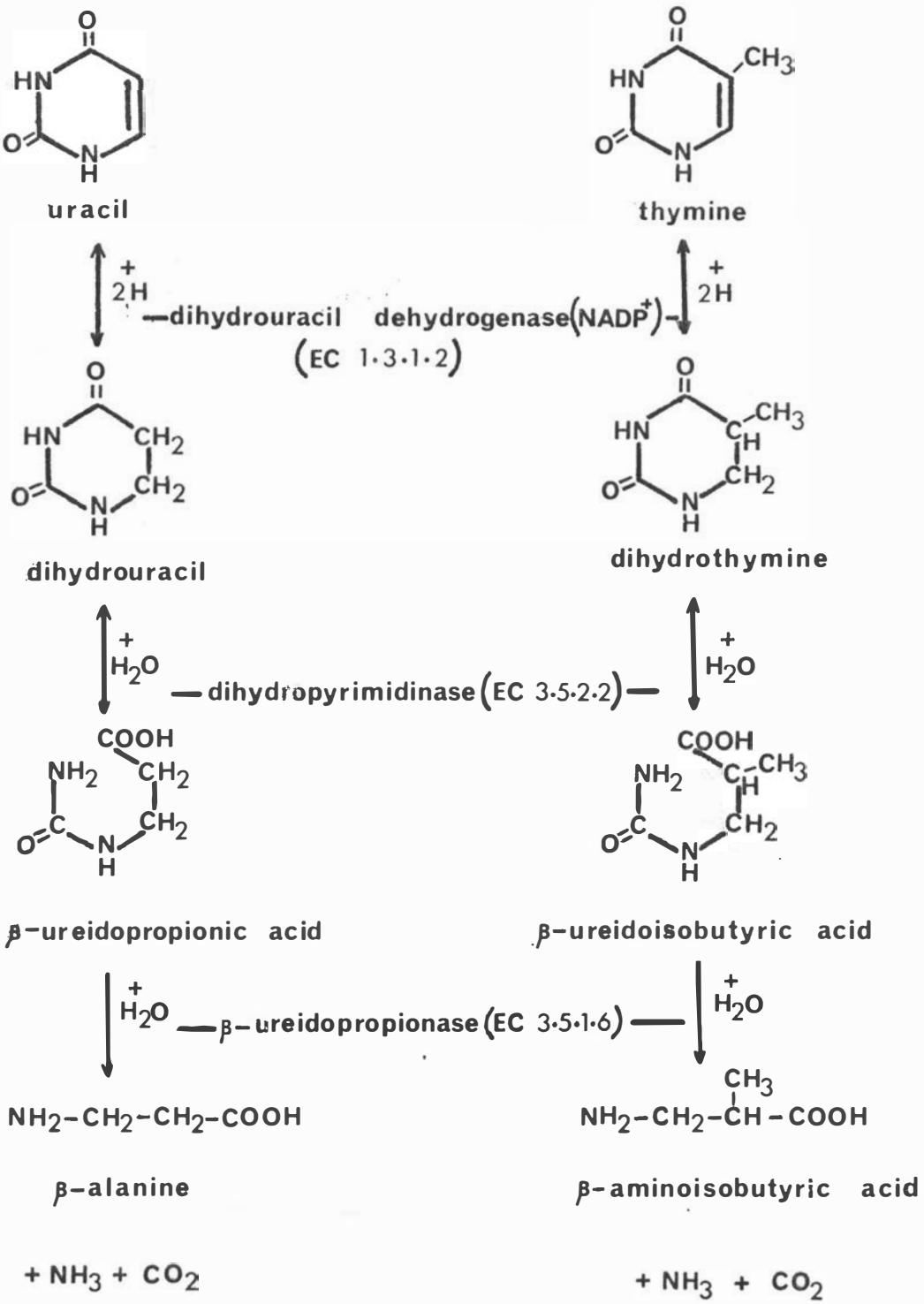


Figure 1.1 Pathway of the reductive catabolism of uracil and thymine

to barbituric acid or methylbarbituric acid. Subsequent hydrolysis yields malonic acid or methylmalonic acid with urea which is then hydrolysed to CO_2 and ammonia.

The oxidative catabolism of uracil to isobarbituric acid and thymine to thymine glycol was first suggested by Cerecedo (1927, 1930, 1931). The findings were based on feeding studies with dogs, where thymine or uracil led to an increase in observation urea excretion. Lara (1952a, b) clearly demonstrated that the pathways proposed by Cerecedo did not apply to thymine-adapted cells of N. corallina. However, he suggested the oxidation of thymine to form barbituric acid, but this seems unlikely from subsequent results (Batt & Woods, 1951; Hayaishi & Kornberg, 1952).

Oxidative catabolism of pyrimidines has been studied principally in certain aerobic soil bacteria (e.g. Nocardia, Mycobacterium, Corynebacterium spp.) which were capable of growing on pyrimidines as the sole source of carbon and nitrogen.

Wang & Lampen (1951) reported that resting cells of an unidentified species of bacterium (tentatively identified as Bacterium sp. by Wang & Lampen, 1952a) oxidised uracil, thymine or cytosine to CO_2 and ammonia. In their later studies using cell-free extracts of this same organism, the authors (1952b) identified barbituric acid as the oxidation product of uracil. The reactions were carried out aerobically in the presence of methylene blue.

Batt & Woods (1951) reported that uracil or thymine, but not cytosine, was oxidized by an unidentified bacterium and found that the oxidation product of thymine had an

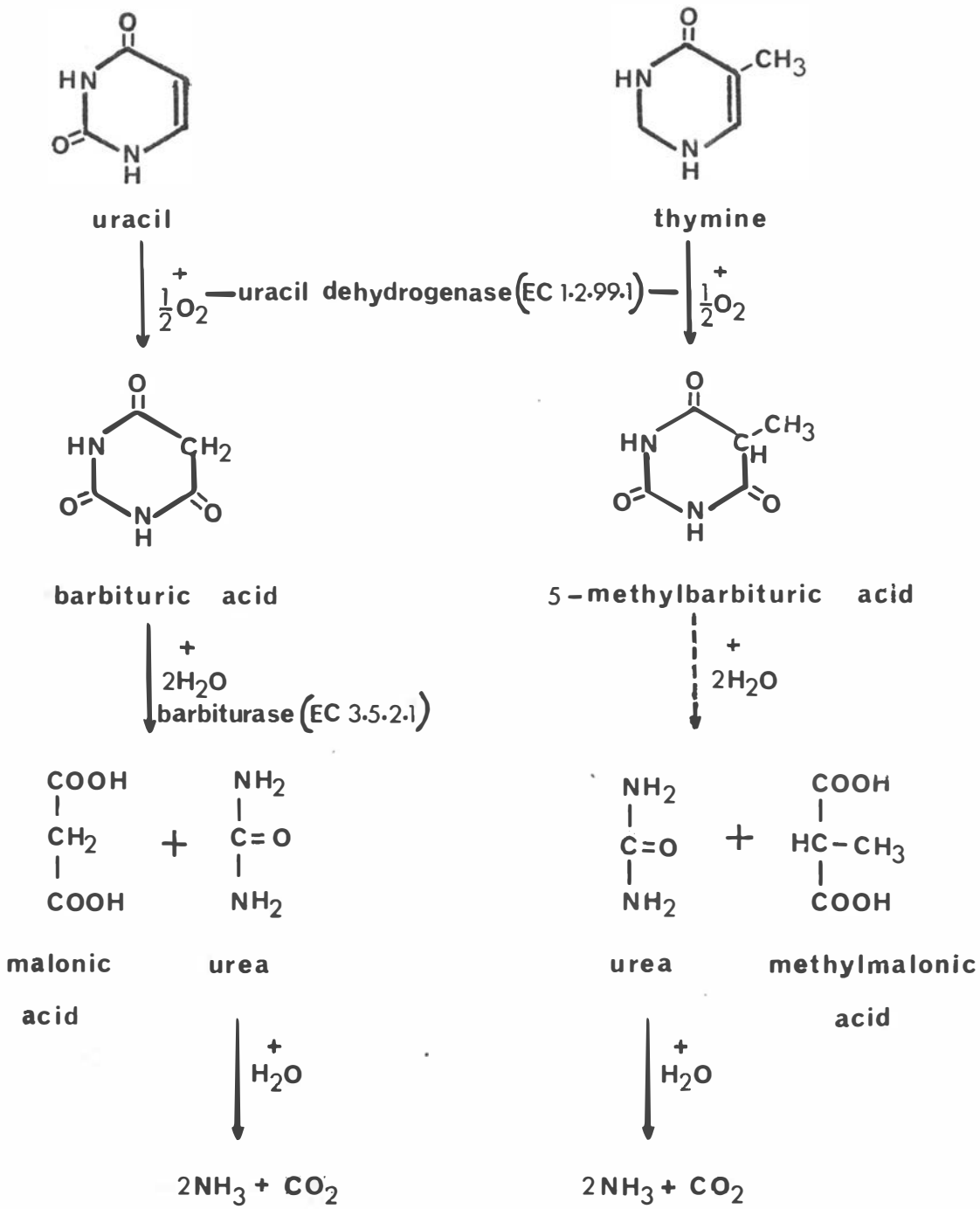


Figure 1.2 Pathway of the oxidative catabolism of uracil and thymine

——→ established reactions

- - - - - → tentative reactions

absorption maximum at 268 nm. Later Hayaishi & Kornberg (1951) characterised the compound as 5-methylbarbituric acid.

Lara (1952a) reported that thymine was oxidized by cells of N. corallina and that barbituric acid was rapidly metabolised. In a subsequent paper, Lara (1952b), described aerobic studies with cell-free extracts of N. corallina which showed that the catabolism of barbituric acid proceeded quantitatively according to the following equations:

$$\text{Barbituric acid} + 2H_2O \longrightarrow \text{Urea} + \text{malonic acid} \qquad (1.1)$$

$$\text{Urea} + H_2O \longrightarrow CO_2 + 2NH_3 \qquad (1.2)$$

In studies by Hayaishi & Kornberg (1951) using a partially purified enzyme from a strain of Mycobacterium, uracil and thymine were oxidized in the presence of methylene blue to barbituric acid and 5-methylbarbituric acid, respectively. A strain of Corynebacterium also metabolised uracil and thymine to the same products.

In further studies (Hayaishi, 1952; Hayaishi & Kornberg, 1952) it was demonstrated that partially purified enzymes from uracil-adapted cells of Mycobacterium, hydrolysed barbituric acid by the reactions (1.1) and (1.2) given previously. Urease, present in crude preparations of the organism, converted the urea to CO_2 and ammonia, but this enzyme was absent in the partially purified enzyme preparation. Malonic acid was produced in 80 - 90% yield from such preparations. The further metabolism of malonic acid could not be demonstrated either in vitro or in vivo, although it is utilised by other bacterial species (Challenger et al.,

1927; Karlson, 1950; Gray, 1952; Hayaishi, 1954). Hayaishi & Kornberg (1952) suggested that a labile compound which yields malonic acid in the isolation procedure may be the true and rapidly metabolized intermediate in the barbiturase reaction. They also reported that 5-methylbarbituric acid was not attacked by the crude or the partially purified preparations although it was actively metabolized by thymine-adapted cells. The metabolic fate of 5-methylbarbituric acid was not known. The authors noted the instability of 5-methylbarbituric acid, which was presumed to be due to auto-oxidation.

In addition to the studies in microorganisms, Nakagawa (1956) reported that in healthy men fed thymine, both thymine and 5-methylbarbituric acid, identified by spectrophotometry, were obtained in urine samples after 24 hours.

Bernabei (1958) was the first author to report methylmalonic acid as an intermediate of thymine metabolism. In his studies, using perfused, isolated rat liver, methylmalonic acid was formed from thymine, and malonic acid from uracil.

Biggs & Doumas (1963) clearly demonstrated that intact cells of Corynebacterium species could convert thymine or 5-methylbarbituric acid to methylmalonic acid and urea, but the yield of methylmalonic acid was so low that it may not be on the main pathway for thymine catabolism. Cell-free extracts were unable to catalyse 5-methylbarbituric acid degradation although they could hydrolyse barbituric acid to malonic acid and urea.

It has not been possible to demonstrate that malonic acid and methylmalonic acid are normal intermediates in vivo. Unpublished work by Pearce (1974) and Husain (1976) suggests

that, in pyrimidine catabolism by N. corallina, these acids are derived from malonyl-CoA and methylmalonyl-CoA. Malonic acid has been observed as a product of barbituric acid in vitro only, and Batt & Woods (1961) were unable to demonstrate an activation system for malonic acid in N. corallina extracts. Neither could they demonstrate growth of the organism on malonate as sole carbon source. N. corallina was, however, able to grow on methylmalonate as sole carbon source.

1.1.2 Uracil dehydrogenase

Induced enzymes for the oxidation of uracil and thymine have been described in several bacterial species isolated by enrichment cultures in which a pyrimidine (either uracil, thymine or cytosine) was the sole carbon and nitrogen source (Hayaishi & Kornberg, 1951, 1952; Batt & Woods, 1951, 1961; Wang & Lampen, 1951, 1952a, b; Lara, 1952a, b).

The enzyme which catalysed the oxidation of uracil or thymine (in the presence of methylene blue and oxygen) to barbituric acid or 5-methylbarbituric acid, respectively, was partially purified from uracil-grown cells of Bacterium sp. and named 'uracil oxidase' by Wang & Lampen (1952b). Since the ratio of the rate of oxidation of uracil to that of thymine remained the same throughout the steps of the purification procedure, they suggested that the same enzyme (uracil oxidase) was responsible for the oxidation of uracil and thymine. They also suggested that methylene blue acted as a hydrogen carrier in the oxidation of uracil in the presence of oxygen. Hayaishi & Kornberg (1951) also obtained the partially purified enzyme from a strain of Corynebacterium and

a strain of Mycobacterium, which were capable of catalysing the oxidation of uracil or thymine to the corresponding barbituric acid. The presence of methylene blue was necessary for the reactions.

Hayaishi & Kornberg (1952) purified the enzyme, 'uracil-thymine oxidase', 2.5 fold (by ammonium sulphate fractionation) from enzyme extracts of a strain of Mycobacterium which was capable of converting uracil and thymine to barbituric acid and 5-methylbarbituric acid, respectively. Their results, which were in agreement with those of Wang & Lampen (1952b), indicated that uracil and thymine were oxidised by the same enzyme. It appears that the 'uracil oxidase' of Wang & Lampen (1952b) and 'uracil-thymine oxidase' of Hayaishi & Kornberg (1952) represent the same enzyme (from different sources). Hayaishi & Kornberg (1952), using partially purified uracil-thymine oxidase studied the specificity of the enzyme for various pyrimidines and reported an optimum pH for thymine and uracil oxidation of approximately 8.5. Michaelis constants of 0.35×10^{-4} and 1.31×10^{-4} M were obtained for thymine and uracil, respectively, at pH 8.5 and 22-25°C. Hayaishi & Kornberg (1952) were unable to suggest a role for the methylene blue in the reaction catalysing the oxidation of thymine or uracil.

Subsequently, the enzymes, 'uracil oxidase' (Wang & Lampen, 1952b) and 'uracil-thymine oxidase' (Hayaishi & Kornberg, 1952) were named uracil dehydrogenase, EC 1.2.99.1 (Barman, 1969; The Commission on Biochemical Nomenclature, 1973).

1.2 Aim of the present investigation

Uracil dehydrogenase which catalyses the oxidative catabolism of uracil or thymine has not been purified or studied in detail. The aims of this investigation were therefore:

1. to purify uracil dehydrogenase from Nocardia corallina grown on thymine as sole source of carbon and nitrogen, and
2. define the properties of the enzyme.

CHAPTER 2

MATERIALS AND METHODS

2.1 Source of materials

(i) Enzymes and proteins

Cytochrome C (horse heart, 90-100% purity) was obtained from Koch-Light Laboratories Ltd., α-chymotrypsinogen A (bovine pancreas, 6x crystallised) from British Drug Houses Ltd. (BDH), and apoferritin from Schwarz/Mann Co. Myoglobin, ovalbumin and bovine serum albumin were obtained from Mann Research Laboratory Inc. (kit of protein mol. wt. markers). Ribulosebiphosphate carboxylase (EC 4.1.1.39) from Spinacia (Ellis, 1973) with a purity greater than 90% was a gift from Dr. J.W. Lyttleton (DSIR, Palmerston North). The following proteins were obtained from Sigma: lactate dehydrogenase (EC 1.1.1.27), a crystalline preparation from rabbit muscle (Sigma Type II); pyruvate kinase (EC 2.7.1.40), a crystalline preparation from rabbit muscle (Sigma Type II); catalase (EC 1.11.1.6), a crystalline (2x crystallised) preparation from bovine liver; D-amino-acid oxidase (EC 1.4.3.3), a crystalline preparation from hog kidney and snake venom (Crotalus atrox).

The apoenzyme of D-amino-acid oxidase was prepared by the method of Friedman (1965), using commercial crystalline D-amino-acid oxidase (Sigma). Preparation of the apoenzyme was carried out at 0-4°C. To 0.25 ml of suspension of crystalline D-amino-acid oxidase (1.25 mg of protein, 21 Sigma units) was added 9.5 ml of 0.1 M-sodium phosphate buffer, pH 7.2, 3.5 ml of saturated ammonium sulphate, and finally with stirring and slowly, 5.6 ml of 0.05 M-sulphuric acid. The mixture

was centrifuged and the supernatant discarded. The precipitate was resuspended in 5.0 ml of saturated ammonium sulphate solution, centrifuged, and the supernatant discarded. The precipitate was then resuspended in 6.0 ml of 0.1 M-sodium phosphate buffer, pH 7.2, to give a clear solution which showed no activity when tested for D-amino-acid oxidase (Section 5.5.3) in the absence of added FAD.

(ii) Other chemicals

Thymine, uracil and other pyrimidine derivatives (except 5-methylbarbituric acid) were obtained from Sigma. NADP was obtained from BDH, NAD and NADH from Sigma. Riboflavin was obtained from BDH, FAD (sodium salt) from Calbiochem and FMN (sodium salt) from C.F. Boehringer & Soehne GmbH Mannheim, Germany. Thiamin hydrochloride was obtained from Fluka A.G. Buchs S.G., Switzerland. The following electron acceptors were obtained from Sigma: Nitro Blue Tetrazolium (NBT), p-Iodonitrotetrazolium Violet (INT), 2,6-dichlorophenolindophenol (DCIP), and phenazine methosulphate (PMS). Methylene blue was obtained from BDH. Acrylamide and N,N′-methylenebisacrylamide were obtained from Bio-Rad Laboratories (Electrophoresis grade) and N,N,N′,N′-tetramethylenediamine from Koch-Light Laboratories Ltd. Amido Schwarz was purchased from BDH and Coomassie Brilliant Blue (R 250) from Colab Laboratories Inc.

Dimethyl-p-phenylene diamine sulphate was obtained from BDH, diethylmethylmalonate from Aldrich Chemical Co. Inc. (U.S.A.), and Blue Dextran from Sigma.

Most other chemicals were of analytical grade but reagent grade materials were used when high purity was not essential.

5-Methylbarbituric acid was prepared by the method of Holmberg (1945). Urea was condensed with diethylmethylmalonate in the presence of sodium methoxide to give the sodium salt of 5-methylbarbituric acid which was twice recrystallised in water. 5-Methylbarbituric acid was obtained by dissolving the sodium salt in a minimum volume of boiling water, acidified to pH 2 with concentrated HCl, and recrystallised from water until the filtrate was chloride free. The final product obtained after washing with absolute ethanol and drying over $CaCl_2$ in a dessicator, had a melting point of 204°C. The ultraviolet absorption spectrum (in 0.1 M-NaOH and in 0.05 M-Tris-HCl,pH 8.85) is shown in Figure 2.1. The molar extinction coefficients in 0.1 M-NaOH (269 nm) and in 0.1 M-phosphate buffer, pH 7.0 (267 nm) were 16170 and 19720 litre·mol^{-1}·cm^{-1}, respectively, (Hayaishi & Kornberg, 1952, obtained corresponding values of 17300 and 19700 litre·mol^{-1}·cm^{-1}).

(iii) Materials used for the purification of uracil dehydrogenase

Fibrous cellulose powder (CF-1) was obtained from Whatman W & R Balston Ltd. The following materials were obtained from Sigma: Sephadex G-200-40 (super fine), Sephadex 4B-200, DEAE-Sephadex A-50-120 and DEAE-cellulose (fine).

Calcium phosphate gel was prepared according to the method of Keilin and Hartree (1938) using 40 g of $CaCl_2.H_2O$ (Univar) and 68 g of $Na_3PO_4.12H_2O$ (Riedel de-Haen). The yield of gel obtained by centrifuging (4200g, 10 min.) was approximately 290g wet wt. The gel was stored at 0-4°C (for not more than one week before use).

Hydroxyapatite was prepared according to the procedure of Tiselius et al. (1956) as described by Bernadi (1971) and stored at 0-4°C in 0.01 M-Na_2HPO_4.

Calcium phosphate gel deposited on cellulose was prepared according to the method of Koike & Hamada (1971). The gel-cellulose was stored at 0-4°C in 0.02 M-sodium phosphate buffer, pH 7.0 (for not more than two weeks before use).

(iv) Solutions

All solutions, buffers and reagents were prepared using deionized distilled water.

Buffer solution, 0.05 M-Tris-HCl, pH 8.85 (25°C), was used in the preparation and purification of uracil dehydrogenase throughout the investigation except where otherwise stated.

2.2 Preparation of columns for enzyme purification

Materials for the column were obtained or prepared as detailed in Section 2.1. Buffer solution, 0.05 M-Tris-HCl, pH 8.85 (prepared as in Section 2.1), was used throughout except where otherwise stated.

2.2.1 Separose-4B column

Sepharose-4B-200 (swollen particle size 40-190 μm diameter) obtained as an aqueous suspension, was equilibrated with buffer, degassed under reduced pressure and poured into a 4.1 cm (diameter) x 80 cm glass column (plugged with glass wool) as described in the manual 'Sephadex-gel filtration in theory and practice' (Pharmacia Fine Chemicals Co.). The column was then equilibrated by eluting with about 3 column

volumes of buffer at 0-4°C. Equilibration was monitored by measuring the conductivity and pH of the eluate and the homogeneity of the packing was checked by passing through Blue Dextran 2000.

2.2.2 DEAE-cellulose column

DEAE-cellulose (fine) was treated successively with 0.5 M-HCl, deionised distilled water, 0.5 M-NaOH and deionised distilled water as described in the information leaflet 'Advanced Ion Exchange Celluloses' (Whatman W & R Balston Ltd.). The resin was then equilibrated with buffer, degassed and the fines removed. The suspension of DEAE-cellulose was transferred into a glass column (plugged with glass wool) (3.5x25 cm or 3.7x52 cm) and allowed to settle by gravity. Equilibration of the column with the appropriate buffer (0.025 M-Na_2HPO_4 in 0.05 M-Tris-HCl, pH 8.85, or 0.05 M-Tris-HCl, pH 8.85) was performed as described for Sepharose-4B (Section 2.2.1).

2.2.3 Column of calcium phosphate gel deposited on cellulose

A suspension of Whatman CF-1 fibrous cellulose powder (40 ml, 10% w/v in 0.02 M-sodium phosphate buffer pH, 7.0) was mixed with a suspension (100 ml) of the gel-cellulose (prepared as described in Section 2.1) with vigorous stirring. This procedure was recommended by Koike and Hamada (1971) to increase the flow rate of the column. The mixture was then diluted with an appropriate volume of 0.01 M-Na_2HPO_4 in 0.05 M-Tris-HCl, pH 8.85, degassed, and poured into a 3.5x30 cm glass column (plugged with glass wool). This was allowed to pack by gravity. Equilibration of the column with 0.01 M-Na_2HPO_4 in 0.05 M-Tris-HCl , pH 8.85, was performed as described for Sepharose-4B (Section 2.2.1).

Hydroxyapatite (prepared as described in Section 2.1) was packed and equilibrated with 0.01 M-sodium phosphate buffer, pH 8.85, in a smaller column (1.8x30 cm).

2.2.4 DEAE-Sephadex A-50 column

DEAE-Sephadex A-50 (particle size 40-120 μm diameter) was swollen in buffer at room temperature (5 days). The gel suspension was then degassed, and packed into a 3.8x30 cm glass column (plugged with glass wool) following the procedure described for Sepharose-4B (Section 2.2.1). The column was equilibrated with 0.10 M-NaCl in 0.05 M-Tris-HCl, pH 8.85.

2.2.5 Sephadex G-200 column

A 2.6x60 cm glass column (plugged with glass wool) was packed with Sephadex G-200 (super fine, particle size 10-40 μm) using the same procedures as described above for DEAE-Sephadex A-50. The homogeneity of the gel bed was checked with Blue Dextran 2000 by the same procedure used for the Sepharose-4B column (Section 2.2.1).

2.3 Analytical methods

2.3.1 Spectrophotometry

The following instruments were used for recording absorption spectra:

(i) Beckman ACTA T.M. III Spectrophotometer (Beckman Instruments, Inc., U.S.A.)

(ii) Perkin Elmer Model 124 Spectrophotometer (Perkin-Elmer, U.S.A.)

(iii) Shimadzu Model MPS-5000 Spectrophotometer (Shimadzu Seisakusho Ltd., Kyoto, Japan)

(iv) Unicam SP. 800 Spectrophotometer (Pye Unicam, England).

2.3.2 Fluorimetry

Fluorescence of flavin solutions was measured with a Turner Model 430 spectrofluorimeter (G.K. Turner Associates, California) equipped with a thermostatically controlled water-bath at 25°C and a recorder (Electronik recorder Model T 02 NI-H, Toshin Elutron Co. Ltd., Japan). The excitation wavelength was set at 460 nm and the emission wavelength at 550 nm. The emission slit width was set at 60 nm. A linear relationship existed between the flavin concentration and fluorescence over the range 0.2 - 1.6 µg of riboflavin/ml in 0.01 M-HCl.

2.3.3 Protein estimation

2.3.3a Standard protein assay

Protein concentrations were routinely determined, following precipitation with trichloroacetic acid using the method of Lowry et al. (1951). Crystalline bovine serum albumin (Cohn Fraction V) was used as a standard (Sigma).

2.3.3b Alternative assays

Alternative procedures for protein estimation included the ultraviolet biuret reaction (Ellman, 1962) using crystalline bovine serum albumin as a standard or the micromethod for the determination of nitrogen (Jaenicke, 1974) using $(NH_4)_2SO_4$ as the nitrogen standard.

2.3.4 Estimation of pyrimidines

Concentrations of the pyrimidines, thymine, uracil, 5-methylbarbituric acid (5-MBA) and barbituric acid (BA) were estimated spectrophotometrically.

(a) In culture medium during growth of N. corallina

In thymine growth medium, thymine and 5-MBA were both present simultaneously. The method used by Batt (1952) for the estimation of binary mixtures of pyrimidines was extended in this work for the differential quantitative estimation of thymine and 5-MBA in a mixture.

Spectra of the four pyrimidines are shown in Figure 2.1. It can be seen that under alkaline conditions thymine but not 5-MBA, absorbs at 295 nm whereas at 270 nm both compounds absorb. In a mixture of thymine and 5-MBA, the concentration of thymine could be estimated from the absorbance at 295 nm. The contribution of this concentration of thymine to the absorbance at 270 nm was calculated and subtracted from the absorbance at this wavelength for the mixture; the difference in absorption at 270 nm was then due to 5-MBA and its concentration was calculated. All the relevant molar extinction coefficients determined are listed in Table 2.1.

For the determination of pyrimidines in a growth medium, an aliquot of the culture supernatant solution was diluted with sodium hydroxide solution (final concentration 0.1 M) to give an absorbance (at 295 nm) between 0.1 and 1.0. The concentrations of thymine and 5-MBA were then determined as above.

(b) In enzyme assay mixtures

In a reaction mixture, containing either thymine or uracil, for the assay of uracil dehydrogenase, a known initial concentration of thymine or uracil was present. This was converted quantitatively to 5-MBA or BA at a rate dependent on the enzyme activity. The extent of this conversion over a particular time interval was measured by the change in

absorbance at a single wavelength. Since the molar extinction coefficients of 5-MBA and BA were much greater than those of thymine and uracil (Table 2.1) at 270 and 255 nm, respectively, the rate of increase in absorbance at this wavelength (at 270 nm or 255 nm for thymine or uracil oxidation) served as the basis for measurements (Hayaishi & Kornberg, 1952). For oxidation of thymine in a cell of 1 cm light path:

$$-\Delta T = \Delta 5\text{-MBA} = \frac{\Delta A_{270}}{12090} \qquad (2.1)$$

or ΔA_{270} of 1 absorbance unit is equivalent to 8.27×10^{-2} µmol of thymine converted/ml

where,

$-\Delta T$ = change in thymine concn. (M)

$\Delta 5\text{-MBA}$ = change in 5-MBA concn. (M)

ΔA_{270} = change in absorbance at 270 nm

12090 = difference between ϵ for 5-MBA and ϵ for thymine at 270 nm in 0.05 M-Tris-HCl, pH 8.85 (Table 2.1).

Similarly, for the oxidation of uracil

$$-\Delta U = \Delta BA = \frac{\Delta A_{255}}{11365} \qquad (2.2)$$

or ΔA_{255} of 1 absorbance unit is equivalent to 8.80×10^{-2} µmol of uracil converted/ml

where,

$-\Delta U$ = change in uracil concn. (M)

ΔBA = change in barbituric acid concn. (M)

ΔA_{255} = change in absorbance at 255 nm

11365 = difference between ϵ for BA and ϵ for uracil at 255 nm in 0.05 M-Tris-HCl, pH 8.85 (Table 2.1).

If both thymine and uracil are present in the reaction mixture, measurement must be made at two wavelengths. These

were set at 270 nm and 250 nm, since, at these wavelengths, the interference of absorbance between thymine oxidation (at 270 nm) and uracil oxidation (at 250 nm) was minimised (see Figure 2.1). A suitable spectrophotometer for simultaneous recording at both wavelengths was not available and a spectral scan was made at intervals (2 min) using the Unicam SP. 800 spectrophotometer.

The concentrations of thymine and uracil were calculated using the molar extinction coefficients of pyrimidines in 0.05 M-Tris-HCl, pH 8.85 (Table 2.1) as follows:

Let,

$$-\Delta T = \Delta 5\text{-MBA}$$

and $$-\Delta U = \Delta BA$$

(symbols defined as in equations 2.1 and 2.2)

ΔA_{270} = absorbance change at 270 nm due to the changes of thymine and uracil concentrations

ΔA_{250} = absorbance change at 250 nm due to changes of thymine and uracil concentrations.

In the molar extinction coefficients given, the superscript refers to the compound (T = thymine, U = uracil, 5-MBA = 5-methylbarbituric acid and BA = barbituric acid) and the subscript refers to the wavelength.

Then, ϵ^{T}_{270} = ϵ for thymine at 270 nm and similarly for all other molar extinction coefficients

$$\Delta A_{270} = \epsilon^{T}_{270}\cdot\Delta T + \epsilon^{MBA}_{270}\cdot\Delta MBA + \epsilon^{U}_{270}\cdot\Delta U + \epsilon^{BA}_{270}\cdot\Delta BA$$

$$\text{Thus, } \Delta A_{270} = (-\Delta T)(\epsilon^{5\text{-MBA}}_{270} - \epsilon^{T}_{270}) + (-\Delta U)(\epsilon^{BA}_{270} - \epsilon^{U}_{270})$$

$$\therefore \Delta A_{270} = 12090\ (-\Delta T) - 3195\ (-\Delta U) \qquad (2.3)$$

Figure 2.1 Absorption spectra of pyrimidines

A, 1.0×10^{-4} M-thymine and 7.5×10^{-5} M-5-methylbarbituric acid dissolved in 0.05 M-Tris-HCl, pH 8.85;

B, 1.0×10^{-4} M-uracil and 7.5×10^{-5} M-barbituric acid dissolved in 0.05 M-Tris-HCl, pH 8.85; and

C, 1.4×10^{-4} M-thymine and 5.7×10^{-5} M-5-methylbarbituric acid dissolved in 0.1 M-NaOH.

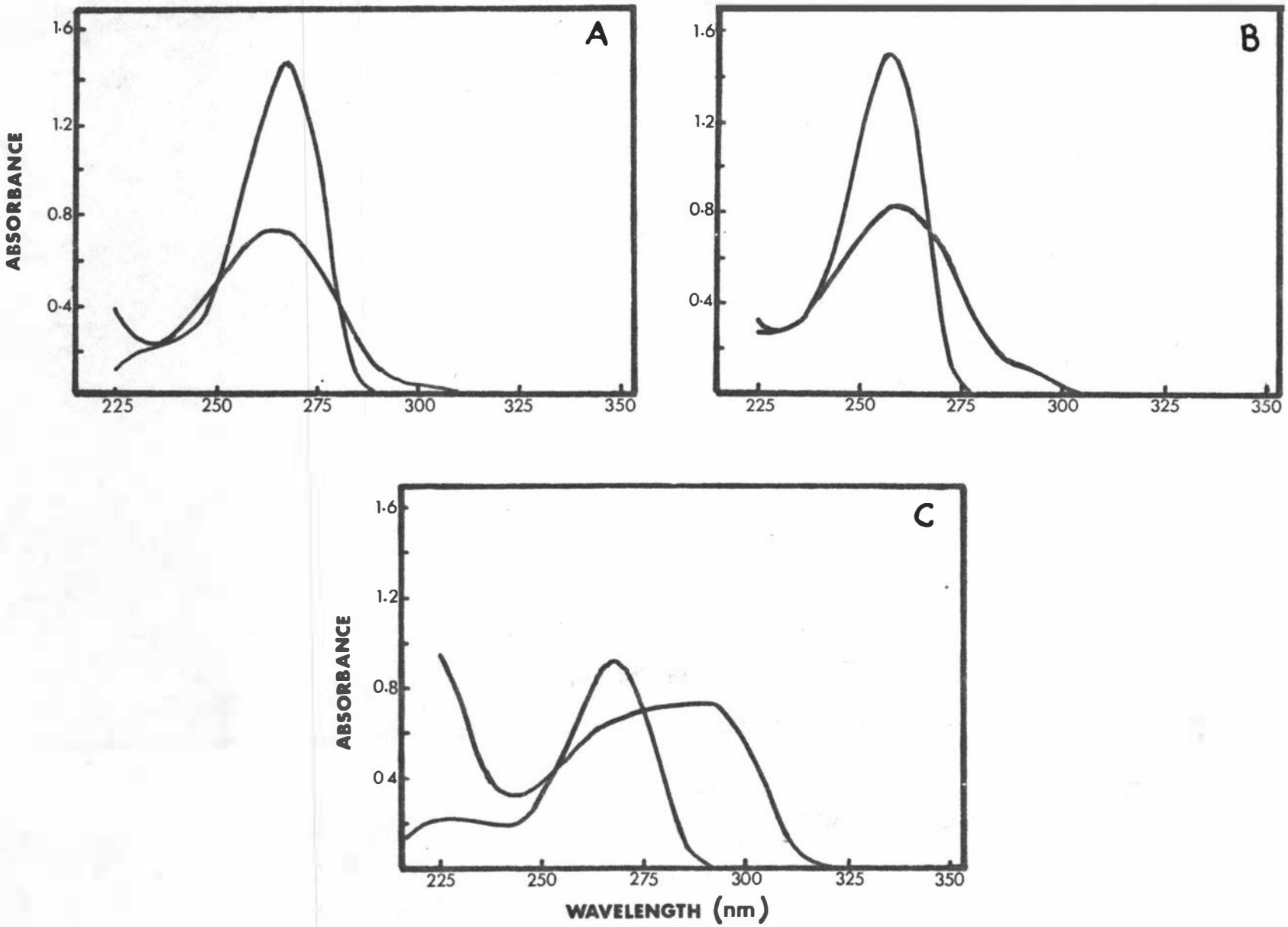

A
B
C
ABSORBANCE
1·6
1·2
0·8
0·4
225
250
275
300
325
350
WAVELENGTH (nm)

TABLE 2.1

MOLAR EXTINCTION COEFFICIENTS OF PYRIMIDINES

The concentration of pyrimidines were as indicated in Figure 2.1.

The values are the absorbance units at the indicated wavelengths in the appropriate mediums calculated for a 1 M solution in a 1 cm light path cell at 25°C.

Pyrimidines	0.05 M-Tris-HCl, pH 8.85 ε at			0.1 M-NaOH ε at	
	270nm	250nm	255nm	270nm	295nm
Thymine	6620	4755	5890	4570	4720
5-Methylbarbituric acid	18710	5945	9790	16050	0
Uracil	5695	6875	7735		
Barbituric acid	2500	16100	19100		

Similarly,

$$\Delta A_{250} = (-\Delta T)(\epsilon^{5\text{-MBA}}_{250} - \epsilon^{T}_{250}) + (-\Delta U)(\epsilon^{BA}_{250} - \epsilon^{U}_{250})$$

$$\Delta A_{250} = 1190\ (-\Delta T) + 9225\ (-\Delta U) \qquad (2.4)$$

Solving ΔT and ΔU from equations 2.3 and 2.4 we obtain:

$$-\Delta T = (8\ \Delta A_{270} + 2.77\ \Delta A_{250}) \times 10^{-5} \qquad (2.5)$$

$$-\Delta U = (10.48\ \Delta A_{250} - \Delta A_{270}) \times 10^{-5} \qquad (2.6)$$

The equations (2.5) and (2.6) were used to calculate the amount of thymine and uracil converted in the assay mixture when both compounds were present.

In the methods described above for determination of the changes in pyrimidine concentrations in enzyme assay mixtures, no account was taken of the ultraviolet absorbance of methylene blue and its reduced form, leuco-methylene blue. However, it is shown in Section 6.1 that the absorbance due to methylene blue and leuco-methylene blue did not change significantly with time under the experimental conditions used.

2.3.5 Assays for uracil dehydrogenase activity

2.3.5a Spectrophotometric assay

(i) Standard enzyme assay

Uracil dehydrogenase was routinely assayed by a spectrophotometric assay adapted from that of Hayaishi & Kornberg (1952). Thymine was oxidised in the presence of methylene blue to give 5-methylbarbituric acid and the reaction was followed by measuring the increase in absorbance at 270 nm due to the formation of the product, 5-methylbarbituric acid, at 25°C. The changes in absorbance were monitored in a 3 ml

quartz cuvette with a 1 cm light path either with a Unicam SP. 800 or a Unicam SP. 500 spectrophotometer equipped with a constant temperature control cuvette compartment. The concentration of methylene blue (2.5×10^{-5} M) chosen, was that which gave the maximum rate of thymine oxidation in air (Section 6.8) and at the optimum pH of 8.85 (Section 6.3). The standard assay mixture (3.0 ml) contained 0.3 µmol of thymine, 0.075 µmol of methylene blue, 150 µmol of Tris-HCl, pH 8.85, and sufficient enzyme to produce a change at 270 nm from 0.005 to 0.045 absorbance units/min at 25°C. The reaction was started by the addition of enzyme.

The enzyme activity was measured over the initial linear portion of the rate curve, the amount of thymine oxidised being calculated as in Section 2.3.4. A unit of enzyme activity is defined as that amount of enzyme which catalysed the oxidation of 1.0 µmol of thymine per min at 25°C in the standard assay mixture. Specific activity is defined as units per mg of protein. Protein was determined by the method of Lowry *et al.* as described in Section 2.3.3a.

(ii) Alternative enzyme assay

For studying the catalytic properties of the enzyme the standard enzyme assay conditions were varied by altering one of the following parameters:- temperature, pH, ionic strength, substrate or nature of electron acceptor. The details of methods are given in the relevant section of Chapter 6. Generally, where the reduced electron acceptor was reoxidised by air, the reaction was followed at 270 nm if thymine was the substrate or at 255 nm for uracil. In some cases (for example in the absence of oxygen or where a

tetrazolium salt was used as the electron acceptor), the reaction was followed at the appropriate wavelength for the electron acceptor.

(iii) Anaerobic enzyme assay

The reduction of various electron acceptors by thymine, catalyzed by uracil dehydrogenase, was studied in a Thunberg cuvette. This was made from a 1 cm spectrophotometric cell fused to a glass tube (1.2 cm x 5 cm) which was in turn joined to the top part of a Thunberg tube. The length of tube was sufficient to prevent the solution in the cuvette from bubbling over into the stopper on evacuation.

For assays with this cell, enzyme solution was placed in the stopper and the other reagents in the cuvette. The stoppered cuvette was then alternately flushed with oxygen-free nitrogen and evacuated using a water-pump for a total of four times to ensure anaerobic conditions. The nitrogen (99.5%, New Zealand Industrial Gas Ltd.) had been bubbled through alkaline pyrogallol solution to remove traces of oxygen as described by Vogel, 1956 . The reaction was then started by tipping in the enzyme from the stopper, and followed at the appropriate wavelength in the spectrophotometer.

2.3.5b Assay of uracil dehydrogenase activity using an oxygen electrode

Oxygen uptake was followed at 25°C using the Clarke Oxygen Electrode unit (Rank Brothers, Bottishham, Cambridge, England) connected to a circulating water bath and attached to a Honeywell recorder (Brown Electronik, Honeywell Control Ltd., Gt. Britain). The reaction mixture in the electrode

chamber contained, in a total of 3.0 ml, 0.125 µmol of methylene blue, 150 µmol of Tris-HCl, pH 8.85, uracil dehydrogenase and (added last) a pyrimidine substrate to initiate the reaction. A potential of 0.6 V was applied between the platinum cathode and the silver reference electrode which was covered with a teflon membrane. The reaction mixture was stirred at high speed using a small magnetic stirrer bar. Before addition of the pyrimidine substrate, the reaction mixture was equilibrated for 10 min at 25°C. The plunger was then inserted and the collar adjusted at the point where the reaction mixture had just entered the small hole in the plunger. The pyrimidine substrate (3-30 µl) was added through the small hole in the plunger using a microsyringe (Scientific Glass Engineering Pty. Ltd., Melbourne, Australia).

The teflon membrane was normally changed after being used for 2-3 days. The surface of the electrodes was cleaned with 15% NH_4OH to remove AgCl deposits and, if necessary, polished with a liquid metal polisher.

The oxygen electrode was calibrated by two methods.
(i) The relative calibration method of Delieu & Walker (1972), in which excess reducing agent (sodium dithionite) was used to de-oxygenate the chamber. The following reaction occurred:

$$Na_2S_2O_4 + O_2 + H_2O \longrightarrow NaHSO_4 + NaHSO_3 \qquad (2.7)$$

The accuracy of the method depended on published figures for the oxygen content of air-saturated water (0.253 µmol/ml at 25°C). The chart recorder was adjusted so that 1 scale division represented 0.01 µmol of oxygen.
(ii) The absolute calibration method of Robinson & Cooper (1970), in which an accurately known limiting concentration

of NADH was used, in the presence of PMS and catalase, to reduce a stoichiometric amount of oxygen. The reactions involved were:

$$PMS + NADH + H^+ \longrightarrow PMSH_2 + NAD^+ \quad (2.8)$$

$$PMSH_2 + O_2 \longrightarrow PMS + H_2O_2 \quad (2.9)$$

$$H_2O_2 \longrightarrow \tfrac{1}{2} O_2 + H_2O \quad (2.10)$$

$$\text{sum: } NADH + H^+ + \tfrac{1}{2} O_2 \longrightarrow NAD^+ + H_2O \quad (2.11)$$

The amount of oxygen consumed was determined from the stoichiometric amount of NADH oxidized. The uptake of oxygen was then expressed as μmol of oxygen/scale division of the chart paper.

2.3.6 Identification and estimation of FAD

2.3.6a Identification of FAD

The following tests were used to determine the nature of the flavin prosthetic group of uracil dehydrogenase. For these tests it was necessary firstly to release the prosthetic group from the enzyme. This was done by heating the purified uracil dehydrogenase (specific activity 8.0 to 10.2 units/mg) for 5 min at 100°C, and the product was referred to as heated uracil dehydrogenase (HUD).

(i) Paper chromatography

Free flavins and standards were separated by descending chromatography in two different solvent systems (Kilgour et al., 1956):

Solvent 1, 5% (w/v) Na_2HPO_4

Solvent 2, butan-1-ol/acetic acid/water (4 : 1 : 5, by vol.); the upper phase was used.

Chromatography was carried out at 28-29°C on Whatman no. 1 paper (46x57 cm sheets, pre-equilibrated in the chromatographic tank), and in darkness in order to minimise the photodegradation of the flavins which were located on chromatograms by their yellow fluorescence under u.v. light (350 nm).

(ii) Fluorescence analysis after phosphodiesterase treatment.

(iii) Reactivation of D-amino-acid oxidase apoenzyme.

Details for the above two tests are given in Sections 5.5.2 and 5.5.3.

2.3.6b Estimation of FAD

(i) Spectrophotometric assay

The FAD content of uracil dehydrogenase was derived from the visible spectrum as detailed in Section 5.4.

(ii) Fluorometric assay

FAD content was estimated as described by Burch (1957). The method was adapted from methods previously described by Bessey et al. (1949) and by Burch et al. (1948). It was based on the fact that an increase in fluorescence occurred when FAD was split to FMN by acid hydrolysis. FAD was extracted from the native enzyme by treatment with cold trichloroacetic acid (10% final concentration) and the protein rapidly removed by centrifugation. One aliquot of the supernatant was then neutralized (non-hydrolyzed control) and was stored for 48 h at 0-4°C while another aliquot was incubated for 48 h at 25°C prior to neutralisation (hydrolysed sample). Bessey et al. (1949) showed that

under these conditions of hydrolysis (10% trichloroacetic acid, 25°C) FAD had a half-life of about 1 h, and hydrolysis was essentially completed in 48 h. Duplicate riboflavin standards were carried through the same procedure as for the enzyme.

The fluorescence of both hydrolyzed samples and non-hydrolyzed controls were measured (Section 2.3.2) both before (F_1) and after (F_2) reduction with sodium dithionite, as described by Burch (1957), in order to correct for any non-flavin fluorescent substances which might be present.

Calculation of FAD content of the original sample was based on the formula of Burch (1957), but omitting the dilution factor used by Burch.

$$\text{Concentration of FAD} = (R_t - R_i)/0.85$$

where R_i = apparent riboflavin concentration in non-hydrolyzed control

= concentration of riboflavin standard

$$\times \frac{(F_1 - F_2) \text{ control}}{(F_1 - F_2) \text{ control standard}}$$

and R_t = apparent riboflavin concentration in hydrolyzed sample

= concentration of riboflavin standard

$$\times \frac{(F_1 - F_2) \text{ sample}}{(F_1 - F_2) \text{ incubated standard}}$$

2.3.7 Metal analyses by atomic absorption spectrophotometry

Analysis of the metallic elements present in the purified uracil dehydrogenase was kindly conducted by Dr. R.D. Reeves, using a Varian Techtron Model AA-3 Atomic

Absorption Spectrophotometer (Techtron Pty. Ltd., Melbourne) with a Varian Techtron CRA-63 Carbon rod atomizer, and a Rikadenki recorder (Kegyo Co., Ltd., Tokyo).

All glassware was washed with detergent and water, rinsed with aqua regia and washed successively with distilled water and deionized water, as a precaution against contamination by metallic ions. Dialysis tubing was cleaned free from metals by the method of McPhie (1971). Standard solutions of 1 µg/ml and 2 µg/ml of salts of Fe, Zn, Mg, Cu, Ni, Co, Mn and Mo (BDH, AnalaR) were prepared in deionized distilled water.

For each metal, appropriate standards, samples and blanks were all analysed in triplicate, on samples of 2 µl applied with an Eppendorf-type microsyringe (Eppendorf Ltd., West Germany). The operating conditions (wavelengths, temperature program, etc.) were selected as optimum for the metal being assayed. The accuracy of each assay was assessed in terms of the standard deviation of triplicate samples.

2.3.8 Amino acid analysis

Analysis of the amino acid composition of the purified uracil dehydrogenase was performed according to the procedure of Spackman, Stein and Moore (1958) using a Beckman 120 C amino acid analyser.

Aliquots (0.2 ml) of purified enzyme (1 mg/ml in 0.025 M-Tris-HCl, pH 8.85; specific activity 10.6 units/mg) were lyophilised in heavy walled Pyrex tubes. After the addition of 2 ml of constant boiling HCl (5.9 M)

the tubes were sealed under a vacuum (0.05 mm Hg) and incubated at 110°C ± 1°C to hydrolyse the protein. Duplicate samples were hydrolysed for 24, 48 and 72 h. After hydrolysis the tubes were opened and the samples dried rapidly in an evacuated dessicator over solid sodium hydroxide. Before analysis each sample was dissolved in 0.1 ml of citrate buffer, pH 2.2 (0.2 M with respect to Na^+).

The hydrolyses and chromatography were kindly performed by Dr. G.G. Midwinter. The analyser was calibrated using a Beckman standard amino acid calibrant mixture (0.05 µmol of each amino acid). Peaks on the effluent curves were integrated manually by multiplying the height of the peak by the width at half the height.

2.3.9 Analysis of labile sulphide

Labile sulphide was determined spectrophotometrically as described by King and Morris (1967). This method had been adapted from that described by Fogo & Popowsky (1949).

The sodium sulphide solution was standardised by oxidation with potassium iodate standard in an alkaline medium as described by Vogel (1961).

2.3.10 Molecular weight estimation

2.3.10a Ultracentrifugation analysis

Sedimentation equilibrium centrifugation of the native enzyme was performed according to the method of Chervenka (1970). The method is a modification of the meniscus depletion technique of the original procedure of Yphantis (1964). Studies were carried out in a Model E Beckman ultracentrifuge using Rayleigh interference optics. The

instrument was kindly operated by the late Dr. J.W. Lyttleton. Measurements of vertical fringe displacements, Δy, of three light fringes at each radial distance, r, were made with a Mann Type 829C Comparator. The average molecular weight was calculated from the slope of a plot of the natural logarithm of the fringe displacement, Δy, against the square of the radial distance, r, by the following equation:

$$\text{mol. wt.} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \cdot \frac{d \ln \Delta y}{dr^2}$$

Where R is the gas content, T is the absolute temperature, ω is the angular velocity, $\bar{v}$ is the partial specific volume of the enzyme, ρ is the solvent density.

The partial specific volume of the enzyme, $\bar{v}$ was determined (according to Cohn & Edsall, 1943) from the amino acid composition (Section 5.3) using the specific volumes of the constituent amino acid residues.

2.3.10b Sephadex G-200 gel filtration

The molecular weight of the native enzyme was estimated on a column (2.6x60 cm) of Sephadex G-200 (prepared as in Section 2.2.5) calibrated at 0-4°C with known molecular weight proteins according to the procedure described by Andrews (1965). The column was equilibrated at 0-4°C with 0.05 M-Tris-HCl, pH 8.85, containing 0.10 N-NaCl. Samples were dissolved in the equilibration buffer (1.5 ml), applied to the column and then eluted with the same buffer using a flow rate of 10-15 ml/h. The effluent was collected in 3 ml fractions. Proteins and Blue Dextran were determined as

follows: uracil dehydrogenase by the standard enzyme assay (Section 2.3.5a); catalase by the disappearance of H_2O_2 followed at 240 nm (Chance & Maehly, 1955); bovine serum albumin, ovalbumin and apoferritin by absorbance at 230 nm; myoglobin by absorbance at 407 nm; ribulosebiphosphate carboxylase by absorbance at 280 nm; and Blue Dextran by absorbance at 620 nm. The elution volume corresponding to maximum concentration of a solution was estimated from an elution diagram by extrapolating both sides of the solute peak to an apex. A calibration curve was prepared by plotting elution volume against the logarithm of the molecular weight. The standard markers were used in various combinations with and without uracil dehydrogenase at levels of 0.3-5.0 mg of each. The molecular weight of uracil dehydrogenase was then determined from its elution volume by use of the calibration curve.

2.3.10c Electrophoresis

Determination of molecular weights of subunits by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS - polyacrylamide gel electrophoresis) is described below in Section 2.3.11b.

2.3.11 Polyacrylamide gel electrophoresis

2.3.11a Polyacrylamide gel electrophoresis of the native enzyme

Analytical gel electrophoresis (Gabriel, 1971) of the purified native enzyme was performed at 0-4°C in a 7.5% acrylamide (0.3% N,N'-methylene-bisacrylamide) gel using 0.1 M-Tris/0.1 M-Glycine buffer, pH 8.7 as the reservoir buffer.

A conventional tube-gel electrophoresis apparatus (based on Davis, 1964) equipped with a constant current power supply unit (Vokam SAE 2761, Shandon; Shandon Scientific Co. Ltd., London) was used. Gels (0.6 x 13.0 cm) were pre-electrophoresed for 1 h at 4 mA per gel and electrophoresis of the purified native enzyme was carried out (at 4 mA per gel) until the dye (bromophenol blue) approached the bottom of the gel. Proteins were stained with 0.25% Coomassie Brilliant Blue in a mixture of 7% acetic acid and 25% methanol for 6 h and destained according to the procedure of Reid & Bieleski (1968). A further gel was stained with Amido Schwarz (1% Amido Schwarz in 7% acetic acid) and destained using the same procedure as used for Coomassie Brilliant Blue. The stained gel was scanned with a Canalco model G gel scanner at 570 nm.

For locating uracil dehydrogenase on the gel, the two following techniques were used:

1. The non-stained gel was frozen by placing on solid carbon dioxide, and then sliced transversely with razor blades into 1 mm slices. Each slice was homogenised with a glass rod in 0.05 M-Tris-HCl, pH 8.85 and the extract, after settling, was used in a standard enzyme assay (Section 2.3.5a).
2. The gel was stained for enzyme activity using the technique based on the transfer of electrons from reduced enzyme to phenazine methosulphate (PMS) and then to Nitro Blue Tetrazolium (NBT), forming an insoluble purple formazan derivative (Quastel, 1957; Nachlas *et al.*, 1960). Uracil dehydrogenase was shown to catalyse such an electron transfer (Section 6.6). A reaction mixture consisting of 50 mM-Tris-HCl, pH 8.85, 0.3 mM-PMS, 0.06 mM-thymine and 0.1 mM-NBT was prepared immediately before use. After electrophoresis, the

gel was removed and washed once in 0.05 M-Tris-HCl, pH 8.85. The gel was incubated in a test tube containing the above assay mixture at room temperature (20-22°C) and in the dark. Deep purple bands indicated the location of uracil dehydrogenase activity. If thymine was excluded from the reaction mixture no band appeared. When sufficient staining had occurred (20-30 min) the gel was removed and washed twice in 0.05 M-Tris-HCl, pH 8.85, before being stored in 7.5% acetic acid. p-Iodonitrotetrazolium Violet (INT) could be used in place of NBT in the above staining procedure.

2.3.11b SDS-polyacrylamide gel electrophoresis

The molecular weight of the subunits of the enzyme was estimated according to the procedure of Weber et al. (1972) by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS).

Electrophoresis was performed in 10% polyacrylamide gels containing 0.1 M-phosphate buffer, pH 7.0 and 0.1% SDS at room temperature (20-22°C). The electrophoresis buffer was 0.1 M-phosphate buffer, pH 7.0, containing 0.1% SDS. Standard proteins and the purified enzyme were heated at 100°C for 2 min in the presence of 1% SDS and 1% 2-mercaptoethanol. Gels (0.6x13.0 cm) were pre-electrophoresed for 3 h at 6 mA per gel and electrophoresis of the sample was carried out (at 6 mA per gel) until the dye (bromophenol blue) approached the bottom of the gel. Proteins on gels were stained with Coomassie Brilliant Blue for 12 h and destained according to the procedure of Reid & Bieleski (1968). A further gel was stained with Amido Schwarz, destained by the procedure as used for Coomassie Brilliant Blue and scanned as described

in Section 2.3.11a.

For the extraction of protein from gels of the native enzyme the slices were homogenized with a tissue grinder (Potter-Elvehjem) in approximately four volumes of 0.05 M-Tris- HCl, pH 8.85. The gel was removed by centrifugation and the extraction repeated a further two times. The combined extracts were concentrated with an ultrafiltration cell over an XM-50 membrane (Amicon corp.). All the above procedures were carried out at 0-4°C.

2.4 Bacteriological methods

2.4.1 Organism

Nocardia corallina, strain S, used in the present investigation was isolated by Batt & Woods (1951) and identified as N. corallina by Professor H.L. Jensen (Batt & Woods, 1961).

The organism was maintained in slope culture at 0-4°C on glucose-yeast extract-agar of the following composition (g/l): KH_2PO_4, 3.40; $(NH_4)_2SO_4$, 3.00; $MgSO_4.7H_2O$, 0.10; thiamin hydrochloride, 0.025; yeast extract (Difco), 0.50; glucose, 7.5; agar (Davis), 20.0. The pH was adjusted to 7.0 with 5 M-NaOH before adding the agar. After heating to dissolve the agar, the medium was dispensed into small screw-cap bottles (10 ml each) and then autoclaved (121°C for 20 min).

Subcultures were made at monthly intervals onto fresh slopes, which were incubated at 30°C for 30 h, then stored at 0-4°C.

2.4.2 Growth in a liquid culture medium

The thymine culture medium contained (g/l): KH_2PO_4, 13.60; $MgSO_4.7\ H_2O$, 0.10; thiamin hydrochloride, 0.03; and thymine, 2.00 adjusted to pH 7.0 with 5 M-NaOH. Aliquots (330 ml) of medium were dispensed into 1 litre conical flasks plugged with cotton wool before autoclaving at 121°C for 20 min.

Bacteria from a fresh slope (which had not been stored at 0-4°C) were transferred to a primary flask containing 330 ml of thymine culture medium and incubated at 30°C with shaking at 150 rev./min on a New Brunswick rotary shaker (Model G-25). Bacterial growth was measured by following the absorbance at 600 nm in a Bausch and Lomb Spectronic 20 Spectrophotometer. The pH of the medium was also measured. Thymine and 5-methylbarbituric acid were estimated in aliquots as described in Section 2.3.4. At a period of approximately 30 h, when the growth had reached the late exponential phase, the organisms were examined using light microscopy and gram staining to check for the presence of contaminants. Aliquots (10 ml) were then subcultured into each of 15 secondary flasks containing the same medium as the primary flask (330 ml/flask). These secondary flasks were incubated under the same conditions as the primary flask. The organisms were harvested early in the stationary growth phase by centrifuging at 5000 g for 15 min at 2°C. The organisms were washed twice with cold 0.05 M-Tris-HCl, pH 8.85, and centrifuged at 10000 g for 10 min and 2°C. The pellets were stored at -20°C until required.

2.4.3 Preparation of cell-free extracts

Various methods of cell disruption were tested to determine the method giving maximum yields of uracil dehydrogenase. The degree of disruption was determined by examination of the disrupted organisms using light microscopy and gram staining and also by assaying for both protein (Section 2.3.3a) and uracil dehydrogenase activity (Section 2.3.5a). Organisms were disrupted in 0.05 M-Tris-HCl, pH 8.85, at 0-4°C and the final extract was obtained by centrifuging at 20000 g for 20 min at 2°C.

(i) Grinding with alumina

The pellet of organisms, alumina and the buffer in the ratio of 1 : 3 : 1.5 (w/w/v) were ground at 0-4°C in a precooled mortar for 5 min. The slurry was diluted with 10 volumes of buffer (w/v) and centrifuged.

(ii) Grinding with glass beads

The pellets, glass beads (for gas chromatography, Mesh 80, BDH) and the buffer in the ratio of 1 : 2.5 : 1 (w/w/v) were mixed in a Sorvall omnimixer. The mixture, in an ice-cooled stainless cup, was stirred for three 5-minute periods (at maximum speed setting) with 5-minute intervals for cooling. It was then diluted with the buffer (4.5 ml/g wet weight of pellets) and centrifuged.

(iii) Disintegration by sonic disintegrator

The pellets were resuspended in buffer at a dilution of 1 : 10 (w/v). The suspension was sonicated (M.S.E. 100 watt ultrasonic disintegrator) at 20 kHz at 0-4°C, for a total sonication time of 40 min, using 15-sec bursts, alternating with a 15-sec cooling period. It was then centrifuged.

(iv) Disintegration with a French pressure apparatus

The operating procedure was as described by French & Milner (1955). The pellets were routinely resuspended in buffer at a dilution of 1 : 15 (w/v), disrupted twice in a French pressure apparatus (Aminco, American Inst. Co. Inc.) at 8000 p.s.i. (5.6×10^7 Pa) and centrifuged. For certain experiments the cell concentration and number of passages through the French pressure apparatus were varied as described in the appropriate sections of the thesis.

CHAPTER 3

GROWTH OF *Nocardia corallina* AND PREPARATION OF CELL-FREE EXTRACTS

3.1 Growth of *N. corallina*

For routine work, batch culture methods in shake flasks were used (as described in Section 2.4.2). Organisms were initially grown on a thymine culture medium in the primary flask until they reached the late exponential phase of growth. Aliquots were then transferred to fresh medium in secondary flasks and harvested at an early stage of the stationary phase. A yield of approximately 1 g wet wt. of organisms per g of thymine was obtained. The yield of organisms obtained using thymine as the sole source of carbon and nitrogen was limited by the low solubility (4 g/l) of thymine. The growth characteristics of *N. corallina* in the secondary flask are shown in Figure 3.1.

3.2 Preparation of cell-free extracts

Using the various methods of disruption (Section 2.4.3) to obtain the maximum yield of uracil dehydrogenase, the levels of the enzyme in the extracts are given in Table 3.1. The specific activity of uracil dehydrogenase was comparable in extracts obtained by all four methods used in this preliminary study. Grinding with glass beads gave the maximum yield of uracil dehydrogenase, but the French pressure apparatus gave a yield approaching that obtained with glass beads and was the most convenient method for routine use.

In subsequent experiments a 2-3 fold increase in yield of enzyme was obtained by using a 1 : 15 (w/v) cell dilution instead of 1 : 10 (w/v) (Table 3.2). Further passages through

Figure 3.1 Growth characteristics of N. corallina in culture medium

N. corallina was grown in thymine culture medium (330 ml) and incubated at 30°C with shaking (150 rev./min). Bacterial growth was measured by following the absorbance at 600 nm (curve A). Thymine and 5-methylbarbituric acid were determined in aliquots as described in Section 2.3.4 (curve B and C, respectively). The pH of the medium during growth was measured (curve D). The cells were harvested near the end of the logarithmic phase of growth at the time indicated by the arrow.

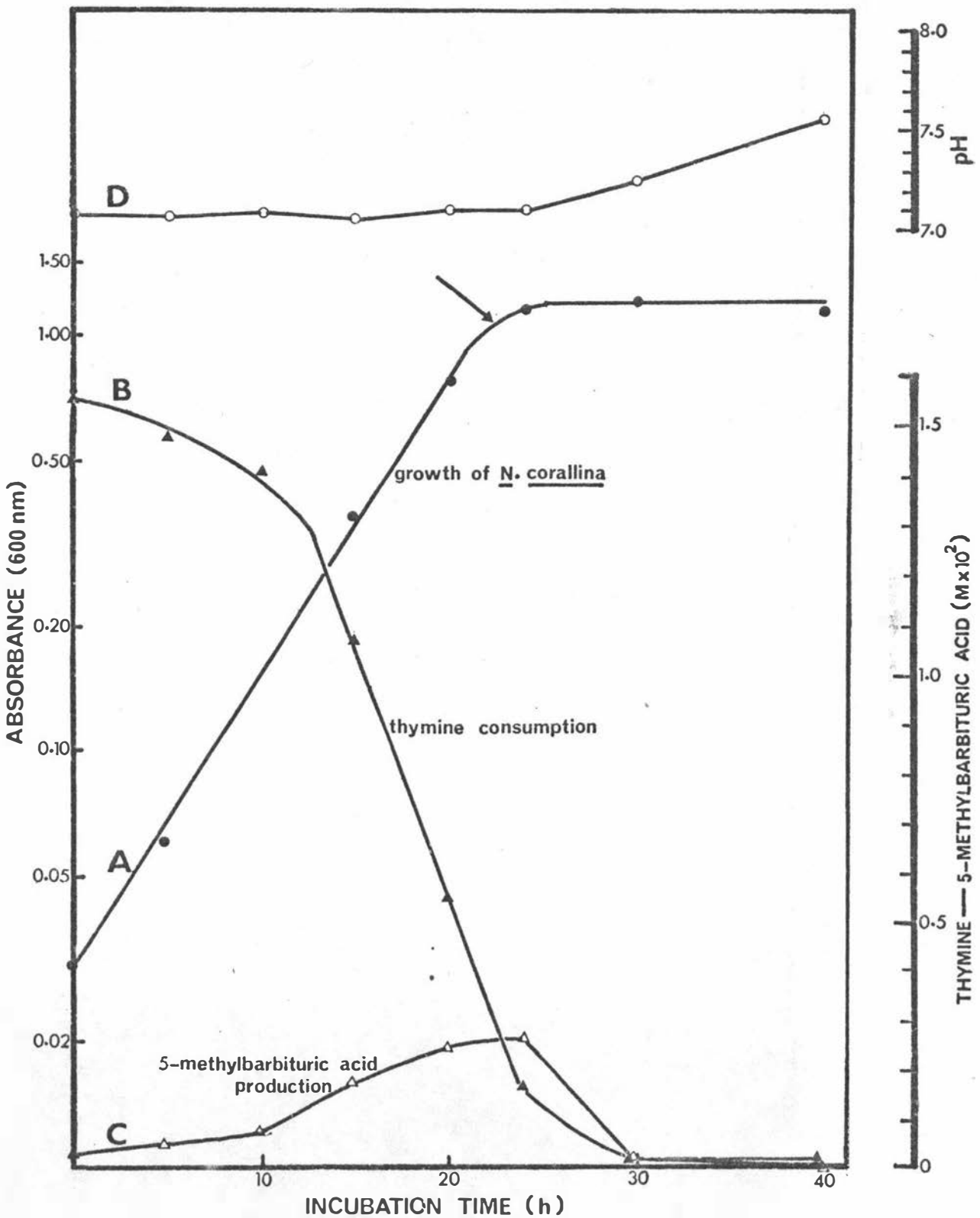

D
B
A
C
growth of N. corallina
thymine consumption
5-methylbarbituric acid production
ABSORBANCE (600 nm)
1.50
1.00
0.50
0.20
0.10
0.05
0.02
INCUBATION TIME (h)
10
20
30
40
pH
8.0
7.5
7.0
THYMINE — 5-METHYLBARBITURIC ACID (M×10²)
1.5
1.0
0.5
0

TABLE 3.1

URACIL DEHYDROGENASE RELEASED FROM N. corallina USING VARIOUS METHODS OF CELL DISRUPTION

Cell-free extracts were prepared (as described in Section 2.4.3) from N. corallina (1 g wet wt. portions of the same batch). The activity of uracil dehydrogenase was determined (Section 2.3.5a) immediately after obtaining the extracts.

Method	Volume (ml)	Total enzyme (units)	Protein (mg/ml)	Total protein (mg)	Specific activity (units/mg)
Grinding with alumina	10.5	2.6	1.65	17.3	0.15
Grinding with glass beads	5.2	4.0	6.54	34.0	0.12
Disintegration by sonic disintegrator	10.5	2.8	1.91	20.1	0.14
Disintegration using a French pressure apparatus[a]	10.5	3.5	2.81	29.5	0.12

[a] Three passages through a French pressure apparatus with pellets suspended in buffer at 1 : 10 ratio (w/v).

the French pressure apparatus up to a total of five, increased the yield of enzyme slightly but the specific activity decreased (Table 3.2). Two passages were therefore used routinely for the preparation of cell-free extracts.

TABLE 3.2

LEVEL OF URACIL DEHYDROGENASE RELEASED FROM *N. corallina* USING A FRENCH PRESSURE APPARATUS

Cell-free extracts were prepared from *N. corallina* (1 g wet wt. portions of the same batch) by passing bacterial suspensions (1 : 15 w/v in 0.05 M-Tris-HCl, pH 8.85) through a French pressure apparatus (as described in Section 2.4.3) up to a total of five passages. The activity of uracil dehydrogenase was determined (Section 2.3.5a) immediately after obtaining the extracts.

Passages through the French pressure apparatus (x times)	Volume (ml)	Total enzyme (units)	Protein (mg/ml)	Total protein (mg)	Specific activity (units/mg)
1	14.3	7.0	2.85	40.8	0.17
2	14.3	8.7	4.51	64.5	0.13
3	14.3	7.9	5.52	78.9	0.10
4	14.3	9.4	6.17	88.2	0.11
5	14.3	9.2	6.42	91.8	0.10

CHAPTER 4

PURIFICATION OF URACIL DEHYDROGENASE FROM N. corallina

4.1 Determination of specific activity of uracil dehydrogenase

The standard assays for determination of enzyme activity and protein concentration are detailed in Section 2.3.5a and 2.3.3a, respectively.

4.2 Purification of uracil dehydrogenase

All steps of the purification procedure described were carried out at 0-4°C.

Step 1 Preparation of cell-free extracts

Cell-free extracts were prepared (as described in Section 3.2) from frozen N. corallina by passing 20 g (wet wt.) aliquots of bacteria suspended in 300 ml of 0.05 M-Tris-HCl, pH 8.85, through a French pressure apparatus. Broken cells were removed by centrifugation (20000 g, 20 min, 2°C). As a routine procedure, four (20 g) batches of cells were extracted and each extract (300 ml) was immediately assayed for uracil dehydrogenase activity and then treated with calcium phosphate gel (step 2). Table 4.1 shows typical results for 4x20 g of cells. The stability of uracil dehydrogenase in cell-free extracts was highly variable. This instability was completely overcome after treatment with calcium phosphate gel but not following ammonium sulphate fractionation.

Step 2 Treatment with calcium phosphate gel

Each extract (fresh; 300 ml) was added slowly (10 min) with mechanical stirring to a separate portion of calcium phosphate gel (110 g wet wt.) which had been aged for 1 week

after preparation (prepared as in Section 2.1). After the addition was completed, stirring was continued using a magnetic stirrer for further 45 min. The suspension was centrifuged (4200 g, 10 min, 2°C) and the supernatant solution which was found to contain less than 3% of total units of enzyme activity was discarded. The gels were stored (2°C) for up to 3 h and then all four lots were combined and extracted by stirring (10 min by manual stirring, 45 min with a magnetic stirrer) with 0.025 M-Na_2HPO_4 in 0.05 M-Tris-HCl, pH 8.85. The preparation was centrifuged (4200 g, 10 min, 2°C) and the supernatant containing enzyme was retained. The gel was extracted, recentrifuged four times and the supernatants were pooled (4000 ml) and assayed for uracil dehydrogenase (Table 4.1).

Step 3 First DEAE-cellulose chromatography

The object of this step was to concentrate the enzyme rapidly and to effect some degree of purification. The combined extracts (4000 ml) from the calcium phosphate gel step were applied to a 3.5x16 cm column of DEAE-cellulose, previously equilibrated with 0.025 M-Na_2HPO_4 in 0.05 M-Tris-HCl, pH 8.85 (Section 2.2.2). Stepwise elution was carried out with 0.05 M-Tris-HCl, pH 8.85, containing initially 0.18 M-NaCl (400 ml) and then with 0.27 M-NaCl (800 ml) to elute the enzyme. The flow rate was 375 ml/h and 10 ml fractions were collected. Fractions with a uracil dehydrogenase activity greater than 0.12 units/ml of eluate were pooled (230 ml) and diluted with 0.05 M-Tris-HCl, pH 8.85, to 500 ml. The diluted enzyme solution was assayed for uracil dehydrogenase activity (Table 4.1).

Step 4 Ammonium sulphate fractionation

The enzyme solution from step 3 (500 ml) was fractionated using ammonium sulphate. Uracil dehydrogenase was recovered in the fraction between 30-60% $(NH_4)_2SO_4$ saturation. Finely ground solid $(NH_4)_2SO_4$ (98 g) was added at the rate of approximately 5 g/min with continuous stirring. After a further 30 min stirring the mixture was centrifuged (9500 g, 15 min, 2°C). The precipitate was discarded and a further 99 g of $(NH_4)_2SO_4$ was added to the supernatant solution with stirring, which was continued for a further 45 min. The precipitate from this step was collected by centrifugation (12500 g, 15 min, 2°C) and redissolved in 0.05 M-Tris-HCl, pH 8.85 (15 ml), to give a clear reddish brown solution which was assayed for uracil dehydrogenase (Table 4.1).

Step 5 Sepharose-4B gel filtration

The preparation (15 ml) obtained from the ammonium sulphate fractionation (step 4) was applied to a 4.1x57 cm column of Sepharose-4B previously equilibrated with 0.05 M-Tris-HCl, pH 8.85 (Section 2.2.1). The column was eluted with the same buffer and 10 ml fractions were collected at a flow rate of 60 ml/h. Protein was estimated by reading the absorbance of alternate fractions at 280 nm. The elution pattern is shown in Figure 4.1. Fractions containing enzyme with activity greater than 0.24 units/ml of eluate were pooled to give a total volume of 210 ml. The enzyme solution was concentrated to 15 ml in an Amicon ultrafiltration cell over an XM-50 membrane (Amicon Corp.) and assayed for uracil dehydrogenase activity (Table 4.1).

Step 6 Second DEAE-cellulose chromatography

The concentrated solution (15 ml) from Sepharose-4B gel filtration (step 5) was applied to a 3.7x43 cm column of DEAE-cellulose, previously equilibrated with 0.05 M-Tris-HCl, pH 8.85 (Section 2.2.2). The column was washed with one resin bed volume of 0.05 M-Tris-HCl, pH 8.85 (400 ml), followed by stepwise elution with 600 ml of 0.19 M-NaCl in 0.05 M-Tris-HCl, pH 8.85, and then by 1000 ml of 0.27 M-NaCl in 0.05 M-Tris-HCl, pH 8.85, to elute the enzyme. The column effluent was monitored at 280 nm and 10 ml fractions were collected (flow rate 90 ml/h). The elution pattern is shown in Figure 4.2. Fractions containing enzyme with activity greater than 0.12 units/ml of eluate were pooled to give a total volume of 366 ml. The enzyme solution was concentrated to approximately 5 ml in an ultrafiltration cell. The concentrated enzyme was desalted by diluting with 10 volumes of 0.05 M-Tris-HCl, pH 8.85, and the enzyme concentrated again. The desalting process was repeated three times and the final enzyme solution (5 ml) was diluted with 0.05 M-Tris-HCl, pH 8.85, to 25 ml and assayed for uracil dehydrogenase activity (Table 4.1).

Step 7 Chromatography with calcium phosphate gel deposited on cellulose

The enzyme solution (25 ml) from the second DEAE-cellulose chromatography (step 6) was applied to a 3.5x28 cm column of calcium phosphate gel deposited on cellulose (prepared as in Section 2.1), previously equilibrated with 0.01 M-Na_2HPO_4 in 0.05 M-Tris-HCl, pH 8.85 (Section 2.2.3). The column was washed with a one third resin bed volume of the Na_2HPO_4/Tris-HCl buffer (90 ml), followed by the elution of the

enzyme with a linear gradient of Na_2HPO_4 in 0.05 M-Tris-HCl, pH 8.85. For production of the continuous gradient, the mixing chamber contained 350 ml of 0.01 M-Na_2HPO_4 in 0.05 M-Tris-HCl, pH 8.85 and the reservoir contained 350 ml of 0.10 M-Na_2HPO_4 in 0.05 M-Tris-HCl, pH 8.85. The column effluent was monitored at 280 nm and 10 ml fractions were collected (flow rate 20 ml/h). The elution pattern is shown in Figure 4.3. Fractions having an enzyme activity at least 0.12 units/ml of eluate were combined (260 ml) and concentrated in an ultrafiltration cell to about 5 ml. The concentrated enzyme was desalted in an ultrafiltration cell with 3x50 ml of 0.05 M-Tris-HCl, pH 8.85, or until the filtrate gave the same conductivity reading as the washing buffer. The reddish brown solution of concentrated enzyme was diluted to 10 ml and assayed for uracil dehydrogenase activity (Table 4.1).

This step was also attempted with a column of hydroxyapatite prepared as in Section 2.2.3 (bed dimensions 1.8x20 cm). The elution followed the same pattern and the same recovery and degree of purification were obtained as with calcium phosphate gel deposited on cellulose, but the flow rate was slow (6 ml/h) for routine use.

Step 8 DEAE-Sephadex A-50 chromatography

The concentrated enzyme solution (10 ml) from calcium phosphate gel adsorption chromatography (step 7) was applied to a 3.8x22.5 cm column DEAE-Sephadex A-50, previously equilibrated with 0.10 M-NaCl in 0.05 M-Tris-HCl, pH 8.85 (Section 2.2.4). The column was washed with a one fourth resin bed volume of the NaCl/Tris-HCl buffer (65 ml), followed by the elution of the enzyme with a linear gradient of NaCl in Tris-HCl,

pH 8.85. For production of the continuous gradient, the mixing chamber contained 270 ml of 0.10 M-NaCl in 0.05 M-Tris-HCl, pH 8.85 and the reservoir contained 270 ml of 0.50 M-NaCl in 0.05 M-Tris-HCl, pH 8.85. The column effluent was monitored at 280 nm and 10 ml fractions were collected (flow rate 20 ml/h). The elution pattern is shown in Figure 4.4. Fractions having an enzyme activity at least 0.24 units/ml of eluate were pooled (94 ml), concentrated in an ultrafiltration cell and diluted to 10 ml. The concentrated solution of enzyme was assayed for uracil dehydrogenase activity (Table 4.1).

Step 9 Sephadex G-200 gel filtration

The concentrated enzyme solution (10 ml) from DEAE-Sephadex A-50 (step 8) was applied to a 3.6x29 cm column of Sephadex G-200, previously equilibrated with 0.05 M-Tris-HCl, pH 8.85 (Section 2.2.5). The enzyme was eluted with the same buffer at a flow rate of 15 ml/h. The column effluent was monitored at 280 nm and 10 ml fractions were collected. The elution pattern (Figure 4.5) showed a single sharp enzyme peak (V_e = 135 ml), slightly after the void volumes (V_o = 120 ml). The enzyme peak was regularly distributed corresponding to the protein peak. Fractions having enzymic activity greater than 0.50 units/ml of eluate were pooled (46 ml) and concentrated in an ultrafiltration cell. The final reddish brown enzyme solution (2.25 ml) was assayed for uracil dehydrogenase (Table 4.1).

An outline of the isolation and purification process is summarised in Table 4.1. Approximately 85 fold purification was obtained with an 18% yield of enzyme.

TABLE 4.1

PURIFICATION OF URACIL DEHYDROGENASE

Step	Preparation	Volume (ml)	Total enzyme (units)	Protein (mg/ml)	Total protein (mg)	Specific activity (units/mg)	Fold
1	Preparation of cell-free extracts	1200	632.8	4.38	5256	0.12	1
2	Treatment with calcium phosphate gel	4000	552.5	0.45	1800	0.31	2.6
3	First DEAE-cellulose chromatography	500	413.8	2.07	1035	0.40	3.3
4	Ammonium sulphate fractionation (30-60% fraction)	15	397.0	17.00	255	1.56	12.9
5	Sepharose-4B gel filtration	15	437.7	15.40	231	1.89	15.7
6	Second DEAE-cellulose chromatography	25	283.8	3.22	80.5	3.53	29.2
7	Chromatography with calcium phosphate gel deposited on cellulose	10	241.9	2.60	26	9.30	77.2
8	DEAE-Sephadex A-50 chromatography	10	161.4	2.12	21.2	7.61	63.3
9	Sephadex G-200 gel filtration	2.25	117.5	5.00	11.3	10.40	86.7

Figure 4.1 Sepharose-4B gel filtration of 30-60% ammonium sulphate fraction from N. corallina

About 255 mg of protein containing 397 units of uracil dehydrogenase was applied to the column (4.1x57 cm) previously equilibrated with 0.05 M-Tris-HCl, pH 8.85. The column was eluted at 4°C with the same buffer. Fractions of 10 ml were collected and aliquots were assayed for uracil dehydrogenase (Section 2.3.5a) and for absorbance at 280 nm. The column void volume (V_o) was 275 ml.

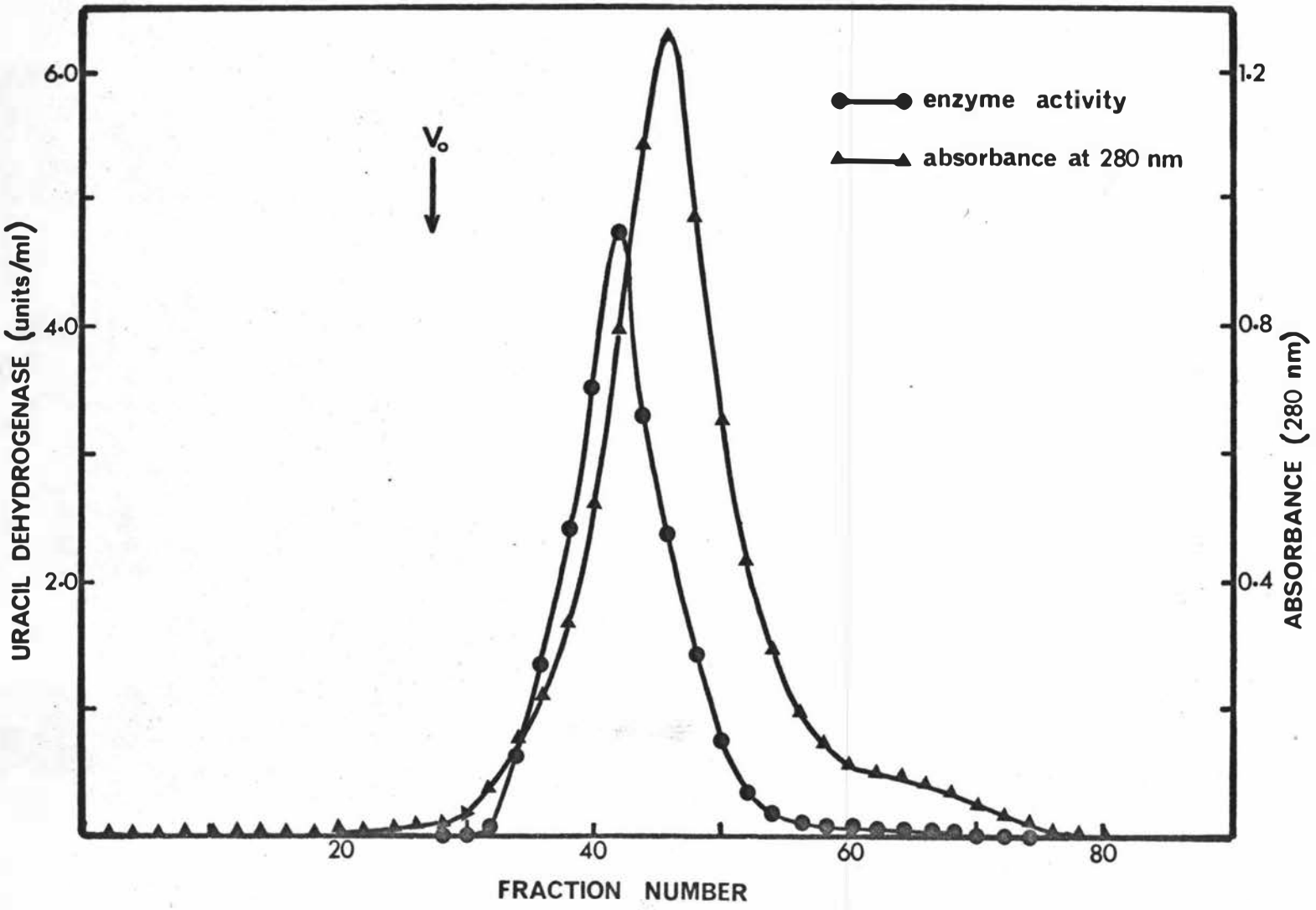

enzyme activity
absorbance at 280 nm
V_o
URACIL DEHYDROGENASE (units/ml)
ABSORBANCE (280 nm)
FRACTION NUMBER
6·0
4·0
2·0
1·2
0·8
0·4
20
40
60
80

Figure 4.2 DEAE-cellulose chromatography of the uracil dehydrogenase preparation from the Sepharose-4B column

About 231 mg of protein containing 438 units of uracil dehydrogenase was applied to the column (3.7x43 cm) previously equilibrated with 0.05 M-Tris-HCl, pH 8.85. After washing at 4°C with 400 ml of the same buffer, the column was eluted with 600 ml of 0.19 M-NaCl in 0.05 M-Tris-HCl, pH 8.85 and then 1000 ml of 0.27 M-NaCl in 0.05 M-Tris-HCl, pH 8.85. Fractions of 10 ml were collected and aliquots were assayed for uracil dehydrogenase (Section 2.3.5a) and for absorbance at 280 nm.

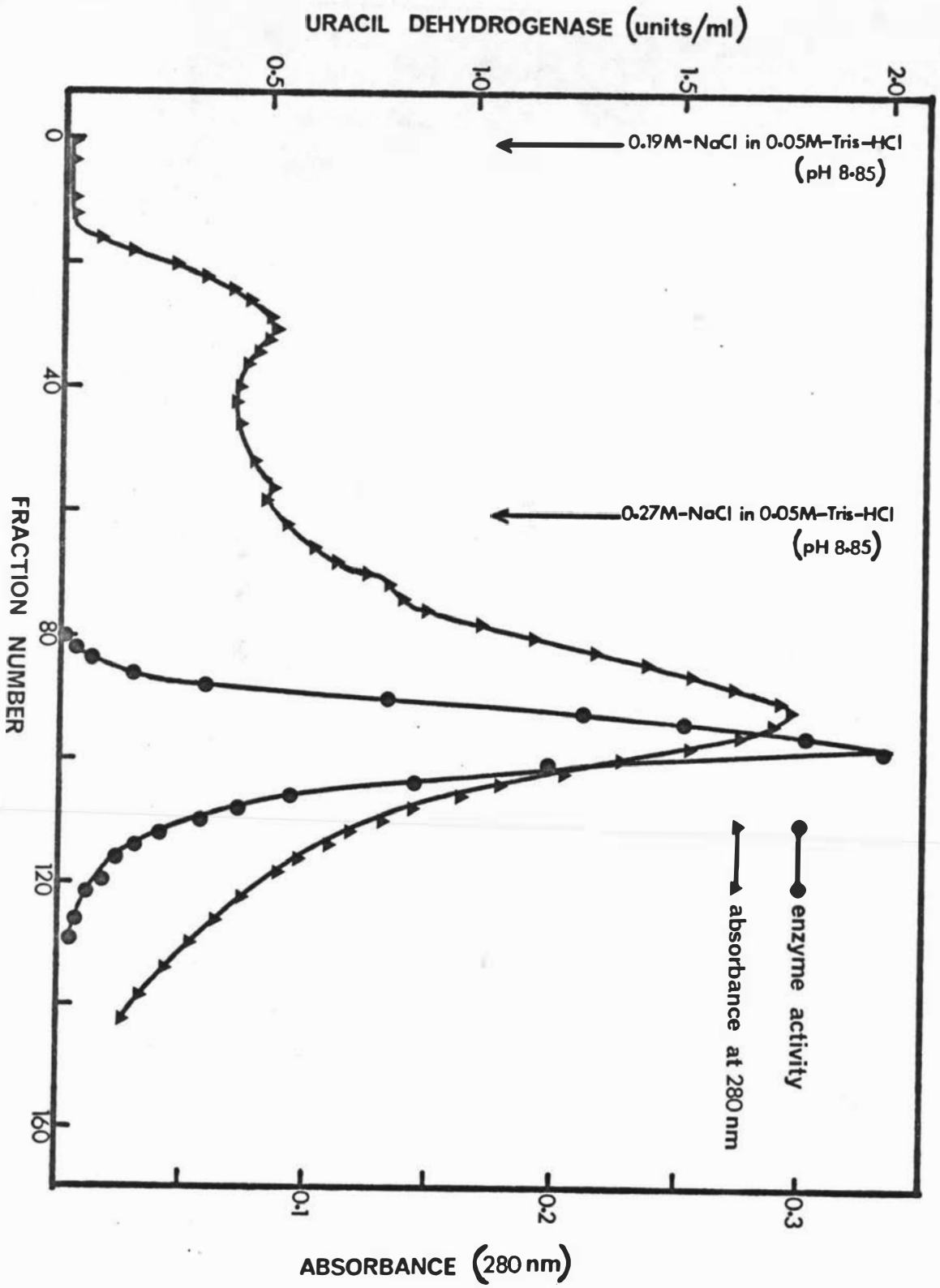

URACIL DEHYDROGENASE (units/ml)
0·5
1·0
1·5
2·0
0·19M-NaCl in 0·05M-Tris-HCl
(pH 8·85)
0·27M-NaCl in 0·05M-Tris-HCl
(pH 8·85)
FRACTION NUMBER
0
40
80
120
160
enzyme activity
absorbance at 280 nm
0·1
0·2
0·3
ABSORBANCE (280 nm)

Figure 4.3 Calcium phosphate chromatography of the uracil dehydrogenase preparation from the DEAE-cellulose column

About 81 mg of protein containing 284 units of uracil dehydrogenase was applied to the column (3.5x28 cm) previously equilibrated with 0.01 M-Na_2HPO_4 in 0.05 M-Tris-HCl, pH 8.85. After washing at 4°C with 90 ml of the equilibrating buffer, the column was eluted with a linear gradient of Na_2HPO_4 (0.01 M to 0.10 M) in 700 ml of 0.05 M-Tris-HCl buffer, pH 8.85. Fractions of 10 ml were collected and aliquots were assayed for uracil dehydrogenase (Section 2.3.5a) and for absorbance at 280 nm.

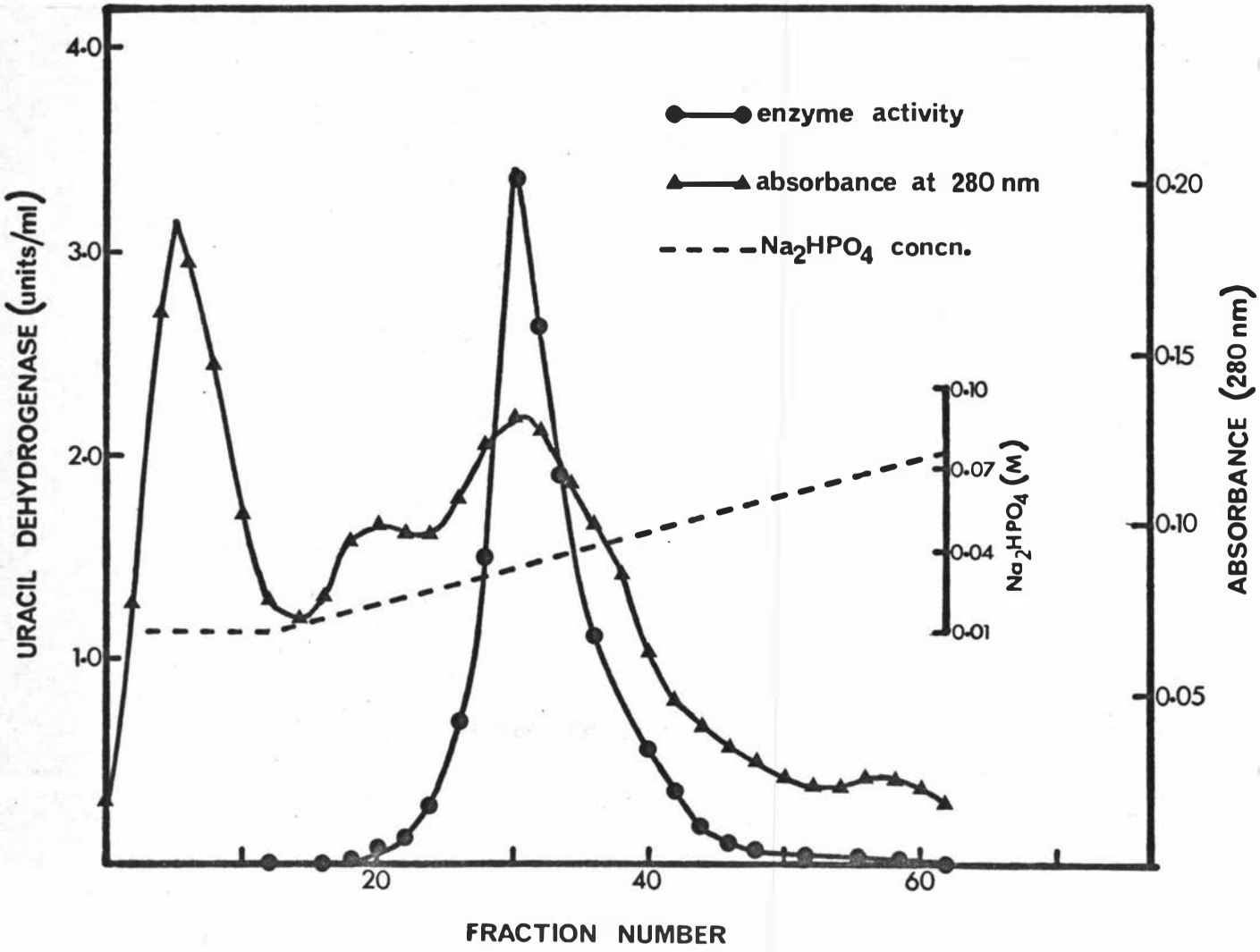

enzyme activity
absorbance at 280 nm
Na_2HPO_4 concn.
URACIL DEHYDROGENASE (units/ml)
ABSORBANCE (280 nm)
Na_2HPO_4 (M)
FRACTION NUMBER
4·0
3·0
2·0
1·0
0·20
0·15
0·10
0·05
0·10
0·07
0·04
0·01
20
40
60

Figure 4.4 DEAE-Sephadex A-50 chromatography of the uracil dehydrogenase preparation from the calcium phosphate column

About 26 mg of protein containing 242 units of uracil dehydrogenase was applied to the column (3.8x22.5 cm) previously equilibrated with 0.10 M-NaCl in 0.05 M-Tris-HCl, pH 8.85. After washing at 4°C with 65 ml of the equilibrating buffer, the column was eluted with a linear gradient of NaCl (0.10 M to 0.50 M) in 540 ml of 0.05 M-Tris-HCl, pH 8.85. Fractions of 10 ml were collected and aliquots were assayed for uracil dehydrogenase (Section 2.3.5a) and for absorbance at 280 nm.

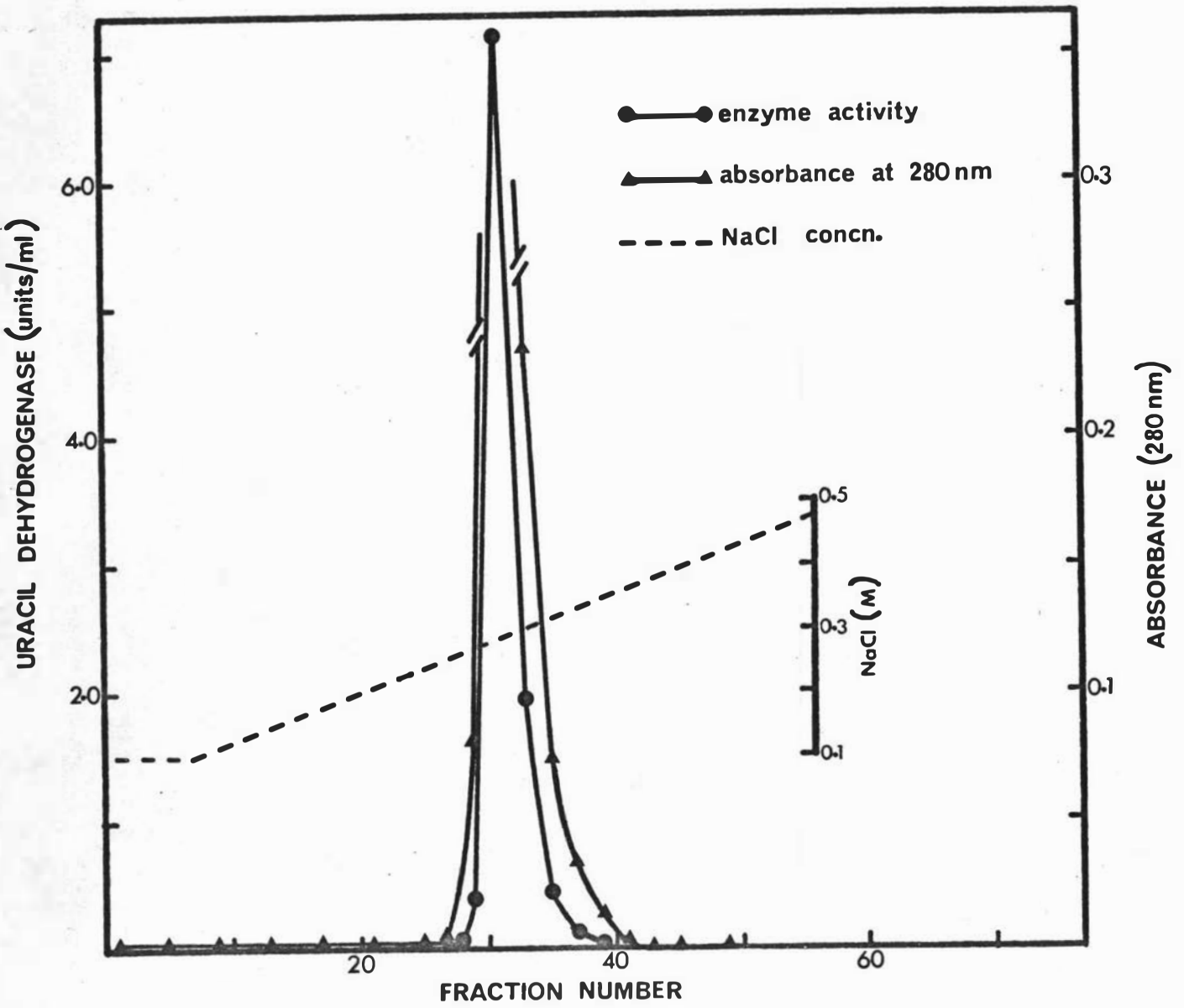
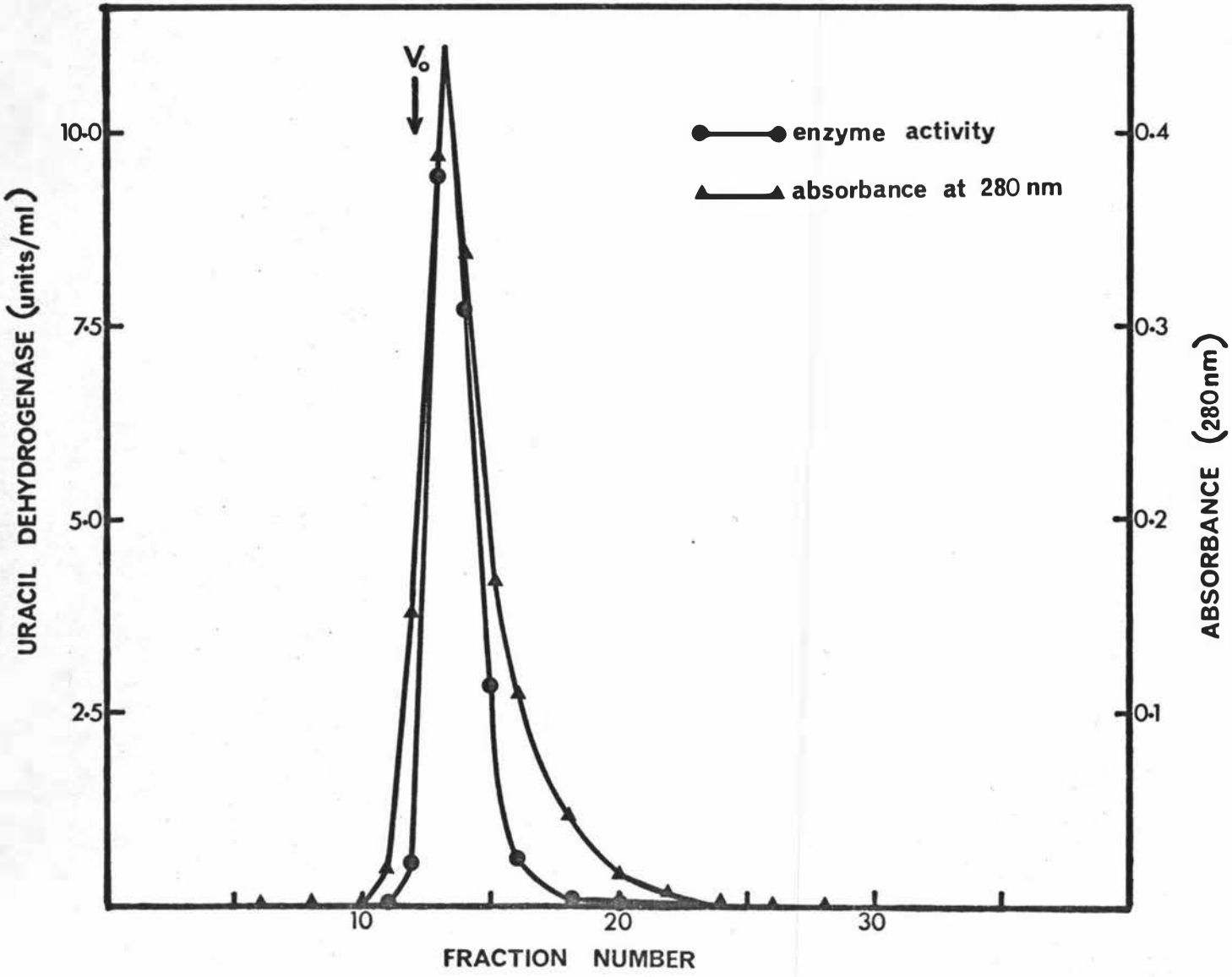

enzyme activity
absorbance at 280 nm
NaCl concn.
URACIL DEHYDROGENASE (units/ml)
ABSORBANCE (280 nm)
NaCl (M)
FRACTION NUMBER
6·0
4·0
2·0
0·3
0·2
0·1
0·5
0·3
0·1
20
40
60

Figure 4.5 Sephadex G-200 gel filtration of the uracil dehydrogenase preparation from the DEAE-Sephadex A-50 column

About 21 mg of protein containing 161 units of uracil dehydrogenase was applied to the column (3.6x29 cm) previously equilibrated with 0.05 M-Tris-HCl, pH 8.85. The column was eluted at 4°C with the same buffer. Fractions of 10 ml were collected and aliquots were assayed for uracil dehydrogenase (Section 2.3.5a) and for absorbance at 280 nm. The column void volume (V_o) was 120 ml.


V_o
enzyme activity
absorbance at 280 nm
URACIL DEHYDROGENASE (units/ml)
ABSORBANCE (280nm)
10·0
7·5
5·0
2·5
0·4
0·3
0·2
0·1
10
20
30
FRACTION NUMBER

4.3 Homogeneity of the purified uracil dehydrogenase

The purified enzyme obtained from the last step of the purification procedure (Section 4.2) was submitted to polyacrylamide gel electrophoresis as described in Section 2.3.11a. On staining with Coomassie Brilliant Blue, the gel showed the pattern of bands illustrated in Figure 4.6.

To locate the enzyme on the gel, two techniques were employed; firstly, by assaying enzymatic activity in extracts of slices cut from the gel and, secondly, staining for enzymatic activity on the gel. The experimental details are described in Section 2.3.11a. The results (Figures 4.7, 4.8) showed that the major band (major enzyme component) and the two slow moving bands (minor enzyme components) all contained enzymatic activity.

Gels which had a high loading of protein showed additional traces of fast moving faint bands which were enzymatically inactive. A spectrophotometric gel scan indicated that these inactive components accounted for not more than 5% of the total protein and in some preparations, less than 2%.

The proteins corresponding to the minor and major bands were extracted from the gels and the subunit structure of the proteins examined by SDS-polyacrylamide gel electrophoresis. The bands showed an identical subunit structure consisting of four polypeptides (Section 5.2, Figure 5.5), thus indicating that the minor bands were derivatives, presumably oligomers, of the major band. Furthermore, these results indicate that it was unlikely that either band was contaminated with non-enzyme protein.

Figure 4.6 Polyacrylamide gel electrophoresis pattern of the native enzyme

The purified uracil dehydrogenase (50 µg; specific activity 10.4 units/mg), was applied to a 7.5% acrylamide gel. The gel and electrode buffer was 0.1 M-Tris/0.1 M-glycine, pH 8.9, and electrophoresis was at a constant current of 4 mA/gel until the tracking dye (indicated by the marker wire) reached the bottom of the gel.

Figure 4.7 Location of uracil dehydrogenase activity after gel electrophoresis by enzyme assays on gel extracts

Procedures are described in Section 2.3.11a. The uracil dehydrogenase preparation (60 µg; specific activity 10.2 units/mg) was applied to the 7.5% acrylamide gel.

A. The uracil dehydrogenase activity profile from the non-stained gel.

B. The gel was stained for protein with Coomassie Brilliant Blue.

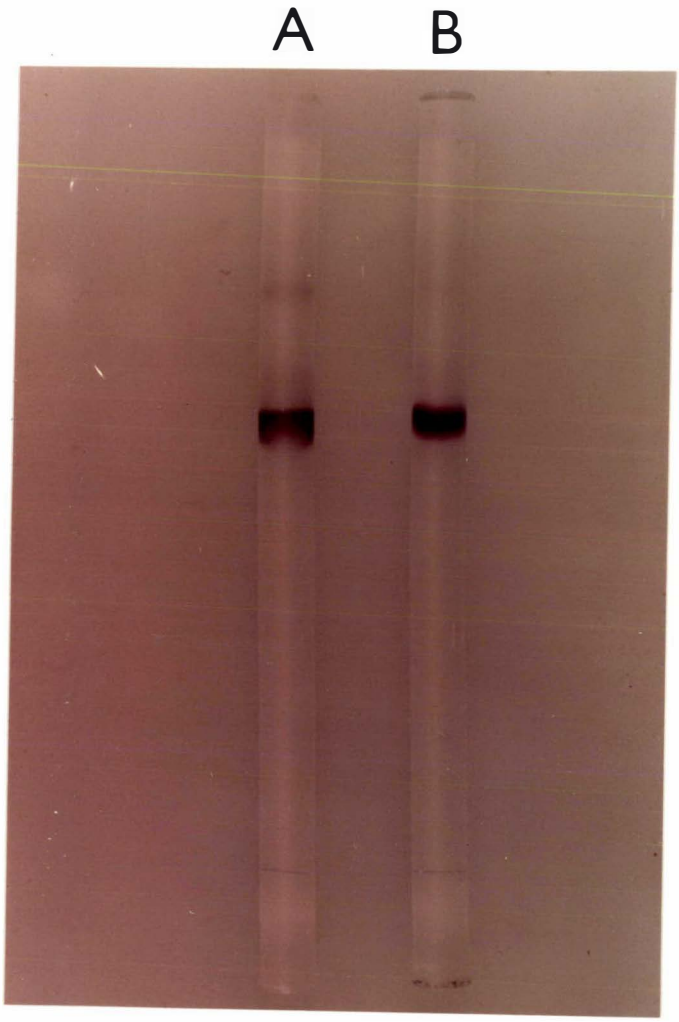
A
B
URACIL DEHYDROGENASE
(units/ml) (10^3)
1·8
1·2
0·6
origin
end
SLICE NUMBER (mm)
–
+
I

Figure 4.8 Location of uracil dehydrogenase activity after gel electrophoresis by activity staining.

Procedures are described in Section 2.3.11a. The uracil dehydrogenase preparation (60 µg; specific activity 10.2 units/mg) was applied to the 5% acrylamide gel.

A. The gel was stained for uracil dehydrogenase activity. The bands identifying uracil dehydrogenase activity result from the reduction of Nitro Blue Tetrazolium by reduced phenazine methosulphate in a reaction which is coupled to the oxidation of thymine by uracil dehydrogenase.

B. The gel was stained for protein with Coomassie Brilliant Blue.


A
B

CHAPTER 5

CHARACTERIZATION OF URACIL DEHYDROGENASE

(CHEMICAL AND PHYSICOCHEMICAL PROPERTIES)

5.1 Molecular weight of the native enzyme

5.1.1 Gel filtration on Sephadex G-200

The molecular weight of uracil dehydrogenase was estimated by gel filtration on a Sephadex G-200 column calibrated with proteins of known molecular weight. Experimental details are described in Section 2.3.10b. The result, shown in Figure 5.1, corresponds to a molecular weight of approximately 300 000 for the native enzyme.

5.1.2. Analytical ultracentrifugation

Sedimentation equilibrium studies of native uracil dehydrogenase (specific activity 10.4 units/mg) by the method described in Section 2.3.10a were performed at a protein concentration of 0.8 mg/ml in 0.025 M-Tris-HCl, pH 8.85. An equilibrium state was reached between 10 and 20 h. The fringe patterns were photographed on to glass plates. Figure 5.2 shows a plot of the natural logarithm of fringe displacement ($\ln \Delta y$) against the square of radial distance (r^2); a slope of 0.96 was obtained. The partial specific volume of the enzyme calculated from the amino acid content was 0.70 g/ml (Section 5.3). From these data, the average molecular weight of the native uracil dehydrogenase was calculated to be 298000 and this value was used throughout the remainder of this thesis.

Figure 5.1 Molecular weight estimation of uracil dehydrogenase by Sephadex G-200 gel filtration

Plot of elution volumes against the logarithms of molecular weights for proteins on a Sephadex G-200 column (2.6x60 cm) at pH 8.85. Experimental details are described in Section 2.3.10b. The arrow indicates the position at which uracil dehydrogenase was eluted. Protein standards: myoglobin, mol. wt. 17800; ovalbumin, mol. wt. 45000; bovine serum albumin, monomer mol. wt. 67000; catalase, mol. wt. 230000-250000; apoferritin, mol. wt. 480000; ribulosebiphosphate carboxylase, mol. wt. 525000 and Blue Dextran, mol. wt. 2000000.

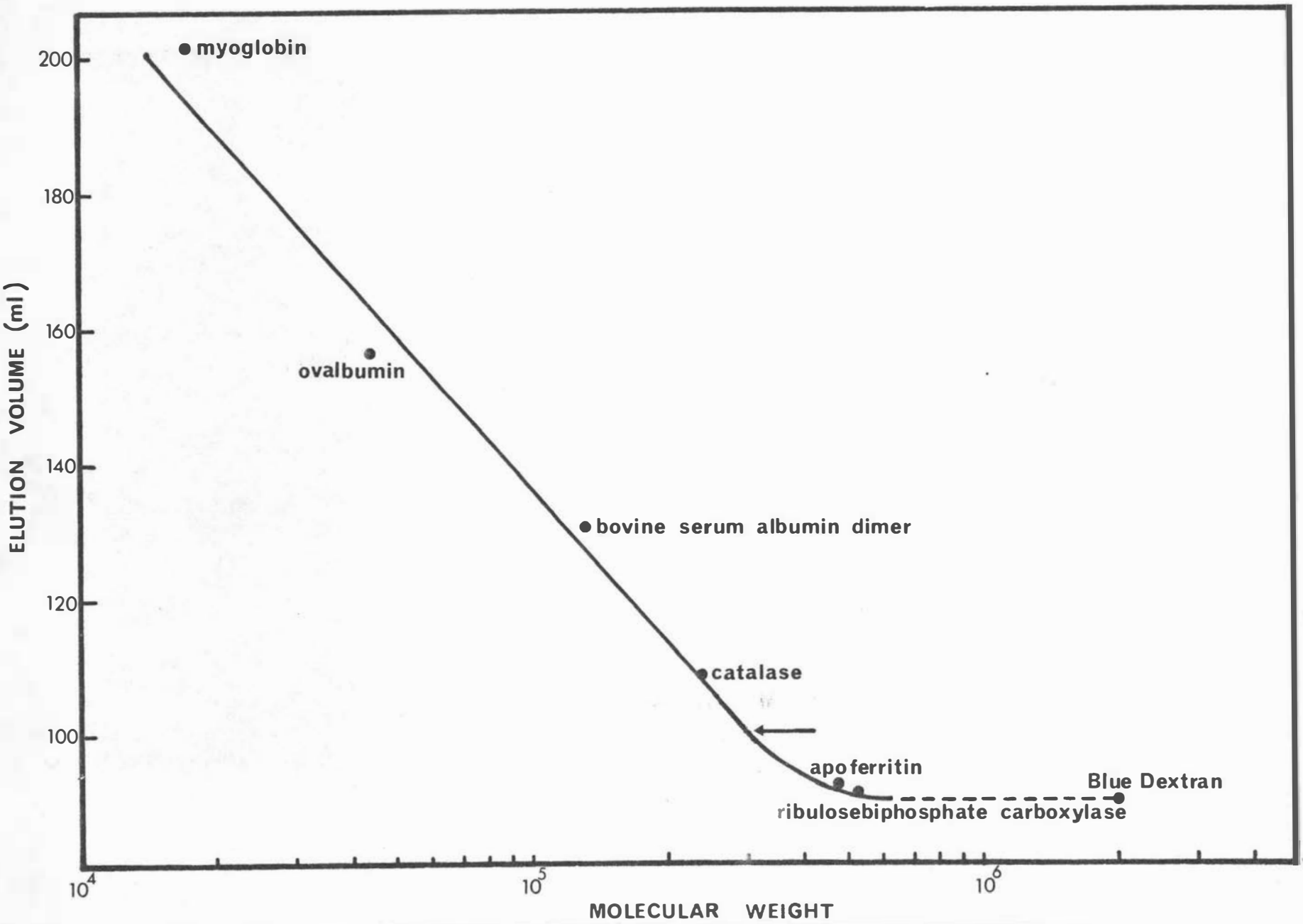
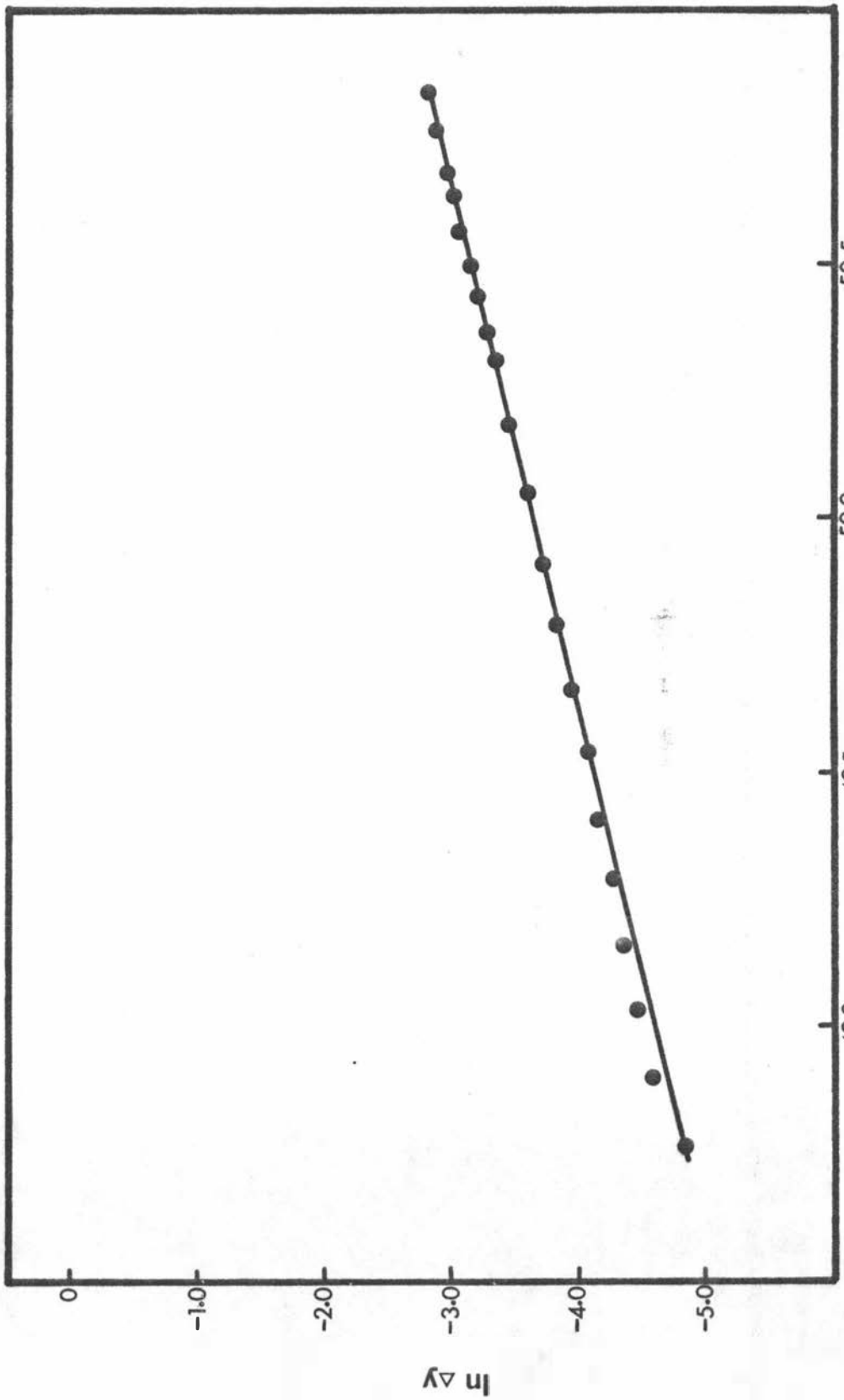

myoglobin
ovalbumin
bovine serum albumin dimer
catalase
apoferritin
Blue Dextran
ribulosebiphosphate carboxylase
ELUTION VOLUME (ml)
200
180
160
140
120
100
10^4
10^5
10^6
MOLECULAR WEIGHT

Figure 5.2 Molecular weight determination of uracil dehydrogenase by sedimentation equilibrium

The purified uracil dehydrogenase (0.8 mg/ml, specific activity 10.4 units/mg) was centrifuged at 6852 rev./min at 15°C for 20 h. The plot represents the natural logarithm of fringe displacement ($\ln \Delta y$) against radial squared (r^2).


0
-1.0
-2.0
-3.0
-4.0
-5.0
ln Δy
49.0
49.5
50.0
50.5
r² (cm²)

5.2 Subunit structure

The subunit structure of uracil dehydrogenase was investigated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS). The electrophoretic system is described in Section 2.3.11b. After heating in the presence of 1% SDS and 1% 2-mercaptoethanol, the purified enzyme showed three distinct components on gel electrophoresis (Figure 5.3). The molecular weights of the three subunits were estimated from the calibration curve (Figure 5.3) to be 92000, 36000 and 21000. A scan of the gel stained with Amido Schwarz showed the relative absorbance of the three stained subunits to be 4.9 : 2.6 : 1.0 (Figure 5.4). Assuming a proportionality between the intensity of staining and protein content, these results suggested that the enzyme subunits were present in the ratio of 1.1 : 1.2 : 1.0. Thus, a likely subunit structure of the enzyme is 2 polypeptides of molecular weight 92000, two polypeptides of molecular weight 36000 and two polypeptides of molecular weight 21000 giving a molecular weight for the native enzyme of 298000.

Since polyacrylamide gel electrophoresis of the native enzyme yielded, in addition to the major band, two minor bands which were enzymatically active, an investigation of the subunit structure of each enzyme component was carried out. Gel electrophoresis of the purified native enzyme was performed, and one gel was stained (Section 2.3.11a) for uracil dehydrogenase activity. Areas corresponding to the two minor bands and the single major band were cut from fourteen gels which had been run in parallel to the stained

gel. These were extracted, concentrated, and reanalyzed for subunit structure by SDS-polyacrylamide gel electrophoresis. The details of procedures are described in Section 2.3.11b. As shown in Figure 5.5, the subunit structure of the minor enzyme components are identical to the major enzyme component. Somewhat unexpectedly the enzyme, after extraction from the gel (polyacrylamide gel electrophoresis of the native enzyme) appeared to contain four subunits of molecular weights 92000, 71000, 36000 and 21000. The origin of the additional 71000 polypeptide will be discussed later (Section 7.2).

5.3 Amino acid composition

The amino acid composition of the purified uracil dehydrogenase was determined as described in Section 2.3.8 and is given in Table 5.1. Duplicate analyses were carried out. The values of amino acid residues per mole of enzyme, as listed in Table 5.1, were based on the molecular weight for the native enzyme of 298000 obtained from sedimentation equilibrium (Section 5.1.2). Allowance was made for the presence of 1 mole of FAD, 4 atoms of Fe and 4 atoms of labile sulphide per 298000 g of enzyme. Tryptophan was destroyed by the acid hydrolysis and the separate determination of tryptophan content was not performed, because of the very limited supply of purified enzyme. In determining the values presented in Table 5.1 the tryptophan content was arbitrarily assumed to be zero. If, e.g., 50 residues of tryptophan were present per mole of enzyme, the values in Table 5.1 would all need to be lowered by about 3%.

Figure 5.3 SDS-polyacrylamide gel electrophoresis of uracil dehydrogenase

Procedures are described in Section 2.3.11b.

A. Electrophoretic pattern of uracil dehydrogenase. The amount of enzyme applied was 20 μg (specific activity 10.4 units/mg).

B. Molecular weight determination of the uracil dehydrogenase subunits.

Protein standards: bovine serum albumin, mol. wt. 67000; ovalbumin, mol. wt. 45000; chymotrypsinogen A, mol. wt. 25000; cytochrome C, mol. wt. 12270. Each protein (20 to 30 μg) was applied to a separate gel. The mobility of uracil dehydrogenase subunits are indicated by arrows.

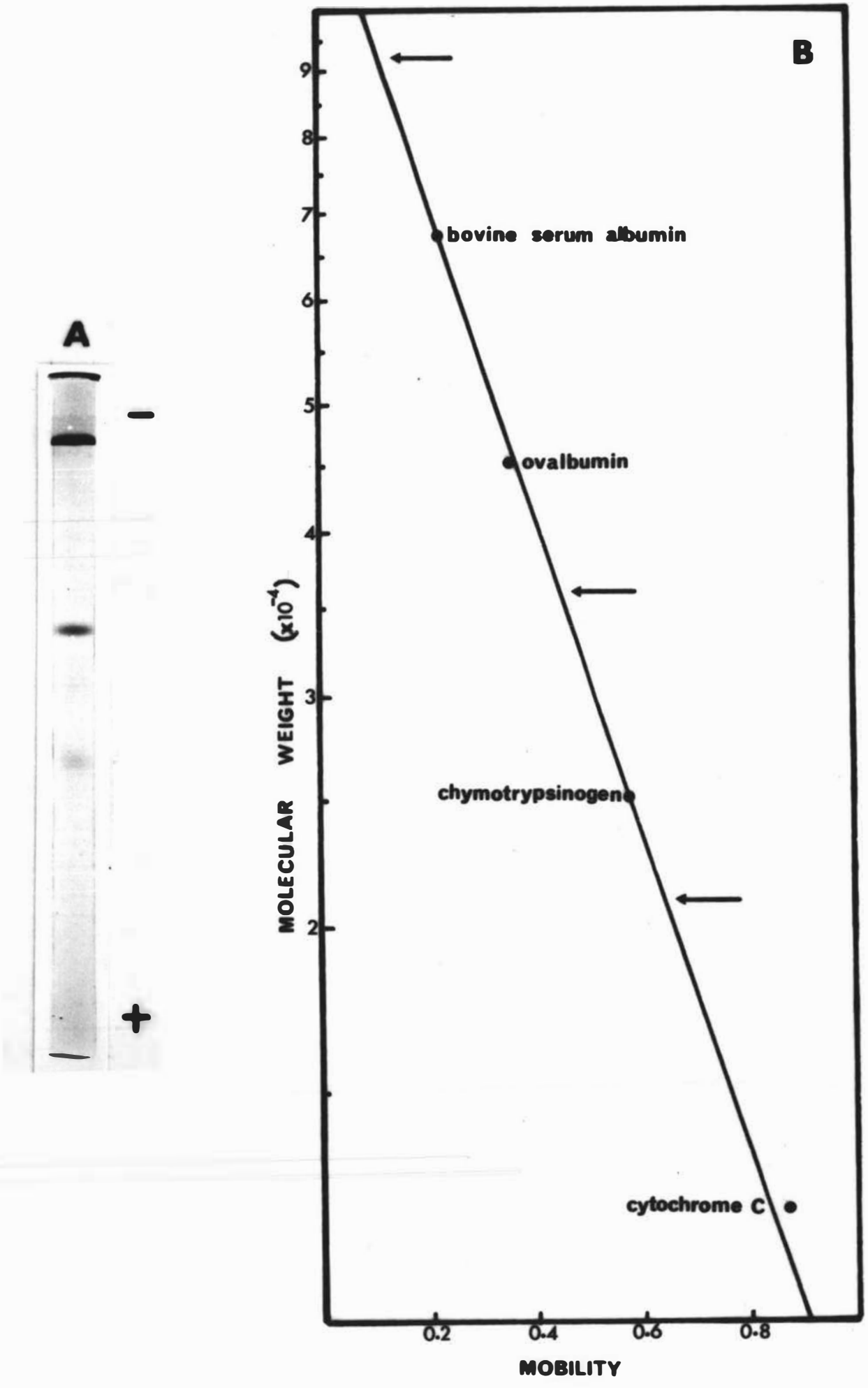

A
B
bovine serum albumin
ovalbumin
chymotrypsinogen
cytochrome C
MOLECULAR WEIGHT (x10⁻⁴)
MOBILITY
9
8
7
6
5
4
3
2
0.2
0.4
0.6
0.8

Figure 5.4 Scanning of SDS-polyacrylamide gel of uracil dehydrogenase

Procedures are described in Section 2.3.11b. Proteins were stained with Amido Schwarz. The amount of the applied enzyme was 20 µg.

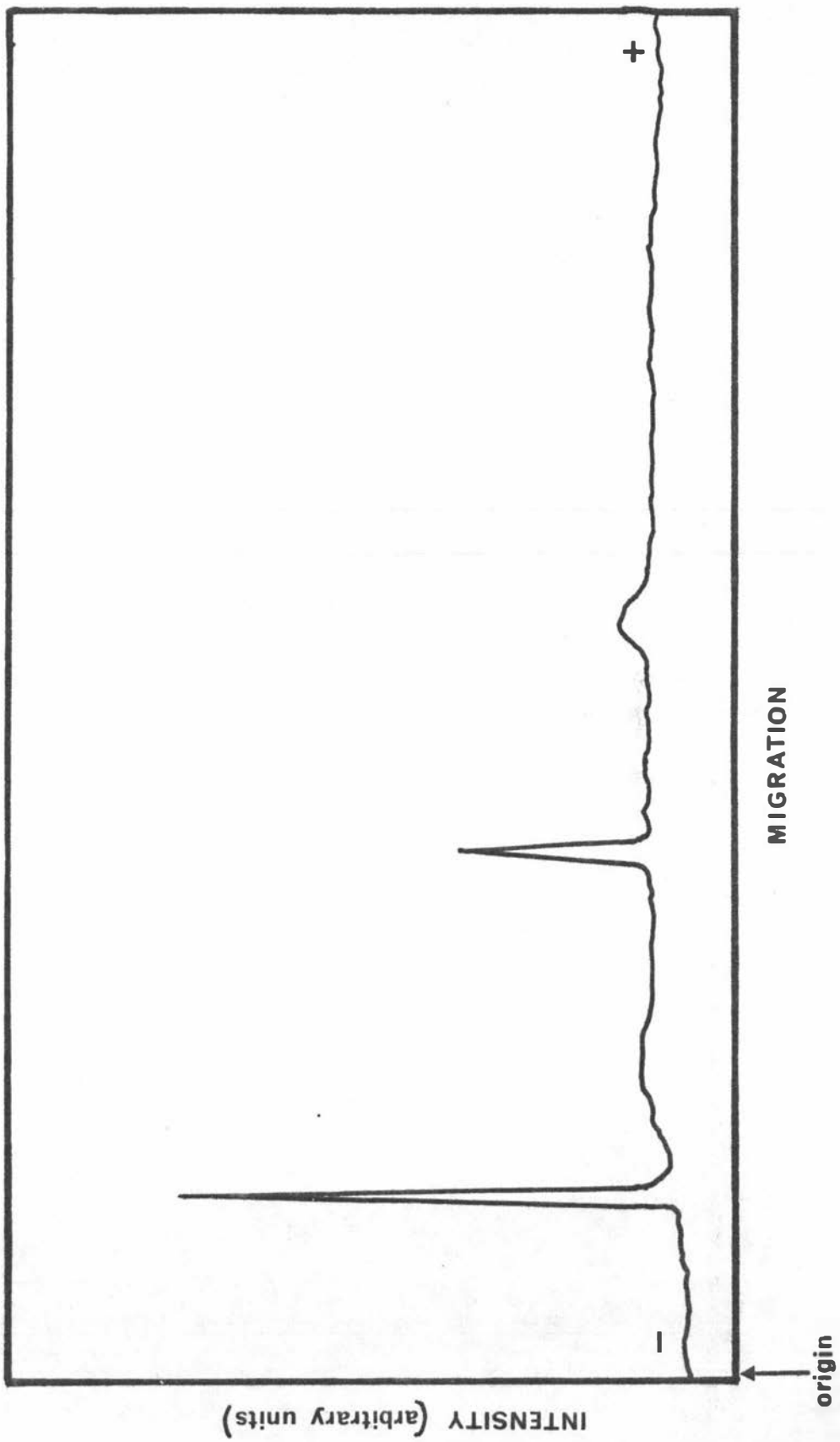

+
MIGRATION
origin
INTENSITY (arbitrary units)

Figure 5.5 SDS-polyacrylamide gel electrophoresis of oligomers of uracil dehydrogenase

Procedures are described in Section 2.3.11b.

A. Electrophoretic pattern of enzyme extracted from minor bands (1) and enzyme extracted from major band (2).

B. Molecular weight determination of the subunits.

Protein standards: bovine serum albumin, mol. wt. 67000; pyruvate kinase, mol. wt. 57000; ovalbumin, mol. wt. 45000; lactate dehydrogenase, mol. wt. 36000. Each protein (20 to 30 µg) was applied to a separate gel. The mobility of uracil dehydrogenase subunits are indicated by arrows.

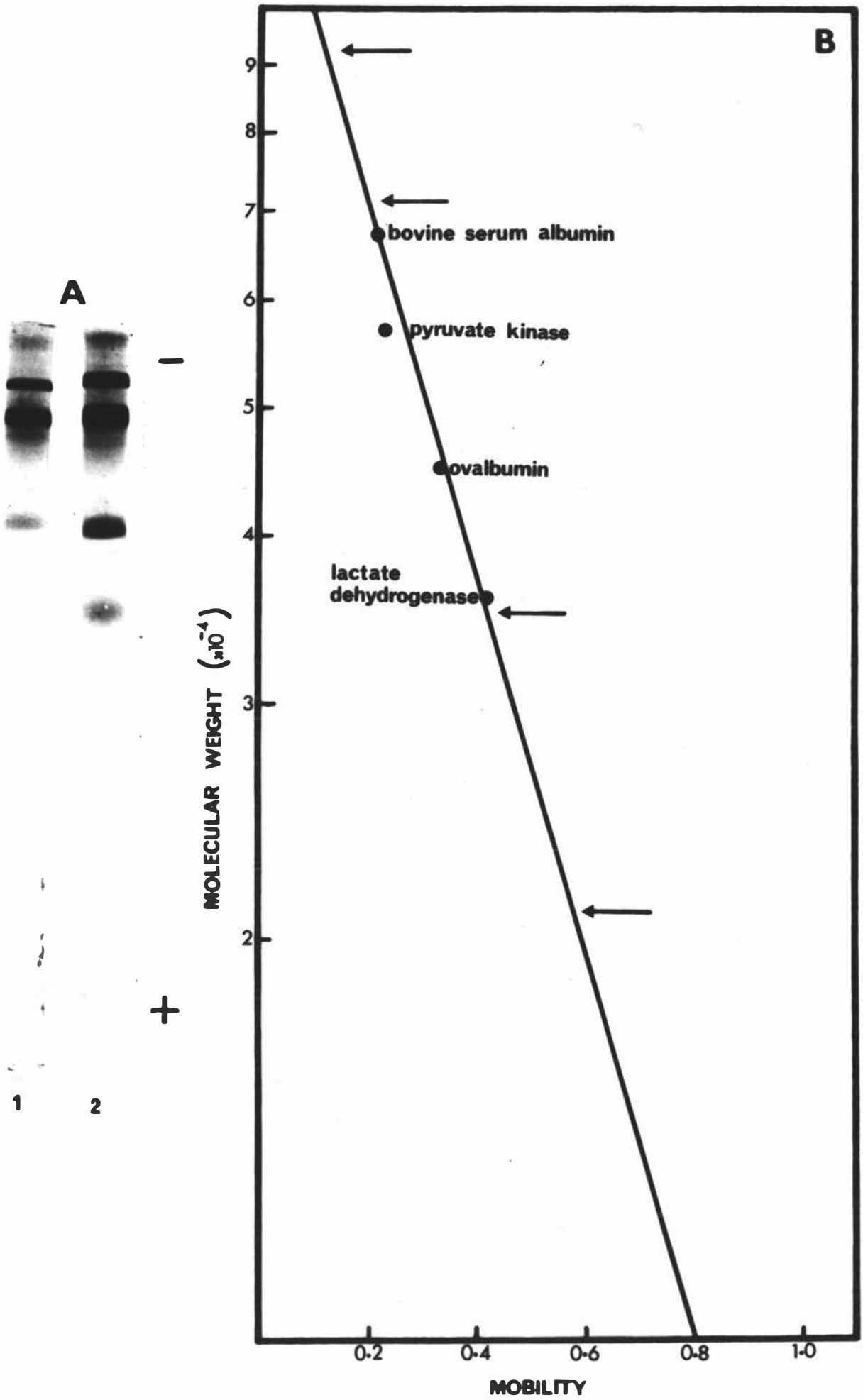

A
B
−
+
1
2
9
8
7
6
5
4
3
2
bovine serum albumin
pyruvate kinase
ovalbumin
lactate
dehydrogenase
MOLECULAR WEIGHT (x10⁻⁴)
0·2
0·4
0·6
0·8
1·0
MOBILITY

TABLE 5.1

AMINO ACID COMPOSITION OF URACIL DEHYDROGENASE FROM N. corallina

Analyses were performed as described in Section 2.3.8. The results are expressed as amino acid residues per mole of uracil dehydrogenase (mol. wt. 298000).

Amino acid	Residues per mole of enzyme (nearest integer)[a]
Lysine	92
Histidine	64
Arginine	132
Aspartic acid[d]	235
Threonine	157[b]
Serine	181[b]
Glutamic acid[d]	281
Proline	149
Glycine	242
Alanine	295
Cystine (half)	462[b]
Valine	46[c]
Methionine	117
Isoleucine	192[c]
Leucine	0
Tyrosine	68[b]
Phenylalanine	75
Tryptophan	not determined
(NH_3)	(142)

[a] Average values of 24, 48 and 72 h hydrolysis were used for calculation

[b] Values were corrected by extrapolating to zero time of hydrolysis

[c] Average values of 48 and 72 h hydrolysis were used for calculation

[d] Amide content was not determined

The relative proportions of cysteine and cystine, glutamate and glutamine, and aspartate and asparagine were not determined.

Following the procedure described by Cohn & Edsall (1943), the partial specific volume, $\bar{v}$, of 0.70 ml/g was calculated from the specific volumes of constituent amino acid residues (without regard to tryptophan content). If allowance was made for the presence of tryptophan at, say, 50 residues/mole of uracil dehydrogenase, the calculated partial specific volume remained unaltered.

From the amino acid and ammonia composition and the presence of 1 mole of FAD, the nitrogen content of purified uracil dehydrogenase was calculated to be 16.7%. This value would not have been altered appreciably (less than 0.6%) by the presence of up to 100 residues of tryptophan/mole of uracil dehydrogenase.

5.4 Spectral characteristics

The absorption spectrum of uracil dehydrogenase, shown in Figure 5.6, gives a maximum at 273 nm and an A_{280}/A_{260} ratio of 1.16. The millimolar extinction coefficient of the enzyme at 280 nm was calculated to be 284 litre$\cdot$mol$^{-1}\cdot$cm^{-1}. The protein concentration was determined by the method of Lowry *et al.* (1951).

The visible and near ultraviolet absorption spectrum of uracil dehydrogenase, shown in Figure 5.7, resemble the spectra of rabbit liver aldehyde oxidase, and shows the absorption in the region from 500 to 700 nm characteristic of

iron containing flavoproteins (Rajagopalan & Handler, 1967). The spectrum of the enzyme with maxima at 325 nm and 445 nm and a pronounced shoulder at approximately 550 nm, is typical of an iron flavoprotein. Minor shoulders are also present at 415 nm and 470 nm. When the enzyme was treated with a small amount of dithionite, the absorbance decreased through the region 400 nm to 600 nm (Figure 5.8).

The ratio A_{450}/A_{550} can be used as a measure of the iron/flavin ratio for iron flavoproteins (Rajagopalan & Handler, 1968), e.g., the A_{450}/A_{550} for bovine milk xanthine oxidase and aldehyde oxidase equals three corresponding to an Fe/FAD ratio of 4, whereas dihydroorotic dehydrogenase exhibits an A_{450}/A_{550} ratio of 5.9 corresponding to an Fe/FAD ratio of 1. The visible spectrum of uracil dehydrogenase is similar to aldehyde oxidase (Figure 5.7) and shows an A_{450}/A_{550} ratio of 3.0 (Table 5.2) indicating that this enzyme has an Fe/FAD ratio of 4. From metal analyses (Section 5.7) it is known that uracil dehydrogenase contains 4 atoms of Fe per mole of enzyme and it could therefore be deduced that uracil dehydrogenase contains 1 mole of FAD per mole of enzyme.

Figure 5.9 shows the absorption spectrum of uracil dehydrogenase, the spectrum of an equivalent amount of FAD (1 mole of FAD/mole of uracil dehydrogenase), and the difference spectrum. The difference spectrum, can be used as a measure of the absorption characteristics of protein-bound iron (Rajagopalan & Handler, 1964). Since the absorbance of 550 nm of uracil dehydrogenase is considered to be due to 4 atoms of Fe per mole of the enzyme (based on the metal analyses, Section 5.7), the molar extinction coefficient of the protein-

TABLE 5.2

CALCULATION OF MOLAR EXTINCTION COEFFICIENTS FOR IRON FROM THE VISIBLE ABSORPTION SPECTRUM OF URACIL DEHYDROGENASE

The absorbances are from the spectrum of uracil dehydrogenase (Figure 5.7). Calculation of the molar extinction coefficients per atom of Fe was based on the molecular weight for the native enzyme of 298000 (Section 5.1.2), an iron content of 4 atoms/mole of enzyme (Section 5.7); and 1 mole of FAD/mole of enzyme; the molar extinction coefficient of 11300 litre/mol/cm was used for FAD at 450 cm (Whitby, 1953). The protein concentration was determined by the method of Lowry et al.

Concn. of enzyme (mg/ml)	Specific activity (units/mg)	A_{450}	A_{550}	$\frac{A_{450}}{A550}$	A_{450} due to FAD	A_{450} due to Fe	ϵ per atom of Fe at 450 nm litre/mol/cm	ϵ per atom of Fe at 550 nm litre/mol/cm
6.61	10.4	0.756	0.249	3.04	0.251	0.505	5689	2805

A_{450} (absorbance at 450 nm) due to FAD = $11300 \times \frac{\text{concn. of enzyme (mg/ml)}}{298000}$

A_{450} due to Fe was taken as A_{450} minus A_{450} due to FAD

ϵ per atom of Fe at 450 nm = $\frac{A_{450} \text{ due to Fe}}{4} \times \frac{298000}{\text{concn. of enzyme (mg/ml)}}$

ϵ per atom of Fe at 550 nm = $\frac{A_{550}}{4} \times \frac{298000}{\text{concn. of enzyme (mg/ml)}}$

Figure 5.6 Absorption spectrum of uracil dehydrogenase

The spectrum of purified uracil dehydrogenase was measured in 0.05 M-Tris-HCl, pH 8.85 (2.280 mg protein/ml, specific activity 10.4 units/mg).

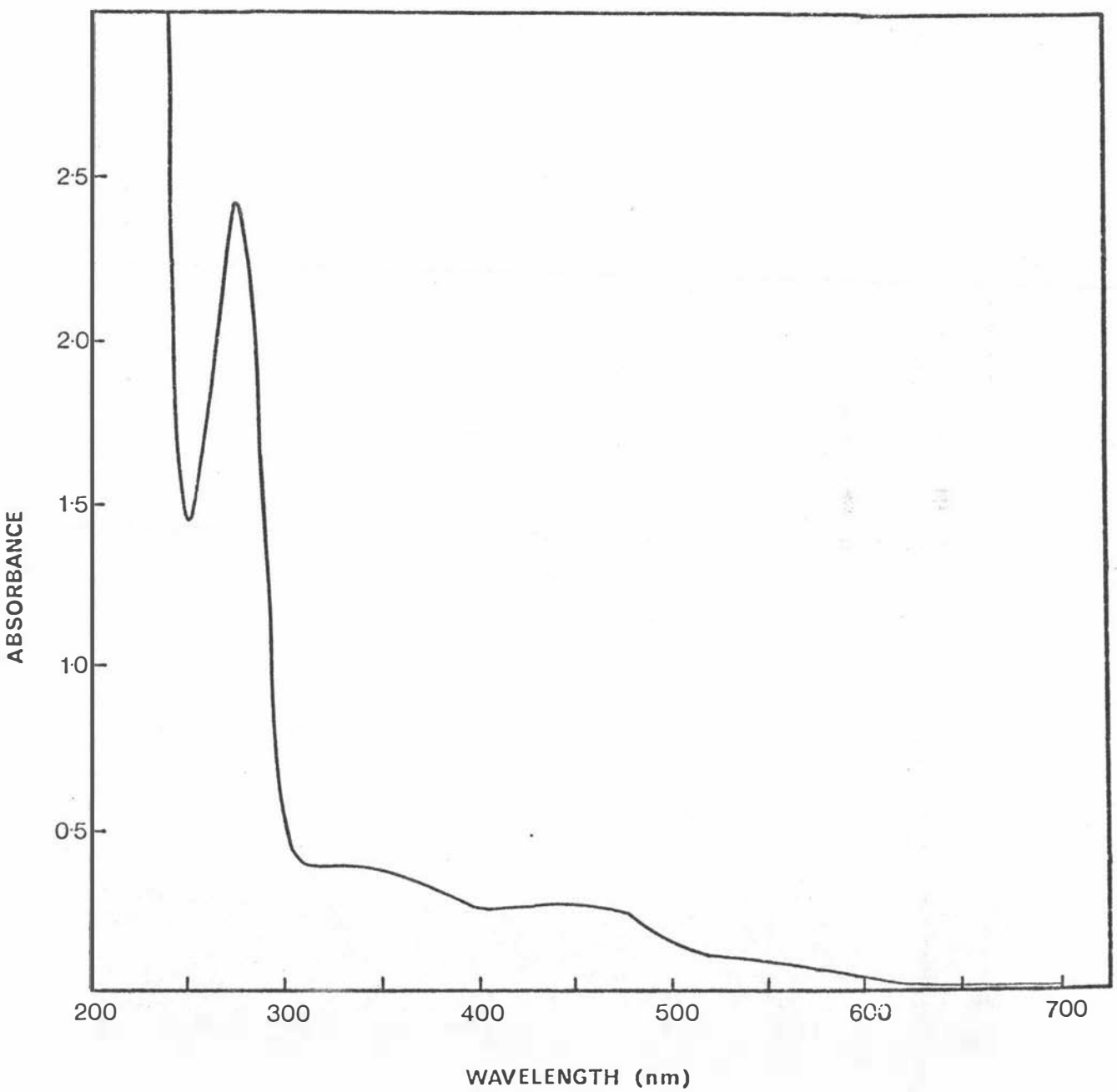

2·5
2·0
1·5
1·0
0·5
ABSORBANCE
200
300
400
500
600
700
WAVELENGTH (nm)

Figure 5.7 Visible absorption spectra of uracil dehydrogenase and aldehyde oxidase

_____, the spectrum of the purified uracil dehydrogenase was measured in 0.05 M-Tris-HCl, pH 8.85 (6.613 mg of protein/ml, specific activity 10.4 units/mg); and _ _ _ _ _, the spectrum of rabbit liver aldehyde oxidase was recorded by Rajagopalan & Handler (1964).

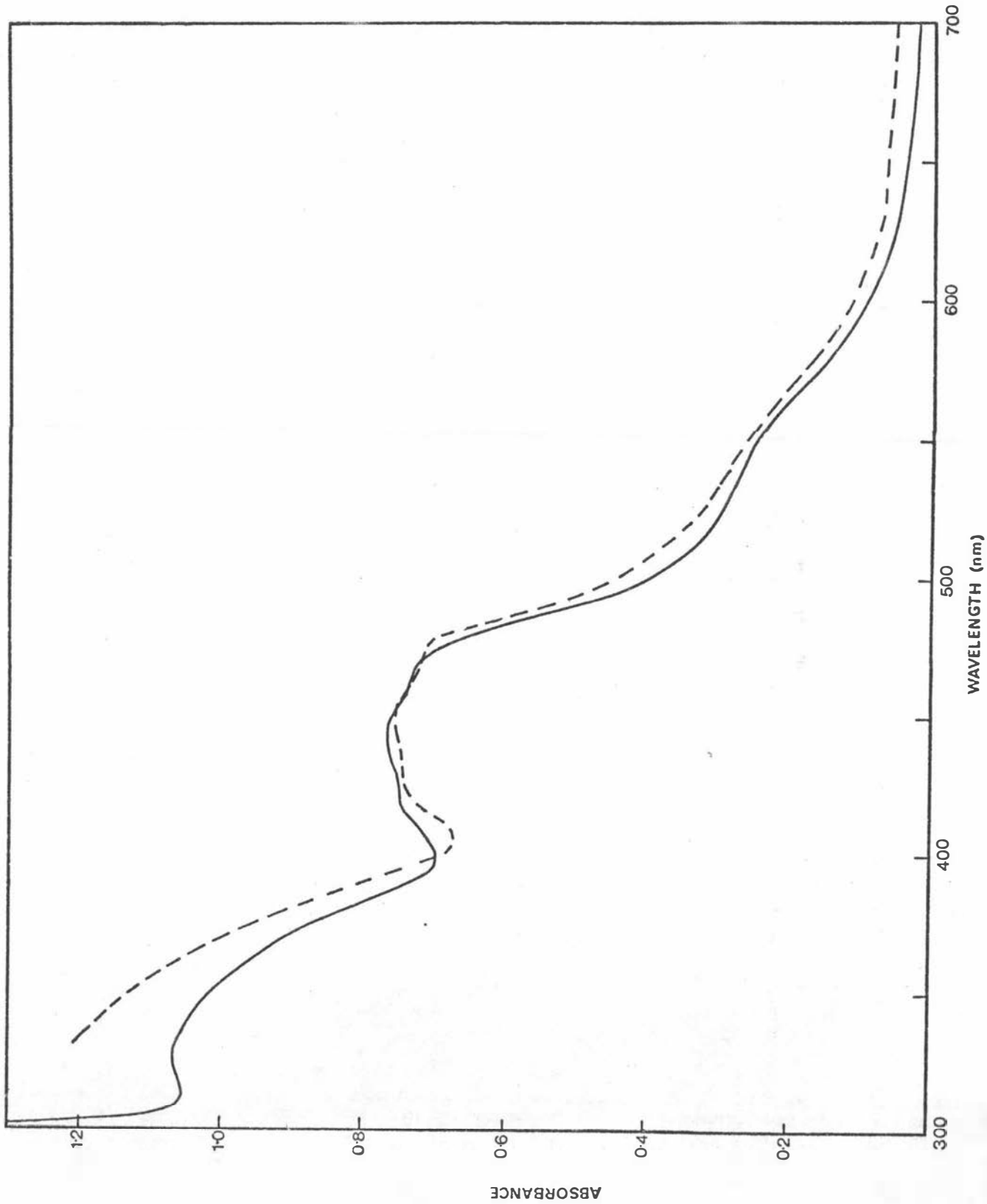

ABSORBANCE
1·2
1·0
0·8
0·6
0·4
0·2
300
400
500
600
700
WAVELENGTH (nm)

Figure 5.8 Effect of dithionite on the absorption spectrum of uracil dehydrogenase

A, the spectrum of the purified uracil dehydrogenase in 0.05 M-Tris-HCl, pH 8.85 (3.442 mg of protein/ml, specific activity 10.4 units/mg). The total volume was 1.2 ml; and

B, the reduced spectrum after addition of 0.5 mg of sodium dithionite.

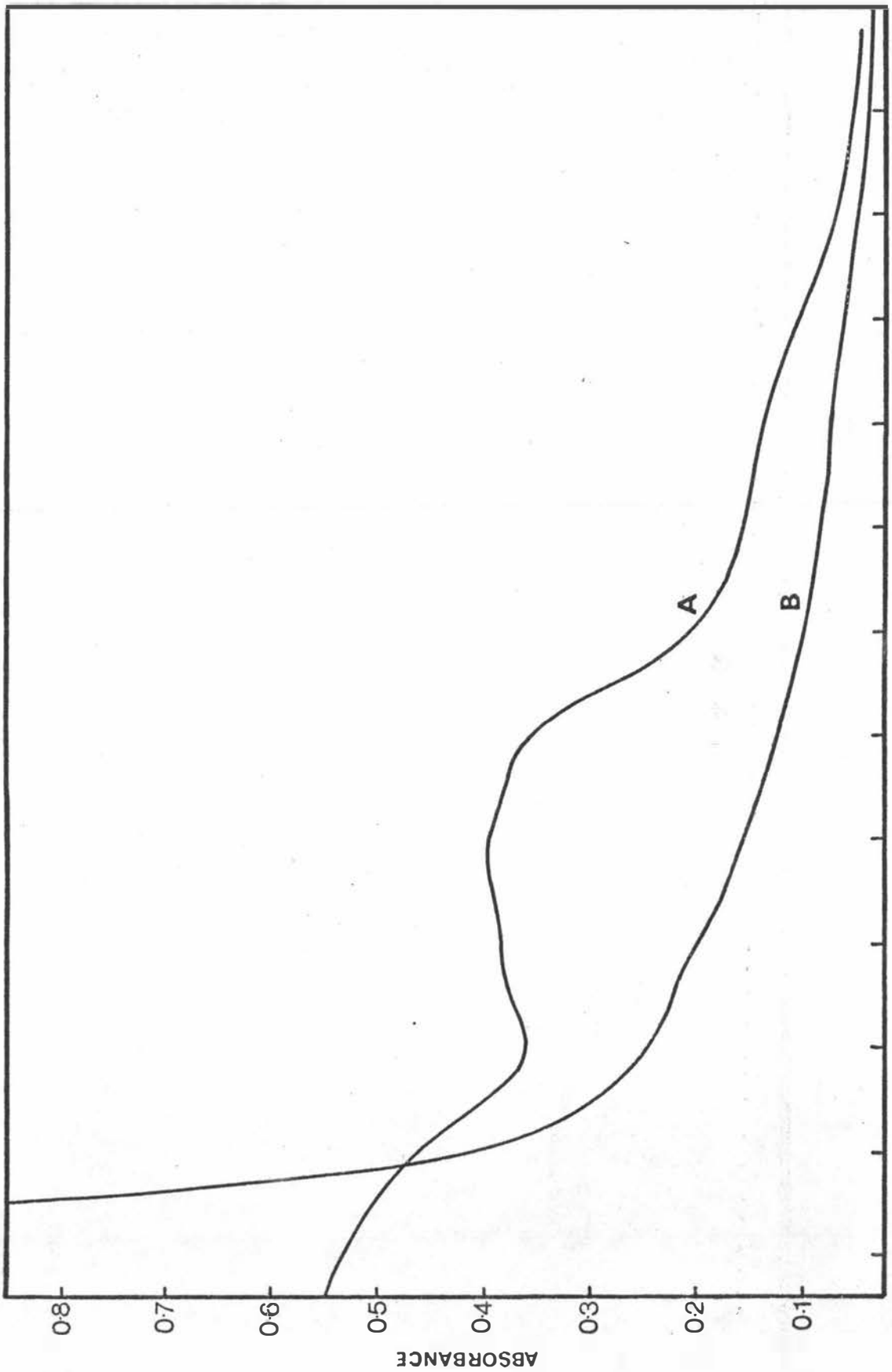
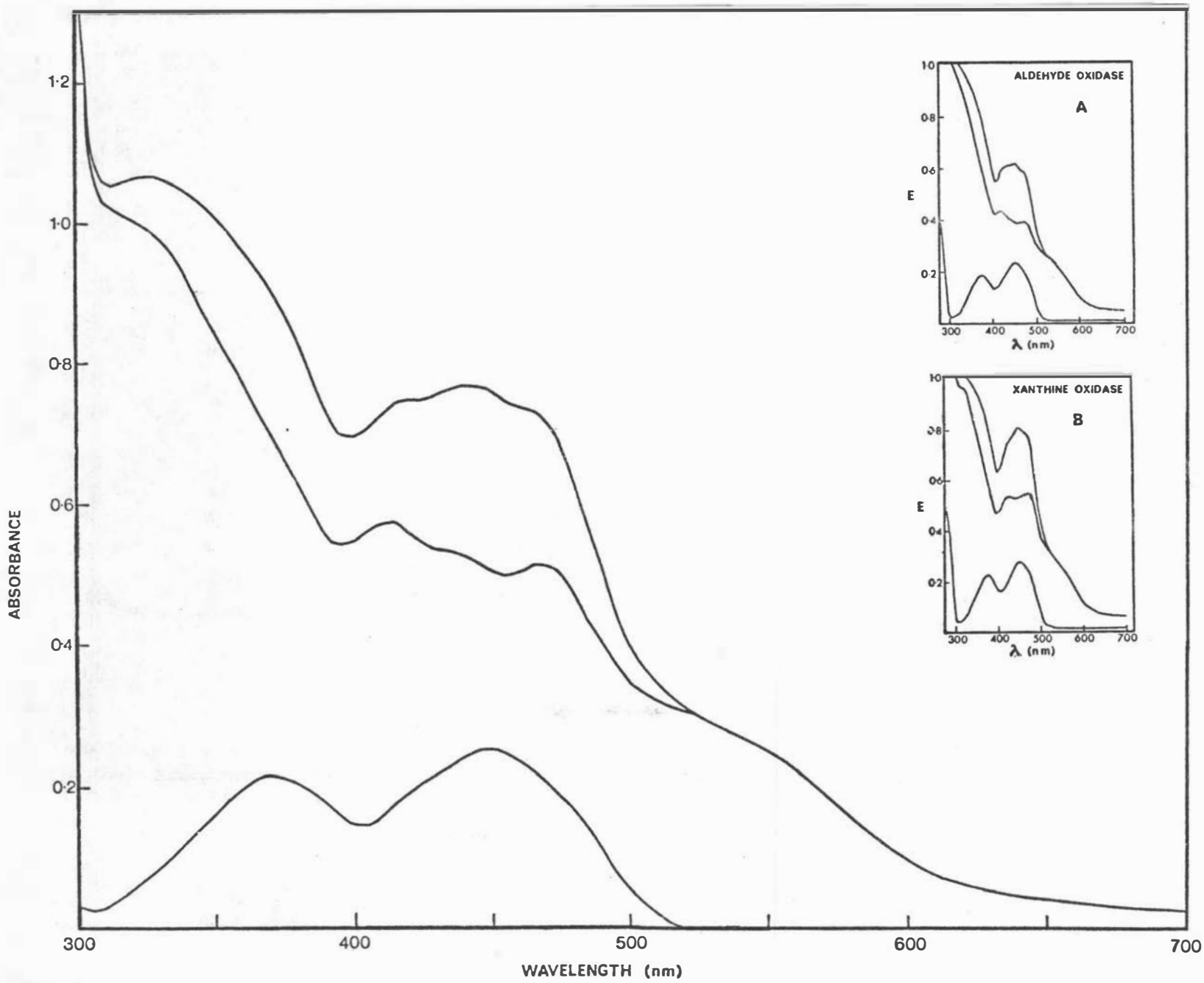

ABSORBANCE
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
350
400
450
500
550
600
WAVELENGTH (nm)
A
B

Figure 5.9 Difference spectrum of uracil dehydrogenase

Top graph: the spectrum of the purified uracil dehydrogenase in 0.05 M-Tris-HCl, pH 8.85 (6.613 mg of protein/ml, specific activity 10.4 units/mg). Bottom graph: the spectrum of the equivalent amount of FAD (1 mole of FAD/mole of uracil dehydrogenase) in 0.05 M-Tris-HCl, pH 8.85. Middle graph: enzyme spectrum minus the flavin spectrum.

Inset A: difference spectrum of aldehyde oxidase. Top graph: oxidised enzyme. Bottom graph: equivalent amount of FAD. Middle graph: enzyme spectrum minus flavin spectrum. Inset B: difference spectrum of xanthine oxidase. Graphs correspond to those for Inset A. (Insets A and B are reproduced from the paper by Rajagopalan & Handler, 1964.)


ABSORBANCE
1·2
1·0
0·8
0·6
0·4
0·2
300
400
500
600
700
WAVELENGTH (nm)
ALDEHYDE OXIDASE
A
E
λ (nm)
XANTHINE OXIDASE
B
E
λ (nm)

bound iron may be calculated. The molar extinction coefficient of protein-bound iron at 450 nm was also calculated, after allowing for the absorbance at 450 nm due to the contribution of FAD (assuming 1 mol of FAD/mol of enzyme). The results are shown in Table 5.2. The molar extinction coefficients per atom of iron of 5700 litre·mol^{-1}·cm^{-1} for Fe at 450 nm and 2800 litre·mol^{-1}·cm^{-1} for Fe at 550 nm were obtained. These values agree well with those reported by Rajagopalan & Handler (1964) for aldehyde oxidase (5000 and 2750), xanthine oxidase (5500 and 2750), and dihydroorotic dehydrogenase (5500 and 2750).

5.5 Identification of flavin prosthetic group

5.5.1 Paper chromatography

Descending paper chromatography of heated uracil dehydrogenase (HUD) and standard flavins was performed as described in Section 2.3.6a. The results are summarised in Table 5.3. In both solvents, heated uracil dehydrogenase revealed a single flavin component, which had an R_F value corresponding to that for FAD. In solvent 1, the compound was retarded compared with standard FAD (probably as a result of interaction with non-flavin components of the heated-enzyme preparation). However, after co-chromatography of standard FAD with heated uracil dehydrogenase in this solvent, only a single spot was observed (apart from minor FAD degradation products) giving an R_F value corresponding to heated uracil dehydrogenase. These results suggest that the prosthetic group from heated uracil dehydrogenase was FAD, with no FMN being detected.

5.5.2 Fluorescence analysis (release of FMN by snake venom)

It is known that flavins characteristically show a greenish-yellow fluorescence in aqueous solution. The fluo-

rescence per mole of riboflavin is equal to that for FMN, but not for FAD. Bessey et al. (1949) reported that the fluorescence of pure FAD in water at a neutral pH was approximately 9% of that of free riboflavin. The increase in fluorescence which occurs when FAD is split to FMN serves as the basis of identifying FAD.

On incubation of FAD with the venom of Crotalus atrox, an increase in fluorescence occurred (Figure 5.10) suggesting the release of FMN by phosphodiesterase (cf. Sullivan, 1968). Uracil dehydrogenase, after heating to liberate the flavin prosthetic group (Section 2.3.6a), showed a similar increase in fluorescence on treatment with the snake venom, but standard FMN showed no net increase above that of the control (Figure 5.10). The results from these analyses were considered to confirm the presence of FAD in uracil dehydrogenase.

5.5.3 Reactivation of D-amino-acid oxidase apoenzyme

The activity of D-amino-acid oxidase can be assayed spectrophotometrically in a coupled system with lactate dehydrogenase according to the following reactions:

$$\text{D-Alanine} + H_2O + O_2 \longrightarrow \text{Pyruvate} + NH_3 + H_2O_2 \quad (5.1)$$

$$\text{Pyruvate} + NADH + H^+ \longrightarrow \text{Lactate} + NAD^+ \quad (5.2)$$

Catalase must be added to remove the H_2O_2.

If the prosthetic group (FAD) of D-amino-acid oxidase is removed, activity is lost. However, the enzyme may be reactivated by adding FAD, but not FMN, to the mixture. This reactivation forms the basis of the quantitative assay of FAD described by De Luca et al. (1956) and was used as the basis of the qualitative assay reported here.

TABLE 5.3

R_F VALUES OF THE FLAVINS AND HEATED URACIL DEHYDROGENASE BY DESCENDING PAPER CHROMATOGRAPHY

Experimental details were described in Section 2.3.6a. Solvent systems: 1, 5% (w/v) Na_2HPO_4 in water; 2, butan-1-ol/acetic acid/water (4:1:5, v/v) (upper phase). The R_F values quoted are from averages from duplicate samples. Previously reported values (ascending chromatography, Dimant et al., 1952) are given in parentheses.

Flavins	Solvent 1 (R_F)[b]	Solvent 2 (R_F)[b]
FMN	0.50 (0.48)	0.13 (0.13)
FAD	0.35 (0.35)	0.04 (0.05)
Riboflavin	0.28 (0.30)	0.32 (0.30)
HUD[a]	0.26	0.04
HUD[a] + FAD	0.29	0.04

[a] the R_F quoted is that of the flavin component

[b] R_F values for the principal flavin component; minor degradation products were also observed with standard FAD, FMN and riboflavin

HUD heated uracil dehydrogenase

Figure 5.10 Effects of Crotalus atrox venom on flavins and heated uracil dehydrogenase

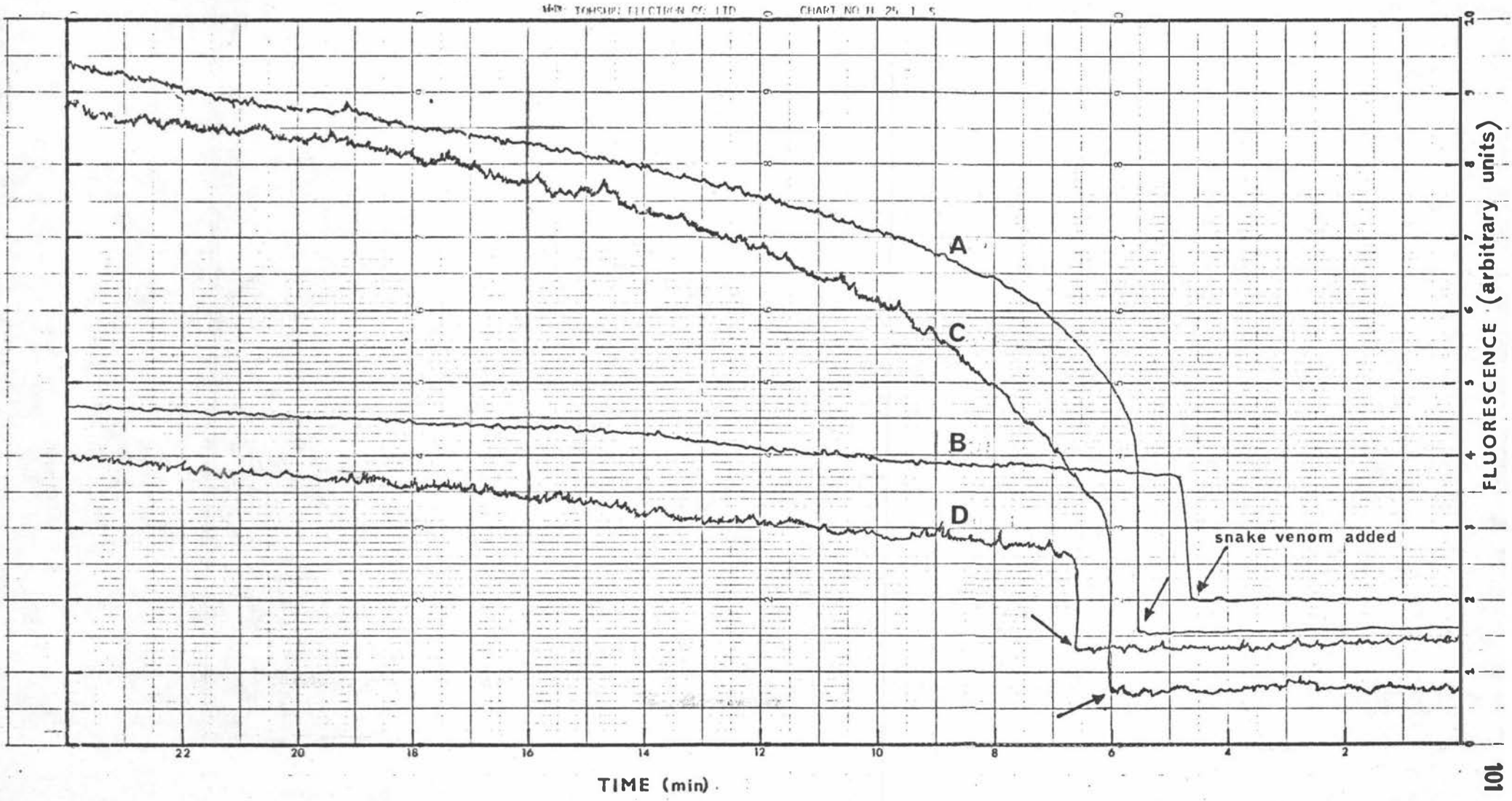
Each fluorimeter tube contained initially, in a total volume of 2.5 ml, 250 µmol of phosphate buffer, pH 7.0, and the sample to be tested. The initial fluorescence of each sample was recorded (Section 2.3.2) and at the points indicated by arrows, 2.0 mg of Crotalus atrox venom (in 50 µl of 0.1 M-phosphate buffer, pH 7.0) was added to each tube, and the change in fluorescence was recorded.

A, FAD (0.1 µg);

B, FMN (0.02 µg);

C, HUD, heated uracil dehydrogenase (0.22 µg); and

D, control (0.1 M-phosphate buffer).


A
C
B
D
snake venom added
FLUORESCENCE (arbitrary units)
TIME (min)
22
20
18
16
14
12
10
8
6
4
2

When FAD was added to the complete system containing D-alanine, NADH, D-amino-acid oxidase apoenzyme, lactate dehydrogenase and catalase, the reaction could be followed by the decrease in absorbance at 340 nm (Figure 5.11). If FMN was added instead of FAD, no decrease in absorbance occurred. If heated uracil dehydrogenase was added, a decrease in absorbance was apparent, indicating the presence of free FAD in the heat-treated uracil dehydrogenase preparation.

5.6 Quantitative determination of FAD

The flavin prosthetic group of the purified uracil dehydrogenase was qualitatively identified as FAD (Section 5.5) and it was quantitatively determined by the following methods.

5.6.1 Spectrophotometric assay

The FAD or FMN content of a simple flavoprotein can normally be determined directly from the absorbance at 448 nm or 450 nm (e.g. Williams & Hager, 1966; Sullivan, 1968). However, examination of the spectrum of uracil dehydrogenase (Section 5.4, Figure 5.7) suggested that the enzyme contained non-heme iron, which contributed to the absorbance at 450 nm. The presence of iron in the enzyme was confirmed by atomic absorption spectrophotometry (Section 5.7). Because of the presence of iron, an alternative method for determining the FAD content from the spectrum of uracil dehydrogenase had to be devised (Section 5.4) and when this was applied it gave an estimate of 1 mole of FAD per mole of enzyme (mol. wt. 298000).

Figure 5.11 Reactivation of D-amino-acid oxidase apoenzyme by FAD and heated uracil dehydrogenase

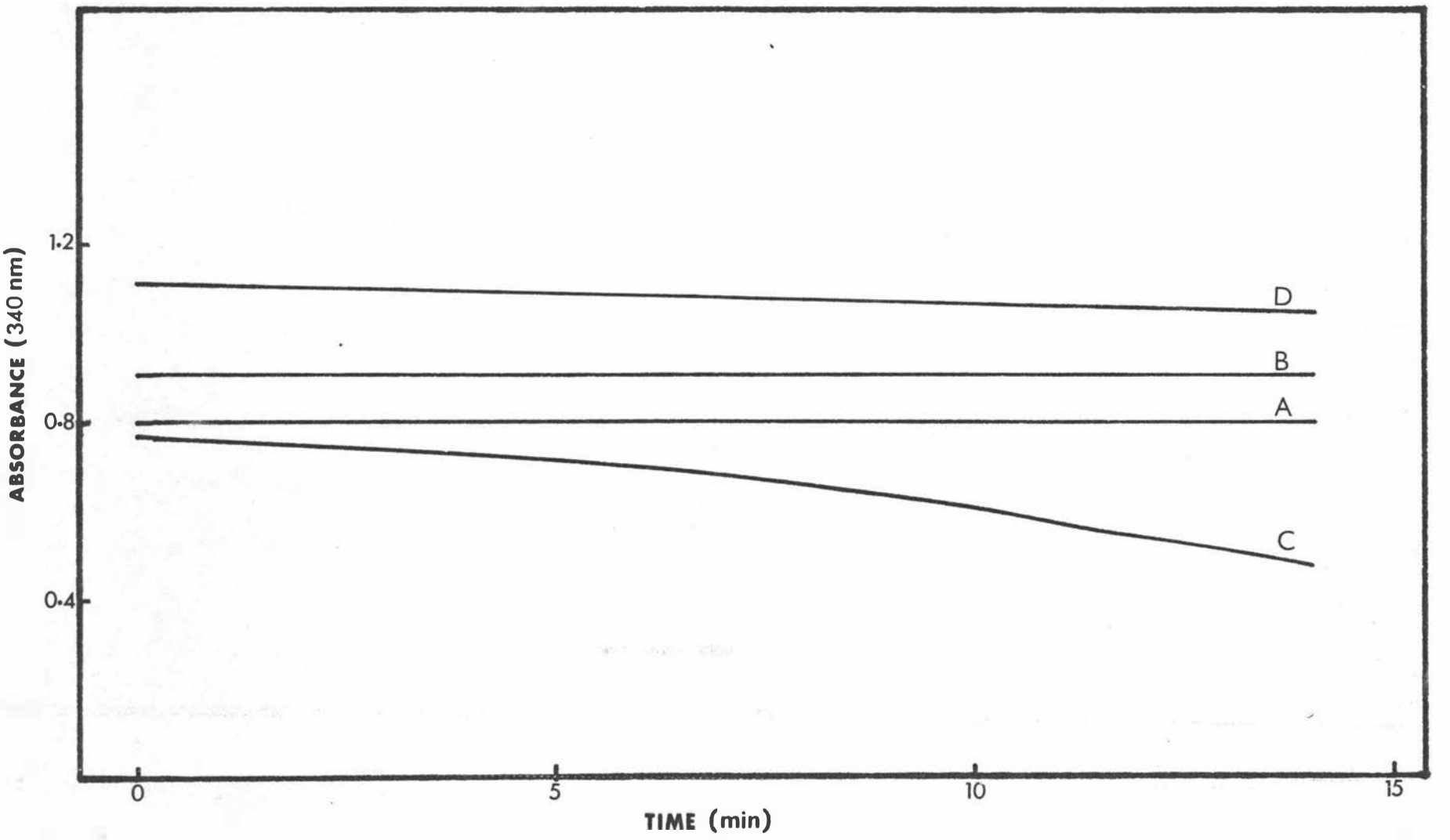
The reaction mixtures in a final volume of 3.0 ml contained 100 μmol of phosphate buffer (pH 7.5), 0.40 μmol of NADH, 150 Sigma units of catalase (5 μg of protein), 81 Sigma units of lactate dehydrogenase (0.1 mg of protein), 115 μmol of DL-alanine, 20 μg of D-amino-acid oxidase apoenzyme (prepared as described in Section 2.1) and the sample to be tested. The incubation was carried out at 25°C and the reaction was started by the addition of alanine.

A, Control (0.1 M-phosphate buffer, pH 7.5);

B, FMN (0.03×10^{-3} μmol);

C, FAD (0.15×10^{-3} μmol); and

D, HUD (heated uracil dehydrogenase, 200 μg).


ABSORBANCE (340 nm)
1·2
0·8
0·4
0
5
10
15
TIME (min)
D
B
A
C

5.6.2 Fluorometric assay

The fluorometric assay used for the determination of FAD in uracil dehydrogenase depends on the increase in fluorescence when FAD is hydrolysed to FMN. The method is described in Section 2.3.6b. As shown in Table 5.4, an increase in fluorescence occurred when uracil dehydrogenase was incubated in 10% trichloroacetic acid, but no increase occurred when riboflavin was treated in the same way. From the value in Table 5.4, the amount of FAD in the 10% trichloroacetic acid supernatant from uracil dehydrogenase may be calculated using the formula in Section 2.3.6b. The value obtained was 0.46 µg of FAD/ml of the original purified enzyme solution. The enzyme concentration was 0.706 mg/ml and using 298000 for the molecular weight of uracil dehydrogenase (Section 5.1.2), an FAD content of 0.25 mol of FAD/mol of uracil dehydrogenase was calculated.

5.7 Metal content

Analysis of the metal content of purified uracil dehydrogenase was performed by the atomic absorption spectrophotometry as described in Section 2.3.7. The standard assays for determination of enzyme activity and protein concentration are described in Sections 2.3.5a and 2.3.3a, respectively.

Concentrated purified uracil dehydrogenase in 0.05 M-Tris-HCl, pH 8.85, (as obtained from Sephadex G-200 in the last step of purification, Section 4.2) had been stored in the frozen state for about six months and was used in the subsequent studies. After thawing, the enzyme had a specific activity of 7.70 units/mg corresponding to approx-

TABLE 5.4

FLUOROMETRIC DETERMINATION OF FAD

Uracil dehydrogenase was a purified preparation (specific activity 10.1 units/mg) of not less than 95% purity on polyacrylamide gel electrophoresis (Section 4.2). The protein concentration was an average of results from the three methods described in Section 2.3.3). To a 0.3 ml sample of enzyme was added 1.2 ml of cold 12.5% trichloroacetic acid. After 15 min at 4°C, the samples were centrifuged. From the supernatant, one aliquot of 0.5 ml was immediately neutralised with 2.5 ml of 0.2 M-K_2HPO_4 and then left for 48 h in the dark at 0-4°C. A second aliquot of 0.5 ml was incubated for 48 h in the dark at 25°C and then neutralised as above. The fluorescence of each sample was measured (Section 2.3.2) both before (F_1) and after (F_2) the addition of 25 µl of a freshly prepared solution of 10% sodium dithionite in 5% sodium bicarbonate. At the same time 0.3 ml aliquots of riboflavin standard (0.5 µg/ml) were treated in the same way through the entire procedure.

Sample	Fluorescence (arbitrary units)			
	Non-hydrolysed Control		Hydrolysed Sample	
	F_1 Before reduction	F_2 After reduction	F_1 Before reduction	F_2 After reduction
Uracil dehydrogenase (0.706 mg protein/ml)	19.5	0.1	78.5	2.5
Riboflavin (0.5 µg/ml, in 0.01 M-HCl)	75.3	0.1	74.5	1.5

imately 74% of the original activity of the freshly prepared enzyme.

Preliminary analysis with a preparation of purified uracil dehydrogenase (5 mg of protein/ml), exhaustively dialysed (at 0-4°C, 95 h) against deionized distilled water, showed that Fe, Cu, Zn and Mg were all present (at > 0.5 atoms/mole of enzyme) but the levels of Ni, Co, Mn and Mo did not differ significantly from the blanks (< 0.2 atoms/mole of enzyme). Dialysis against deionized distilled water caused precipitation of the enzyme and complete loss of activity in the standard enzyme assay. Replicate metal analyses showed only poor agreement with this turbid suspension, possibly due to the difficulty of accurately pipetting 2 µl of samples of a non-homogeneous suspension. Therefore, in subsequent analyses, the enzyme was dialysed against Tris-HCl, pH 8.85, in which uracil dehydrogenase remained soluble.

Estimation of Fe, Zn, Mg and Cu in the purified uracil dehydrogenase was performed. Samples were prepared in the following ways and each sample was assayed for enzyme activity (standard enzyme assay, Section 2.3.5a) to determine whether any of the dialysis procedures affected the activity. Protein was determined by the method of Lowry et al. (Section 2.3.3a).

(1) Concentrated purified enzyme (0.30 ml, 2.25 mg of protein/ml, specific activity 7.70 units/mg) was diluted with approximately three volumes of 0.025 M-Tris-HCl, pH 8.85. The final preparation was assayed for the enzyme activity and stored (0-4°C, 110 h) during the preparation of

enzyme in subsequent preparations. The enzyme after storage showed full enzymic activity i.e. 100% of the original activity.

(2) Enzyme diluted as in (1) (a final volume of approximately 1.2 ml) was dialysed (0-4°C, 110 h) against 0.025 M-Tris-HCl, pH 8.85 (8x1 litre). The enzyme after dialysis again retained full enzymic activity (100% original activity).

(3) Concentrated enzyme (0.3 ml) as in (1) was diluted with approximately three volumes of 0.025 M-Tris-HCl, pH 8.85, containing the chelating agents, 2 mM-EDTA and 1 mM-1, 10-phenanthroline and assayed for the enzyme activity. The enzymic activity of this preparation was not affected by the presence of chelating agents under the conditions used. It was then dialysed (0-4°C) against 0.025 M-Tris-HCl, pH 8.85 containing 2 mM-EDTA and 1 mM-1,10-phenanthroline (5x1 litre) and then dialysed (0-4°C) against 0.025 M-Tris-HCl, pH 8.85 (3x1 litre). The latter dialysis with buffer was to remove the chelating agents. The total dialysis time was 110 h. The enzyme preparation after dialysis contained 102% original activity.

Samples prepared as by the three procedures were then used for atomic absorption spectrophotometric analyses. The results are shown in Table 5.5. The amounts are expressed as atoms of the respective metal per mole of uracil dehydrogenase (based on mol. wt. 298000 of uracil dehydrogenase obtained from ultracentrifugation, Section 5.1.2).

It was clear that dialysis against buffer (0.025 M-Tris-HCl, pH 8.85) resulted in a partial loss of Zn. Dialysis against buffer containing chelating agents (0.025 M-Tris-

HCl, pH 8.85 containing 2 mM-EDTA and 1 mM-1,10-phenanthroline) however, removed most of the Cu and Zn and about a half of the Mg, without affecting the Fe content. Enzyme activity was not affected by either dialysis procedure.

These results indicate that the purified enzyme contained, per mole of protein, 4 atoms of tightly bound Fe, 2 atoms of loosely bound Zn, 1 atom of rather loosely bound Mg and a fractional amount of loosely bound Cu.

The results also show that the Zn, Mg and Cu are not required for enzyme activity. They do not show, however, whether Fe is required for activity, since Fe could not be removed by the dialysis procedures used.

5.8 Labile sulphide content

Labile sulphide was determined (Section 2.3.9) using Na_2S as the standard sulphide. Bovine serum albumin (which contains cysteine but no labile sulphide) gave no colour reaction under the assay conditions. From a calibration curve, an amount of uracil dehydrogenase (specific activity 10.2 units/mg) containing 0.342 mg of protein gave an absorbance equivalent to 4.75nmol of sulphide. This was calculated to be 4.1 moles of sulphide per mole of uracil dehydrogenase of mol. wt. 298000 (Section 5.1.2).

TABLE 5.5

ESTIMATION OF THE IRON, ZINC, MAGNESIUM AND COPPER CONTENT IN THE PURIFIED URACIL DEHYDROGENASE

Atomic absorption spectrophotometric analyses for metallic content of purified enzyme preparations (specific activity 7.70 units/mg): (1), purified enzyme (0.506 mg of protein/ml); (2), enzyme (0.553 mg of protein/ml) dialysed against 0.025 M-Tris-HCl, pH 8.85; and (3), enzyme (0.525mg of protein/ml) dialysed against 0.025 M-Tris-HCl, pH 8.85 containing 2 mM-EDTA and 1 mM-1,10-phenanthroline and then dialysed against 0.025 M-Tris-HCl, pH 8.85. The details of the preparation of enzyme samples are described in the text. Results were based on a molecular weight for the native uracil dehydrogenase of 298000 (Section 5.1.2).

Metallic ions	Metal content (atoms/mole of enzyme)[a]		
	(1)	(2)	(3)
Fe	4.09 ± 0.2	4.12 ± 0.2	4.08 ± 0.2
Zn	2.89 ± 0.1	2.06 ± 0.1	0.31 ± 0.1
Mg	0.90 ± 0.1	0.93 ± 0.1	0.55 ± 0.1
Cu	0.44 ± 0.03	0.43 ± 0.03	0.11 ± 0.03

[a] The metal content of each sample was corrected by subtracting the amount present in the appropriate blank. Each value is expressed as the mean of three assays ± the standard deviation.

CHAPTER 6

CHARACTERIZATION OF URACIL DEHYDROGENASE (CATALYTIC ACTIVITY)

6.1 Validity of the enzyme assay

The assay of uracil dehydrogenase activity was based on the change in absorbance at the optimum wavelength for the particular pyrimidine used (255 nm for uracil, 270 nm for thymine). This assay is valid, only if the change in absorbance is due solely to oxidation of the pyrimidine. Since methylene blue shows significant absorption in the region 250-270 nm (see Figure 6.1), it was necessary either to show that reduction of methylene blue did not contribute appreciably to the observed changes in absorbance, or to correct for changes in absorbance with methylene blue present.

(i) Aerobic assay

Under aerobic conditions reduced methylene blue is rapidly re-oxidised by molecular oxygen (Reid, 1930; Macrae, 1931; Jones, 1969). Accordingly, during the oxidation of thymine or uracil by uracil dehydrogenase, a steady state concentration where the rate of reduction of methylene blue and the rate of re-oxidation of the reduced product (leuco-methylene blue) are equal may be expected. The situation was examined by following the absorbance of a standard uracil dehydrogenase assay reaction mixture at 666 nm (the absorption maximum for methylene blue in 0.05 M-Tris-HCl, pH 8.85, Figure 6.1) while the rate of thymine oxidation was determined at 270 nm in an identical reaction system.

Figure 6.2 shows that for two different rates of thymine oxidation by uracil dehydrogenase, a steady state methylene

blue oxidation-reduction level (measured at 660 nm) was reached within 1 min. In most enzyme assays, the change in absorbance at 270 nm was linear for a period of several minutes (see Section 6.2) and it was not necessary to follow the absorbance within the first minute. During the period of linear enzyme reaction, the concentrations of oxidised and reduced forms of methylene blue did not change, and the observed changes in absorbance at 270 nm were considered to be due solely to the conversion of thymine to 5-methylbarbituric acid.

At the higher enzyme concentration used (Figure 6.2, B), the uracil dehydrogenase level was 0.011 units/ml and the steady state level of methylene blue was 3% reduced (reduced methylene blue concentration 0.75×10^{-6}M). In Figure 6.2, A, the uracil dehydrogenase level was 0.003 units/ml and the steady state level of methylene blue was 2% reduced (reduced methylene blue concentration 0.5×10^{-6}M). The results showed that the steady state levels of methylene blue and reduced methylene blue depended on the rate of enzyme reaction. In most of the experiments reported in this chapter (Chapter 6), the level of enzyme was not greater than 0.003 units/ml; hence the concentration of methylene blue would differ, in the steady state, by not more than 2% from the concentration added (2.5×10^{-5}M).

(ii) Anaerobic assay

Under anaerobic conditions, methylene blue was reduced to leuco-methylene blue during thymine oxidation. The absorbance changes at 270 nm due to methylene blue and its reduced form were examined. Since reduced methylene blue could not

be obtained pure for spectrophotometry, its absorption spectrum was obtained indirectly by difference spectrum in the presence of pyrimidines by the following procedure. Methylene blue was completely reduced by thymine with uracil dehydrogenase in the absence of air under the experimental conditions described for Figure 6.3. Assuming a stoichiometric relationship between thymine oxidised and methylene blue reduced, and since the initial concentration of thymine and methylene blue are known, the amounts of thymine and 5-methylbarbituric acid in the resulting reaction mixture (after complete reduction of the methylene blue) could be determined from the known amount of methylene blue reduced. The combined spectrum related to the amounts of thymine and 5-methylbarbituric acid in the mixture could be deduced from their standard spectra (Section 2.3.4, Figure 2.1). The difference between this calculated spectrum and the observed spectrum in the reaction mixture provided a difference spectrum due to reduced methylene blue. This is shown in Figure 6.3. The spectrum of the equivalent amount of methylene blue is also shown in Figure 6.3. From this figure it can be seen that no change in absorbance at 270 nm occurred when methylene blue was reduced. Accordingly, the observed absorbance change at 270 nm was assumed to be due solely to the conversion of thymine to 5-methylbarbituric acid.

Methylene blue reduction would interfere with the measurement of uracil oxidation in the absence of oxygen, since uracil oxidation is followed at 255 nm. Anaerobic studies were therefore confined to assays with thymine as the substrate.

Figure 6.1 Absorption spectrum of methylene blue

The spectrum of methylene blue (2.5×10^{-5} M) was measured in 0.05 M-Tris-HCl, pH 8.85.

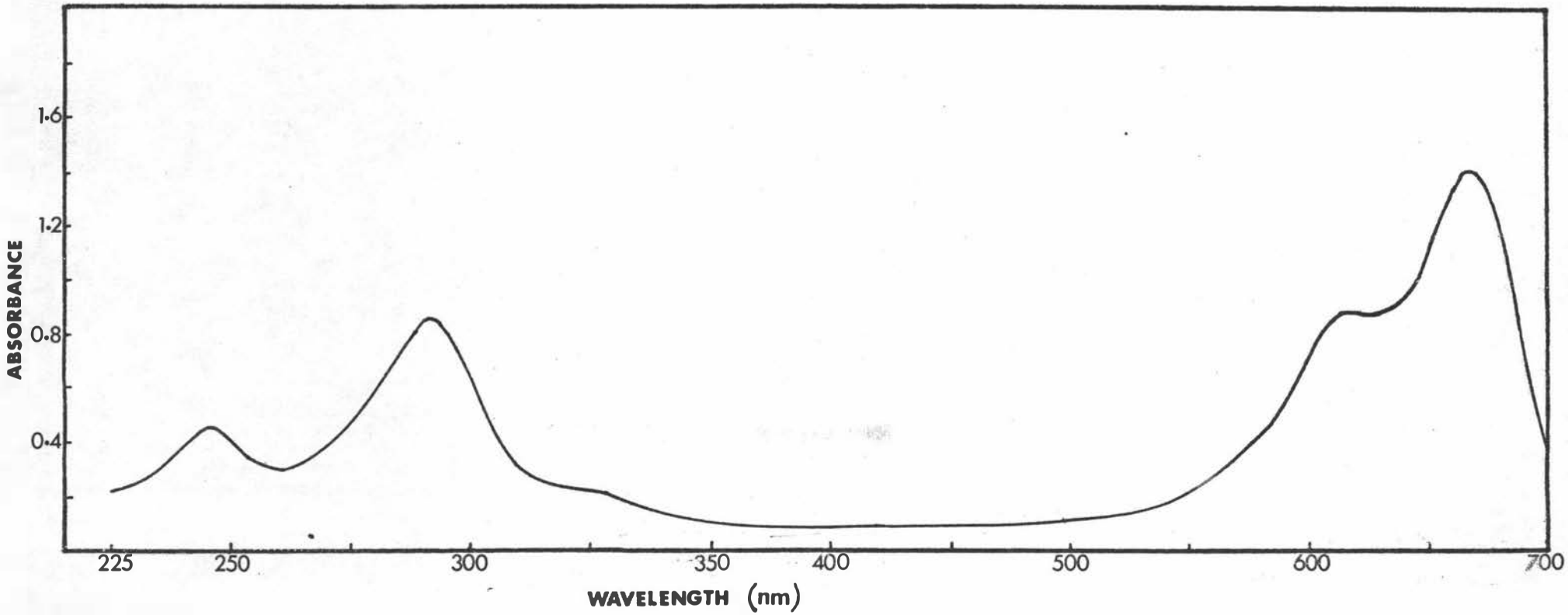
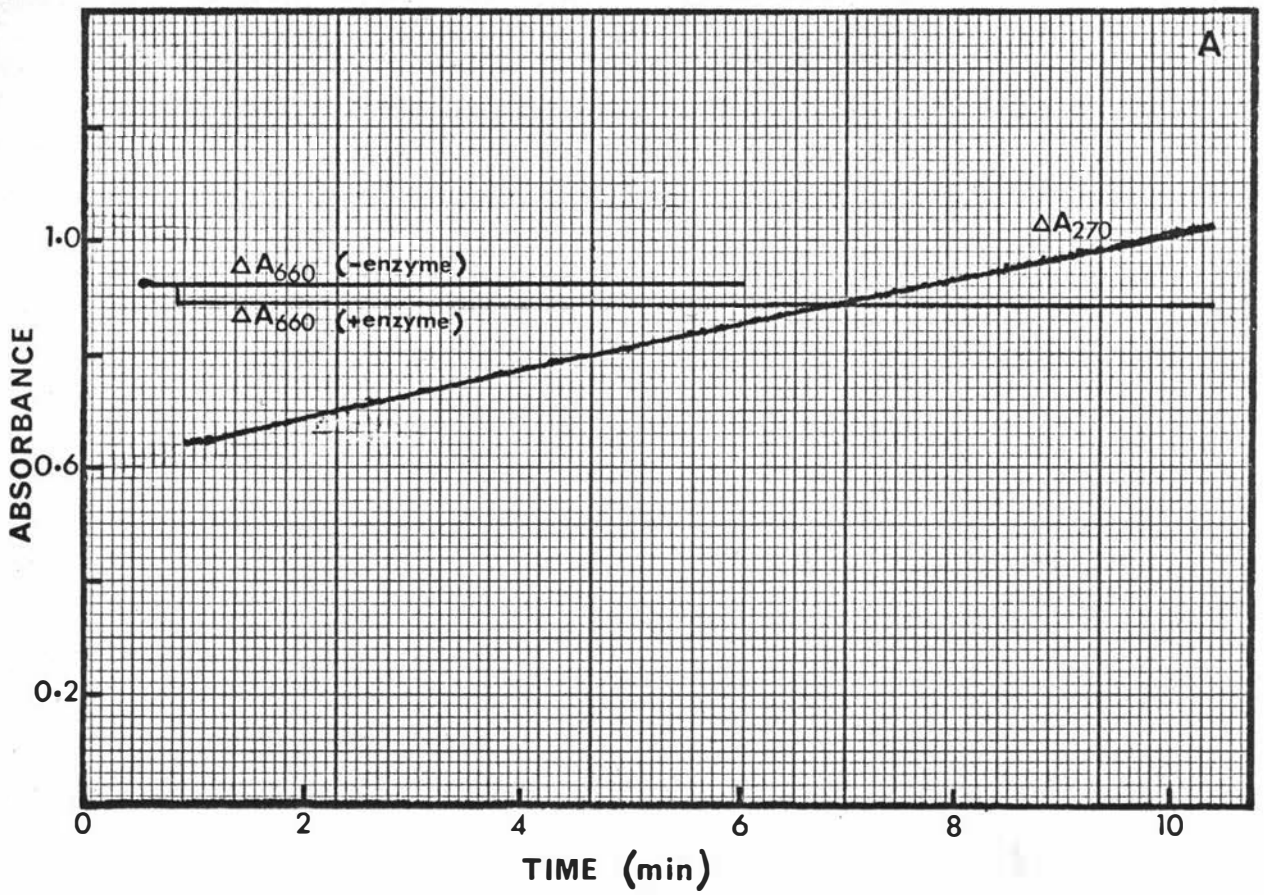

ABSORBANCE
1·6
1·2
0·8
0·4
225
250
300
350
400
500
600
700
WAVELENGTH (nm)

Figure 6.2 Absorbance changes during the oxidation of thymine by uracil dehydrogenase under aerobic conditions in the presence of methylene blue

The standard enzyme assay conditions (Section 2.3.5a) were used. The reaction was started by the addition of the enzyme. The change in absorbance at 270 nm (ΔA_{270}) was recorded. In an identical reaction mixture the absorbance change at 660 nm (ΔA_{660}) was recorded, before and after the addition of enzyme.

A. The amount of enzyme used in the assay mixture was 0.009 units (0.88 µg of protein; specific activity 10.2 units/mg).

B. The amount of enzyme in the assay mixture used was 0.033 unit (3.24 µg of protein; specific activity 10.2 units/mg).


A
ΔA270
ΔA660 (-enzyme)
ΔA660 (+enzyme)
ABSORBANCE
1.0
0.6
0.2
0
2
4
6
8
10
TIME (min)

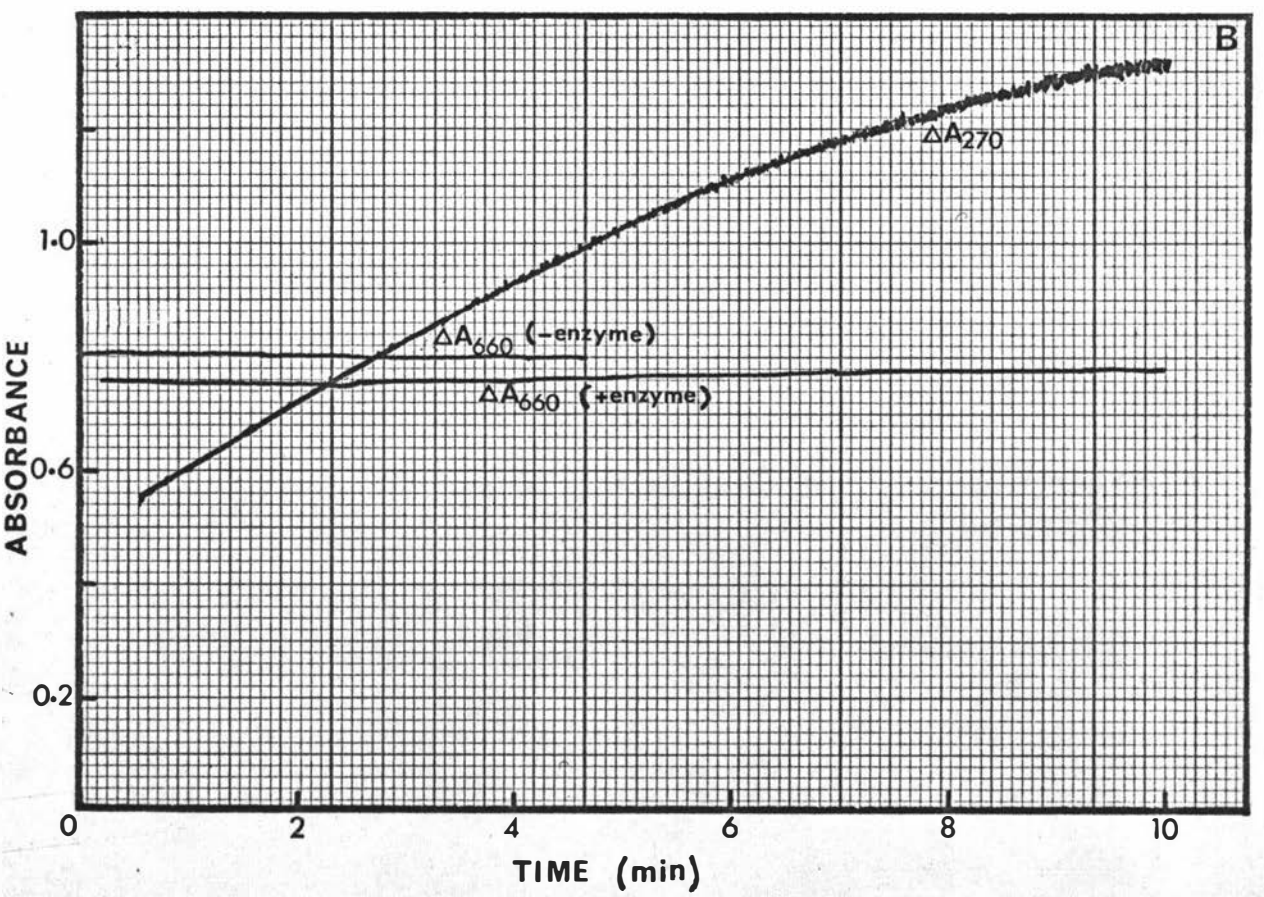

B
ΔA270
ΔA660 (-enzyme)
ΔA660 (+enzyme)
ABSORBANCE
1.0
0.6
0.2
0
2
4
6
8
10
TIME (min)

Figure 6.3 Spectral analysis to determine the absorption spectrum of reduced methylene blue

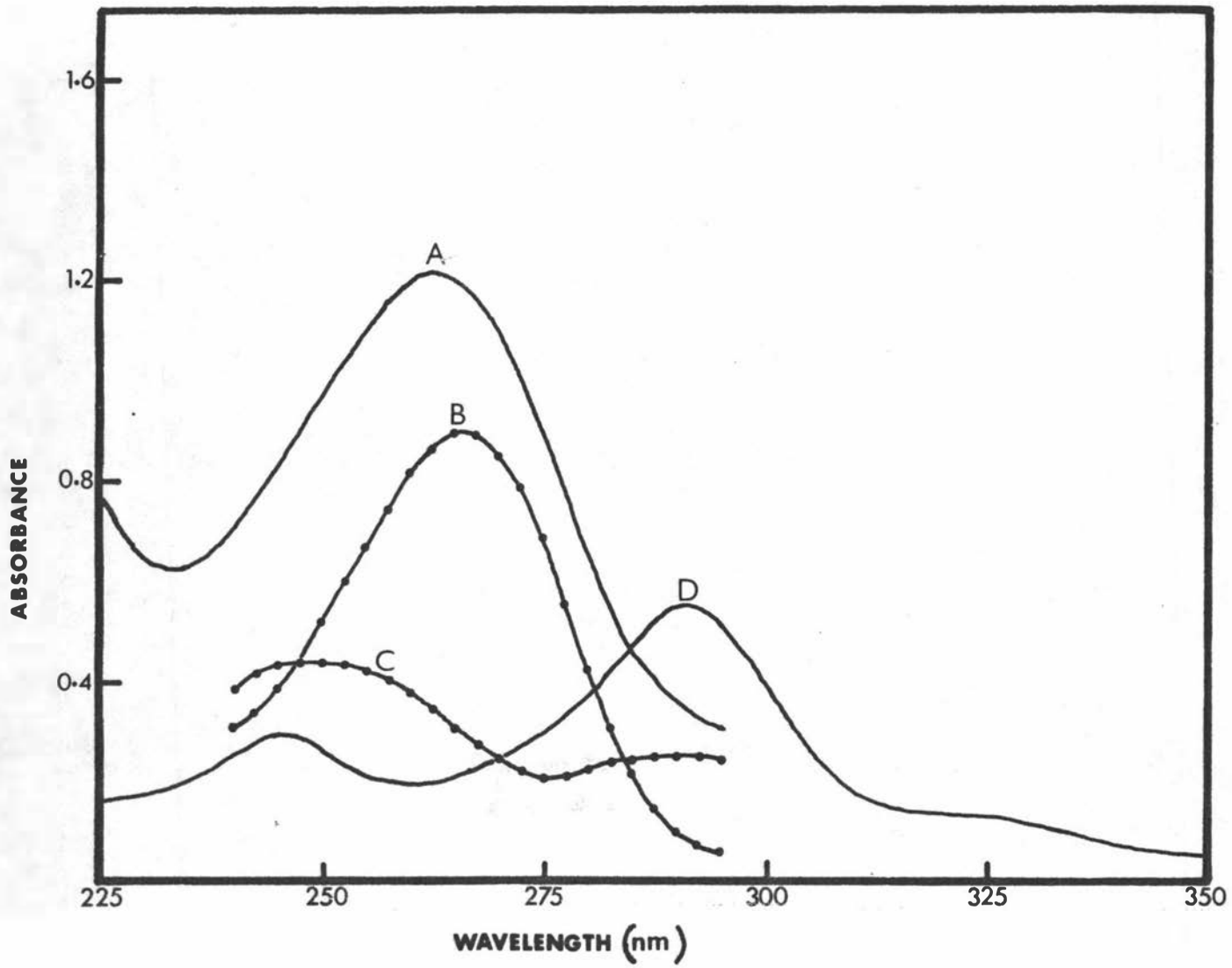
The reaction mixture (3.0 ml) in a Thunberg cuvette contained 0.3 µmol of thymine, 0.055 µmol of methylene blue, and 150 µmol of Tris-HCl, pH 8.85. Uracil dehydrogenase (5 µl, containing 4.94 µg of protein, specific activity 10.4 units/mg) was placed in the hollow stopper. The cuvette was alternately flushed with nitrogen and evacuated (Section 2.3.5a) a total of four times. After the initial spectrum in the region 650 nm to 700 nm was recorded, enzyme was added by tipping the cuvette and the rate of thymine oxidation was followed at 270 nm. The reaction was completed after 16 min at 25°C, as judged by the absorbance at 650-700 nm, and the absorption spectrum in the region 225 nm to 300 nm was recorded (curve A). Thus, 0.055 µmol of methylene blue had been reduced and 0.055 µmol of thymine was oxidised to 5-methylbarbituric acid (assuming a stoichiometric reaction between thymine and methylene blue).

Curve A shows the spectrum of the reaction mixture containing thymine, 5-methylbarbituric acid and reduced methylene blue (at 16 min).

Curve B shows the calculated spectrum of a solution containing thymine and 5-methylbarbituric acid at the concentrations calculated to be present in A.

Curve C shows the difference spectrum, A-B, which is due to reduced methylene blue.

Curve D shows the spectrum of a solution containing methylene blue at the concentration used in this experiment.


1·6
1·2
0·8
0·4
ABSORBANCE
A
B
C
D
225
250
275
300
325
350
WAVELENGTH (nm)

6.2 Linearity of the enzyme assays

In studies with the partially purified enzyme using standard assay conditions, the reaction rate was proportional to the amount of enzyme over the range of enzyme concentrations used (Figure 6.4). The assay of enzyme activity was also linear with time for at least 15 min, or until approximately 14% of the thymine had been converted to 5-methylbarbituric acid (Figure 6.5) with 0.001 units/ml in the assay mixture. Thus, over a range of enzyme concentrations from 0.005 down to 0.0005 units/ml, the reaction was linear for from 3 to 30 min.

6.3 Effects of pH and ionic strength on enzyme activity

(i) Optimum pH

Figure 6.6 shows that optimum pH at 25°C for the purified enzyme with thymine as a substrate was at pH 8.85. The pH-activity curve is a symmetrical bell-shaped curve with high activity (over 95% of maximum activity) in the range of pH 8.6 to 9.0 and a sharp maximum activity at pH 8.85. In the region of pH overlap between the two buffers, Tris-HCl and Glycine-NaOH, identical activities were observed with the two systems, suggesting that neither buffer affected the activity of the enzyme at the concentration used. Higher concentrations of Tris-HCl, pH 8.85 (up to 0.10 M or ionic strength of 0.085) were not inhibitory (Figure 6.7).

(ii) Ionic strength

The ionic strength of the standard enzyme assay reaction mixture of 0.043 was optimal for activity (Figure 6.7). Within the range of ionic strength 0.02 to 0.09 at pH 8.85,

Figure 6.4 Proportionality between enzyme concentration and the rate of enzyme reaction

The enzyme was assayed under standard assay conditions (Section 2.3.5a). The enzyme used was the partially purified enzyme after treatment with calcium phosphate gel (Section 4.2, step 2) which had a specific activity of 0.21 units/mg.

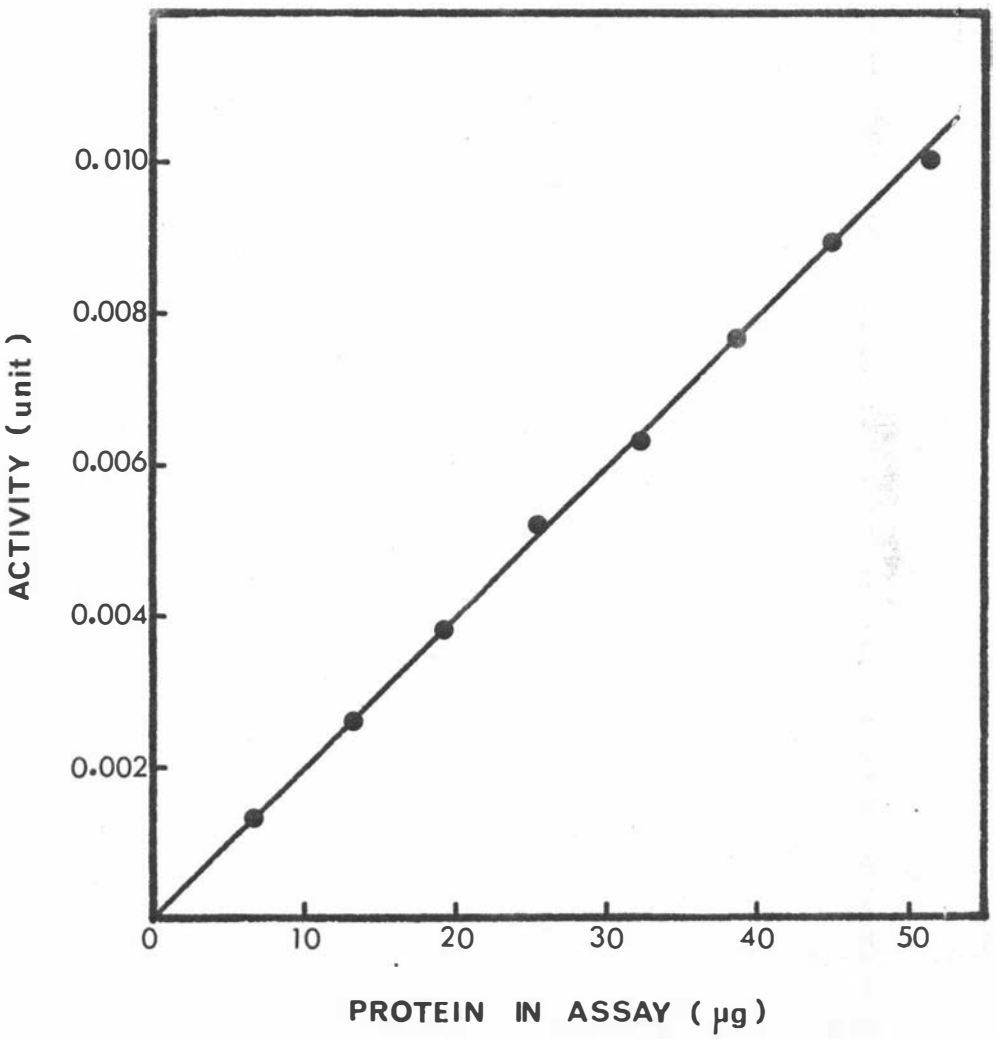

0.010
0.008
0.006
0.004
0.002
ACTIVITY (unit)
0
10
20
30
40
50
PROTEIN IN ASSAY (μg)

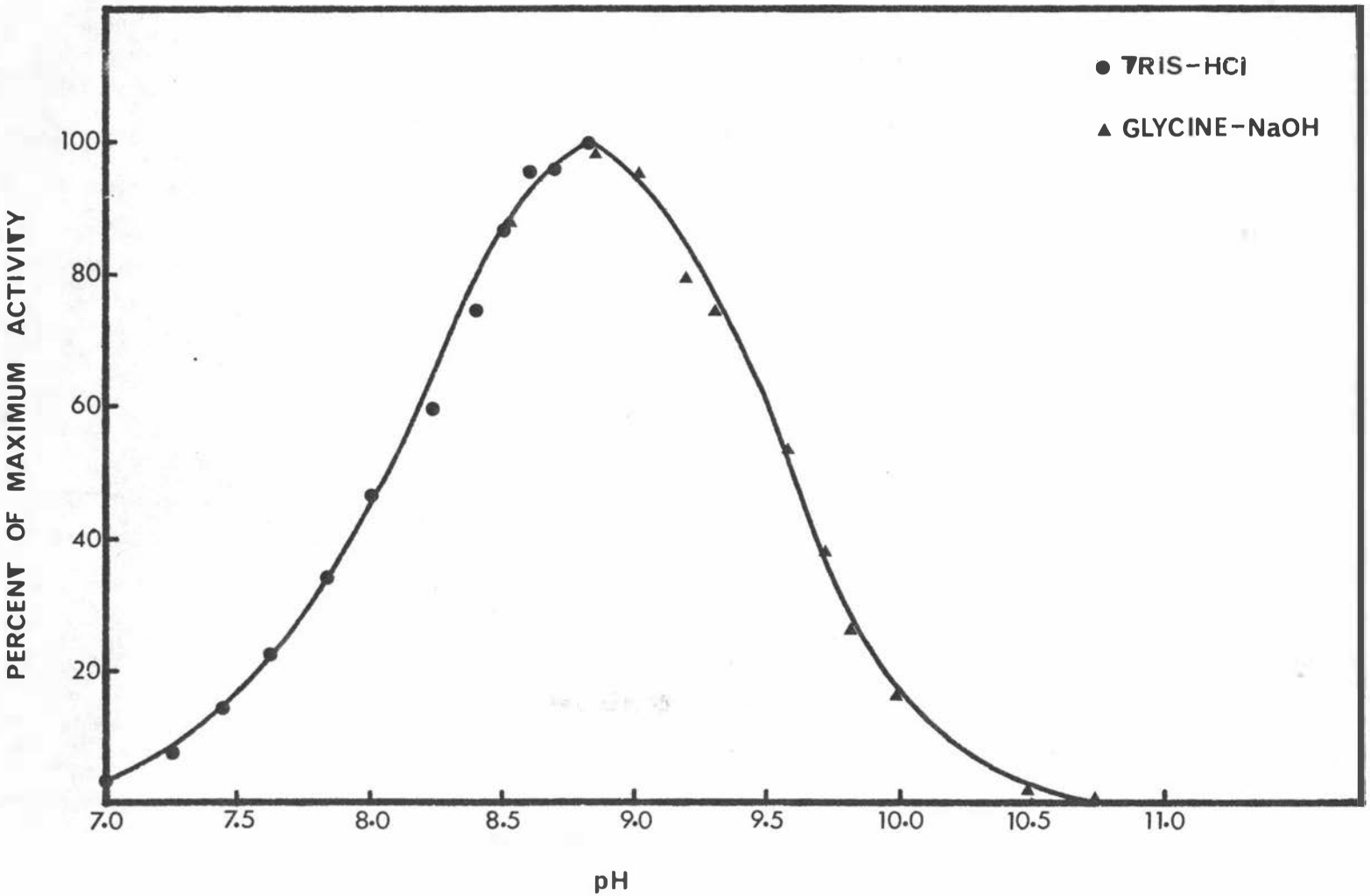
Figure 6.5 Linearity of the enzymic reaction with time

The enzyme sample was the same as that used in the experiment which gave the results shown in Figure 6.4. The amount of the enzyme in the assay was 14.65 µg of protein (specific activity 0.21 units/mg). The enzyme was assayed under standard assay conditions (Section 2.3.5a) (0.3 µmol of thymine initially present).

CONVERSION OF THYMINE (%)
50
40
30
20
10
THYMINE CONVERTED (μmol)
0.15
0.10
0.05
0.025
0
10
20
30
40
50
60
TIME (min)

Figure 6.6 Effect of pH on the rate of the enzymic reaction

Standard enzyme assay conditions (Section 2.3.5a) were used except that 0.05M of the indicated buffers and pH (25°C) (prepared as described by Dawson, 1969) were substituted for the standard 0.05 M-Tris-HCl, pH 8.85. Purified enzyme (1.1 µg of protein in 3 ml total, specific activity 8.7 units/mg in the standard assay) was used. The values plotted are means of duplicates whose results did not differ by more than ± 5%. The results are expressed as a percentage of the activity observed in the standard assay.


● TRIS-HCl
▲ GLYCINE-NaOH
PERCENT OF MAXIMUM ACTIVITY
100
80
60
40
20
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
11.0
pH

the activity was little affected (95-100% maximum activity). Outside this range, the activity was somewhat lower at ionic strength down to 0.01 and a steady decrease occurred above 0.09 ionic strength. Activity was reduced to about 50% of the maximum at an ionic strength 0.45.

6.4 Stability of the enzyme

(i) Storage

The stability during storage of the crude enzyme from cell-free extracts was studied at 0-4°C and pH 8.85 as described in the legend to Figure 6.8. The crude enzyme could be kept under these conditions for 10 h with less than 10% loss of activity. The enzyme had lost half of its activity after 30 h. However, the stability of the enzyme in cell-free extracts was highly variable. The concentrated purified enzyme (5 mg of protein/ml, specific activity 10.4 units/mg) could be stored in the frozen state at -20°C in 0.05 M-Tris-HCl, pH 8.85, for over 6 months with less than 25% loss of activity. A dilute solution (0.1 mg of protein/ml) of thawed purified enzyme which had previously been stored at -20°C for 6 months lost a further 30% of its activity after 3 weeks at 0-4°C, pH 8.85.

(ii) pH

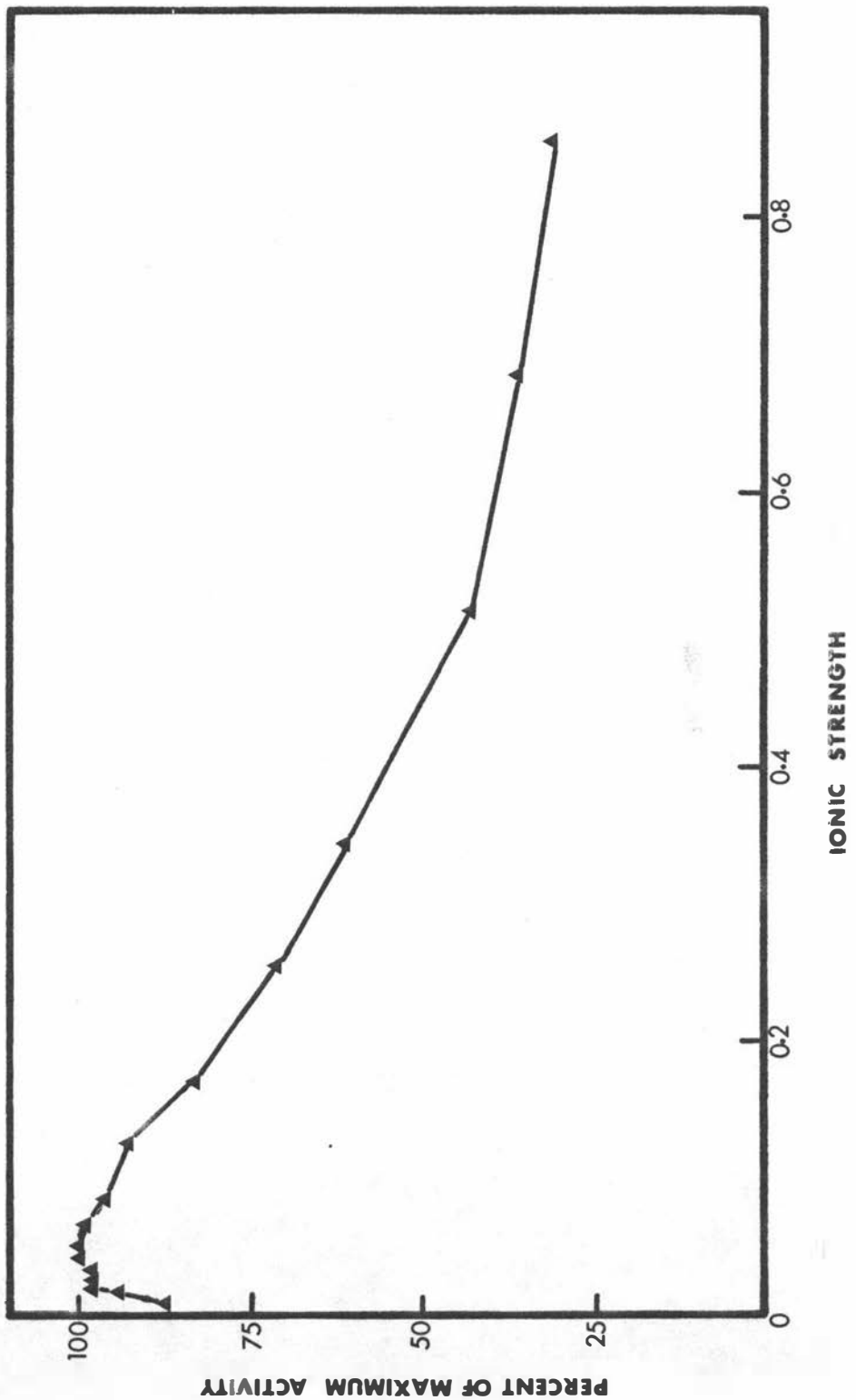
The stability of the purified enzyme in the pH range 7.5 to 10.0 was examined using two buffer systems, Tris-HCl and Glycine-NaOH at 0-4°C. Results and experimental details are shown in Figure 6.9. Decreasing the pH decreases the stability of the enzyme and the enzyme is more stable at pH values above 8.5 with a slow loss of activity. The remaining activity after storage at pH 10.0 for 70 h was 96% of the

Figure 6.7 Effect of ionic strength on the rate of enzymic reaction

The assay conditions were those of the standard enzyme assay (Section 2.3.5a), except that the ionic strength of the assay mixture was varied as follows: Tris-HCl, prepared by diluting 1 M-Tris-HCl, pH 8.85 (25°C) solution (approx. 1.5 M-Tris was adjusted to pH 8.85 at 25°C with HCl solution and diluted to 1.0 M) to the appropriate final ionic strength.

The enzyme sample was the same as that used in the experiment which gave the results shown in Figure 6.7 (1.1 µg of protein in 3 ml assay).

Plotted points are the means of duplicates, which did not differ by more than about 5%, and are expressed as a percentage of the activity observed in the standard assay.


PERCENT OF MAXIMUM ACTIVITY
100
75
50
25
0
0.2
0.4
0.6
0.8
IONIC STRENGTH

original activity and 21% at pH 7.5.

(iii) Temperature

The temperature stability and heat inactivation of the purified enzyme at pH 8.85 was studied at the various temperatures shown in Figures 6.10 and 6.11. At 40°C, the enzyme lost only 5% of its original activity in 90 min. At 60°C, 50% loss occurred in about 10 min, while at 70°C, over 90% inactivation occurred in this time.

6.5 Pyrimidine specificity

The specificity of uracil dehydrogenase for different pyrimidines was studied by two methods.

(i) Spectrophotometry

Any substrate of uracil dehydrogenase which absorbs in the ultraviolet spectral region should show a change in its absorption spectrum during an enzymic reaction. To test potential substrates by this method, standard enzyme assay conditions were used except that thymine was replaced by the test compound (0.10 mM final concentration except where indicated). The amount of enzyme used in each assay was 1.7 µg of purified uracil dehydrogenase (specific activity 8.88 units/mg). The reaction mixture was scanned (between 225 and 450 nm) before and after the incubation period of 30 min. The reference cuvette contained the same mixture but without the compound to be tested. No changes were observed in the absorption spectrum with any of the following compounds: cytosine, 6-aminouracil (0.05 mM), 2-thiouracil, 6-methyluracil, thymidine, thymidine-5-monophosphate, uridine and uridine-5-monophosphate. This suggests that none of these compounds were substrates for uracil

dehydrogenase.

Dihydrouracil and dihydrothymine showed no absorbance in the region 250-450 nm before or after incubation.

(ii) Measurement of oxygen uptake

The enzyme was tested, using the oxygen electrode, for the ability to catalyze the oxidation of various pyrimidines listed in Table 6.1. The oxygen electrode was calibrated using the method of Delieu & Walker (Section 2.3.5b). Among 24 compounds tested, only seven were associated with oxygen uptake in the assay system. For each of these seven compounds, changes in the ultraviolet absorption spectra during the reaction with uracil dehydrogenase (Figure 6.12) confirmed that they were substrates. The seven active substrates were, apart from uracil itself, all 5-substituted uracils. The nature of the substituent group (R) on the 5-position of the pyrimidine determined the rate of reaction and the following order of reactivity was established for the compounds.

Nature of R group: CH_3 > Br > I > H > F > OH > CH_2OH

With R = NO_2 or COOH, no activity was detected.

6.6 Electron acceptor specificity

The activity of uracil dehydrogenase with various electron acceptors was investigated by following the change in absorbance under anaerobic conditions unless otherwise indicated (Table 6.2). When thymine was omitted from the incubation mixture no absorbance change was observed with any

Figure 6.8 Effect of storage on the stability of uracil dehydrogenase in cell-free extracts

Cell-free extracts (2.8 mg/ml in 0.05 M-Tris-HCl, pH 8.85; specific activity 0.12 units/mg) prepared with a French pressure apparatus (Section 3.2) were held at 0-4°C. At 10 h intervals up to 70 h, aliquots were taken and assayed for the enzymic activity using the standard enzyme assay (Section 2.3.5a).

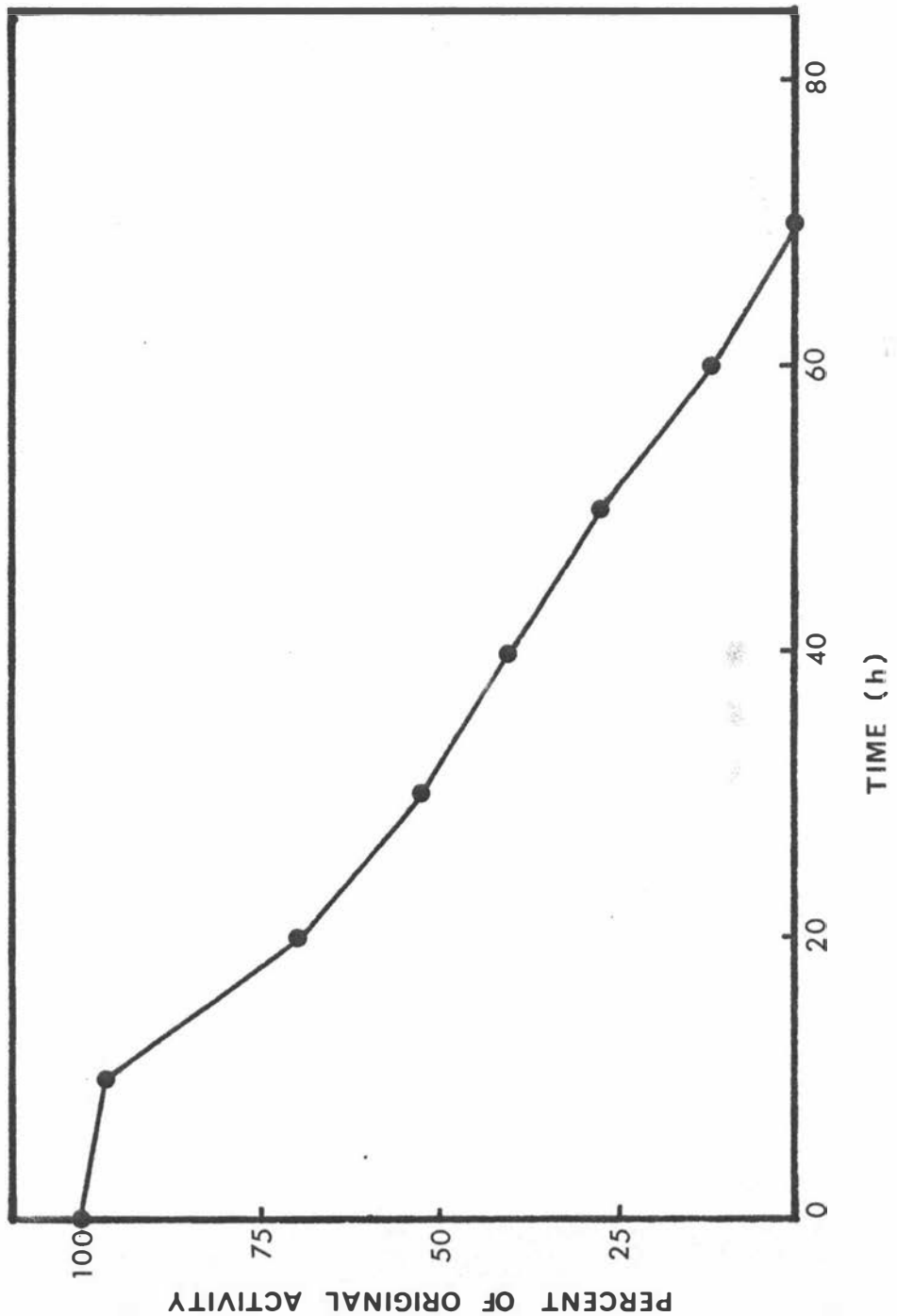

PERCENT OF ORIGINAL ACTIVITY
100
75
50
25
0
20
40
60
80
TIME (h)

Figure 6.9 Effect of pH on the stability of the purified enzyme

The purified enzyme (final concentration 0.01 mg/ml, specific activity 10.2 units/mg) was incubated at 0-4°C at the appropriate pH using the buffer systems: 0.05 M-Tris-HCl buffer, pH 7.5-8.5 and 0.05 M-Glycine-NaOH buffer, pH 9.0-10.0 (prepared as described by Dawson, 1969). At the times shown, aliquots (100 µl) of the enzyme were taken and assayed using the standard procedures (Section 2.3.5a).

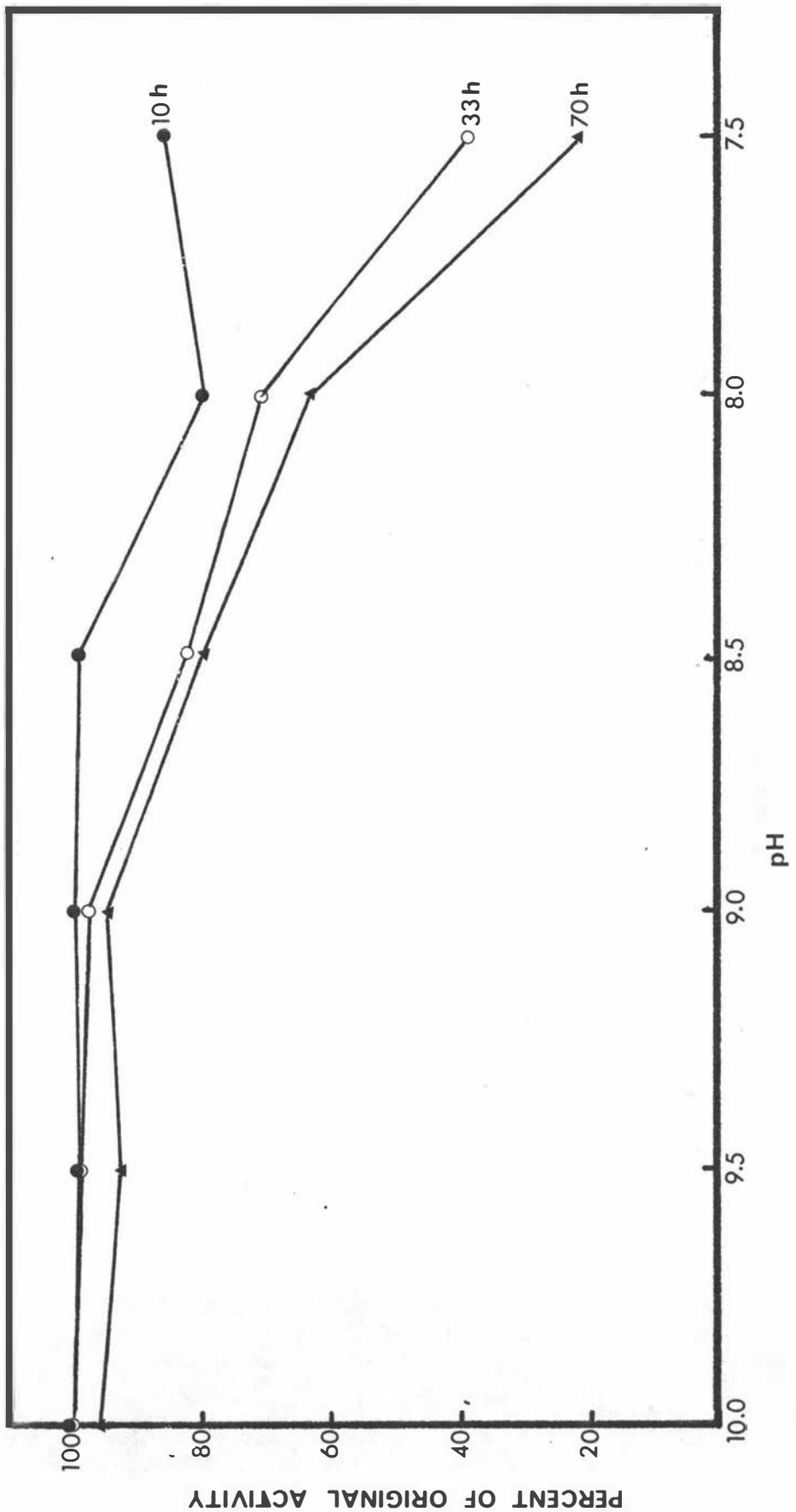

10 h
33 h
70 h
100
80
60
40
20
7.5
8.0
8.5
9.0
9.5
10.0
pH
PERCENT OF ORIGINAL ACTIVITY

Figure 6.10 Temperature stability of the purified enzyme

The purified enzyme (final concentration 0.011 mg/ml, specific activity 10.2 units/mg) was incubated at the indicated temperature for 10 min in 0.05 M-Tris-HCl, pH 8.85. After incubation, the enzyme was cooled to 0°C in an ice bath and aliquots (100 µl) were assayed immediately for enzymic activity. Standard assay conditions were used (Section 2.3.5a).

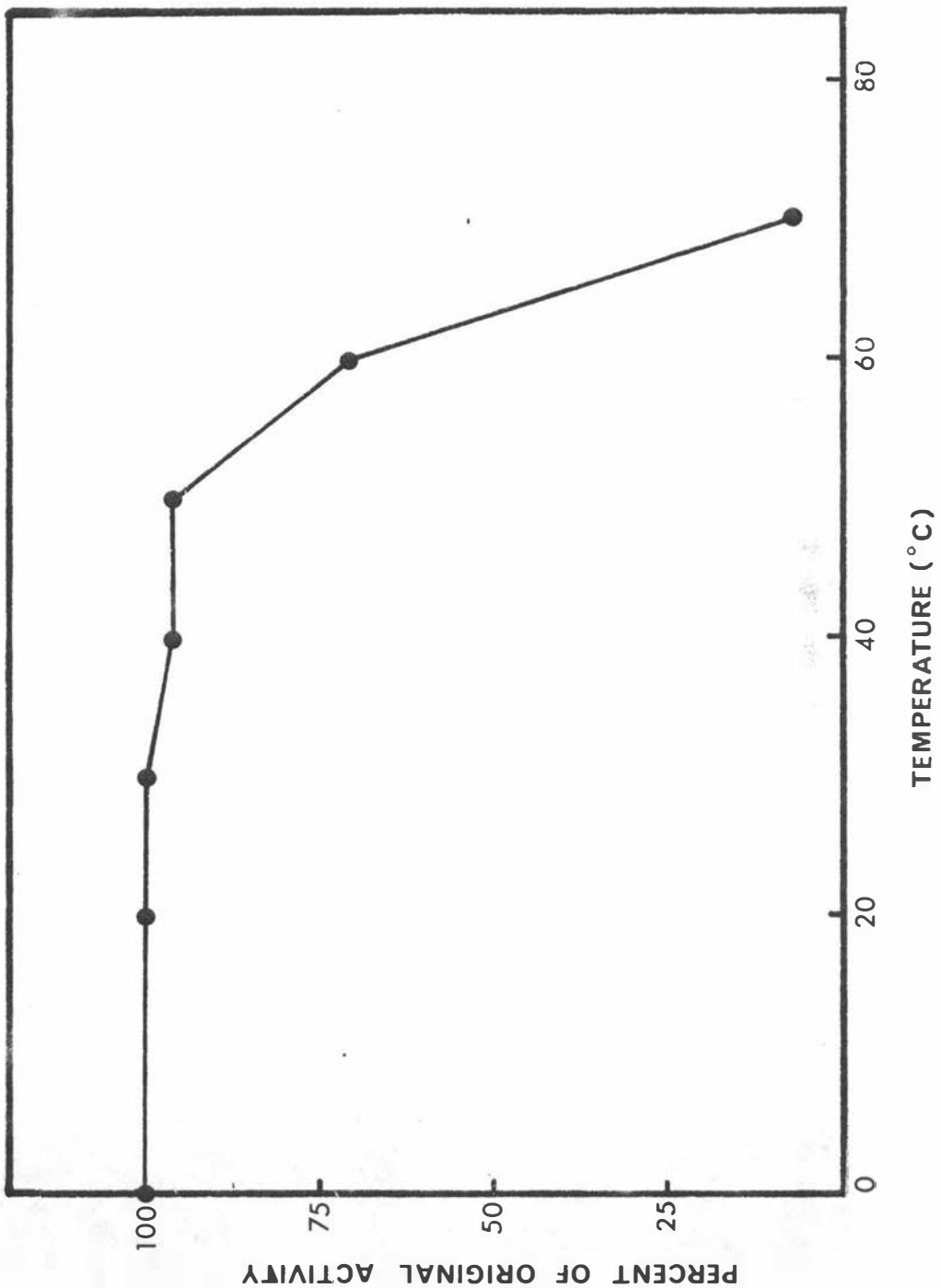

PERCENT OF ORIGINAL ACTIVITY
100
75
50
25
0
20
40
60
80
TEMPERATURE (°C)

Figure 6.11 Heat inactivation of the purified enzyme

The purified enzyme (0.012 mg/ml) was held in 0.05 M-Tris-HCl, pH 8.85, at the indicated temperature for up to 90 min. At the appropriate time intervals, aliquots (100 µl) of enzyme were taken, cooled to 0°C in an ice bath and immediately assayed for enzymic activity. Standard assay conditions were used (Section 2.3.5a).

40°c
50°c
60°c
100
75
50
25
0
20
40
60
80
100
TIME (min)
PERCENT OF ORIGINAL ACTIVITY

of the electron acceptors tested. In the presence of thymine, seven of the electron acceptors were reduced. Benzoquinone and phenazine methosulphate (PMS) were the most efficient acceptors. Dichlorophenol-indophenol, NAD^+, $NADP^+$ were not reduced (Table 6.2).

The reaction rate with Nitro Blue Tetrazolium or p-Iodonitrotetrazolium Violet was increased approximately two-fold (not shown in the table) by inclusion of PMS (0.15 μmol) in the assay mixture. The combination of PMS and tetrazolium salt was used for locating uracil dehydrogenase on polyacrylamide gels (Section 2.3.11a).

The oxidation of thymine by PMS in the presence of uracil dehydrogenase was also assayed by following the aerobic formation of 5-methylbarbituric acid at 270 nm, as in the standard assay with methylene blue (Section 2.3.5a). Over the range of PMS concentration 0.82×10^{-5} to 5.0×10^{-5} M the reaction rate was constant indicating that the enzyme was already operating at its maximum rate, V. This rate with PMS was twice the value of V obtained with methylene blue under identical assay condition (Section 6.8).

6.7 Specific activity and calculation of enzyme concentration

Freshly purified enzyme had a specific activity in the standard assay (Section 2.3.5a) from 10.0 to 10.4 units/mg. If it is assumed, basing on polyacrylamide gel electrophoresis of the native enzyme (Section 4.3) that the enzyme of 10.4 units/mg was 98% pure, then the pure native enzyme would have a specific activity of 10.6 units/mg. As

TABLE 6.1

SUBSTRATE SPECIFICITY OF URACIL DEHYDROGENASE

The assay mixture contained, in a total volume (before adding the compound to be tested) of 3.0 ml, the purified enzyme (10 µg of protein, specific activity 10.4 units/mg), 0.125 µmol of methylene blue, 150 µmol of Tris-HCl, pH 8.85, and 23 µg of catalase (675 Sigma units). The reaction was started by the addition of the compound to be tested (0.3 µmol in 0.03 ml of water). Incubation was at 25°C, in an oxygen electrode cell (as described in Section 2.3.5b). For the five most active substrates, the reaction was allowed to run to completion and then two further successive additions of test compound were made (each 0.3 µmol of substrate). The oxygen uptake is expressed as the average initial rate for the three successive assays. In the case of a non-substrate, no reaction occurred and further additions of 1.2 µmol and then 1.5 µmol of the test compound were made (giving a final concentration of 1.0 mM).

Compound tested	Oxygen uptake (nmol of O_2/min)
Thymine	94
Uracil	32
5-Bromouracil	50
5-Iodouracil	37
5-Fluorouracil	16
5-Hydroxyuracil (isobarbituric acid)	4.2
5-Hydroxymethyluracil	0.5
5-Nitrouracil	0
Isoorotic acid (uracil-5-carboxylic acid)	0
Cytosine	0
5-Methylcytosine	0
2-Thiouracil	0
Thymidine	0
Thymidine monophosphate	0
Uridine	0
Uridine monophosphate	0
Dihydrothymine	0
Dihydrouracil	0
6-Aminouracil	0
6-Methyluracil	0
Orotic acid	0
Barbituric acid	0
2-Thiobarbituric acid	0
Alloxan	0

Figure 6.12 Spectral changes during the oxidation of various pyrimidines

Reaction mixtures were taken from Table 6.1. A separate 0 min sample was set up for each substrate (0.1 mM-substrate in buffer and methylene blue as in Table 6.1, but without enzyme). Reaction samples were all diluted after incubation with buffer and methylene blue (concentrations as in Table 6.1) to a level equivalent to 0.1 mM initial substrate concentration. Spectra of 0 min samples and of diluted incubation samples were recorded immediately in a Unicam SP. 800 recording spectrophotometer with buffer and methylene blue (concentrations as in Table 6.1) in the reference tube.

Incubation times: a and d, 15 min; b, c and e, 25 min; f, 90 min; and g, 180 min.

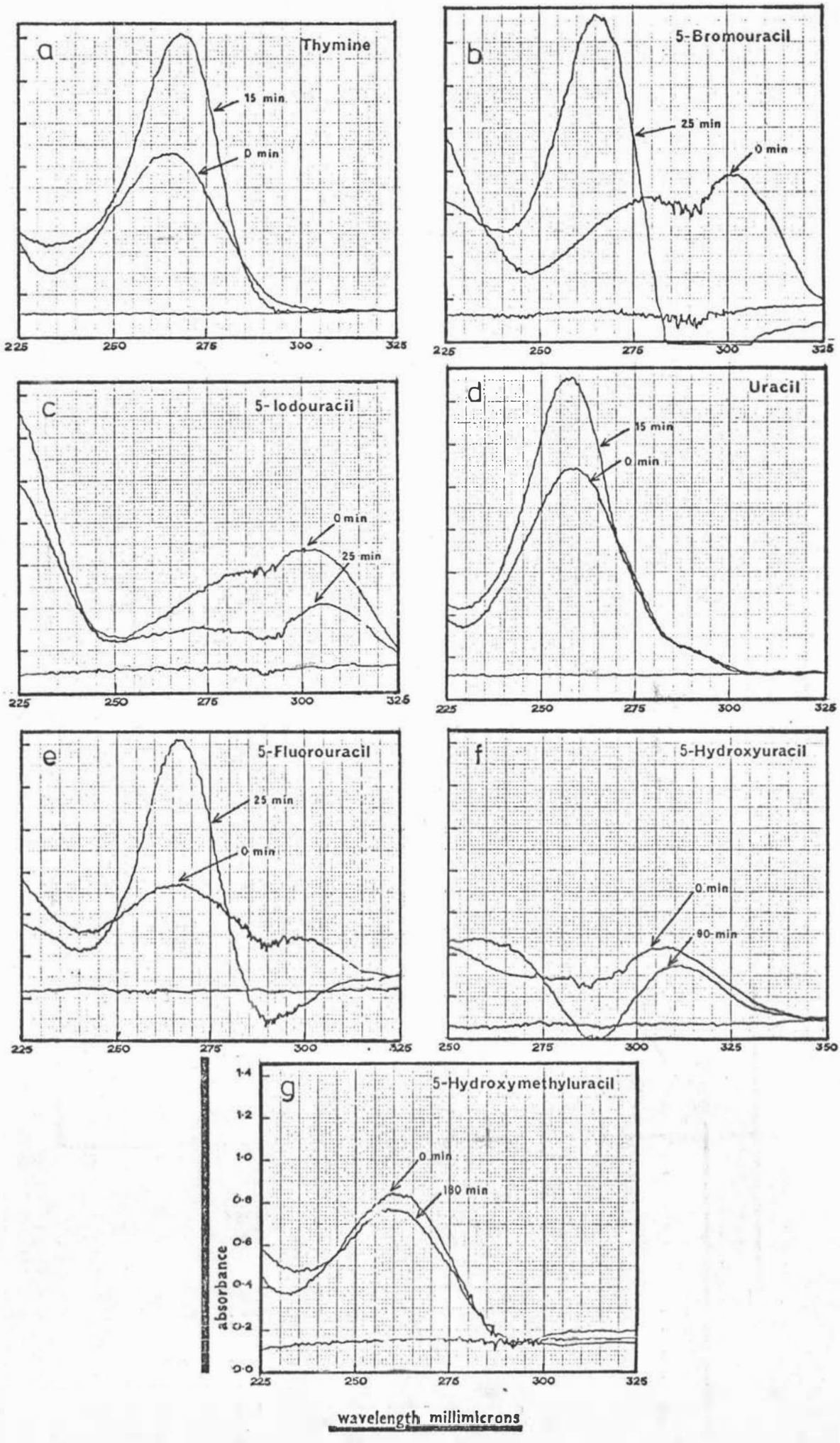

a
Thymine
15 min
0 min
225
250
275
300
325
b
5-Bromouracil
25 min
0 min
225
250
275
300
325
c
5-Iodouracil
0 min
25 min
225
250
275
300
325
d
Uracil
15 min
0 min
225
250
275
300
325
e
5-Fluorouracil
25 min
0 min
225
250
275
300
325
f
5-Hydroxyuracil
0 min
90 min
250
275
300
325
350
g
5-Hydroxymethyluracil
0 min
180 min
absorbance
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
225
250
275
300
325
wavelength millimicrons

TABLE 6.2

SPECIFICITY OF URACIL DEHYDROGENASE FOR ELECTRON ACCEPTORS

The assay reaction mixture contained, in a total volume of 3 ml, 150 µmol of Tris-HCl, pH 8.85; 0.3 µmol of thymine, the indicated amount of electron acceptor and the purified enzyme (0.1 ml containing 3.8 µg of protein; specific activity 8.9 units/mg). The reactions were carried out anaerobically in a Thunberg cuvette (Section 2.3.5a) unless otherwise indicated and were started by the addition of enzyme for the incubation at 25°C. The change in absorbance was followed at the appropriate wavelength over a period of 10 or 20 min.

Electron acceptor		λ (nm)	$\Delta\epsilon$[b]	Absorbance change	
Name	µmol			10 min	20 min
$K_3Fe(CN)_6$	1.00	420	1020		-0.03
Cytochrome C	0.15	550	21200		+0.10
1,4-Benzoquinone	3.00	300	2318[c]	+0.5	
1,4-Naphthoquinone	3.00	340			+0.10
2,4-Dichlorophenol-indophenol	0.15	600	21000		0
PMS	0.15	388	20500	-0.5	
Nitro Blue Tetrazolium[a]	5.00	550	d		+0.16
p-Iodonitrotetrazolium Violet[a]	5.00	500	d	+0.30	
NAD^+	0.30	340	6220		0
$NADP^+$	0.30	340	6200		0

[a] reaction carried out aerobically (Section 2.3.5a)

[b] difference between molar extinction coefficient of reduced and oxidised form at the wavelength cited (Dixon, 1971).

[c] $\Delta\epsilon$ at 295 nm

[d] reduced product insoluble

described in Section 6.4.1, the enzyme slowly lost activity on storage. For this reason, the concentration of enzyme in subsequent studies (Section 6.8) was calculated from the result of the standard enzyme assay performed at that time. Thus, if the standard assay gives an activity of X units/ml, then the concentration of active enzyme protein (at 100% pure) in the assay mixture would be $\frac{X}{10.6}$ mg/ml.

6.8 Effect of substrate concentration

The enzyme concentrations in this section were calculated as described in Section 6.7.

a. Thymine

The activity of uracil dehydrogenase was determined at various thymine concentrations with methylene blue as the alternate substrate. The methylene blue concentration used (2.5×10^{-5} M) gave the maximum reaction rate with 1.0×10^{-4} M-thymine in air (see d. in this section). The result is shown in Figure 6.13. The enzyme exhibited normal Michaelis-Menten saturation (hyperbolic) kinetics. An analysis of the data by the double reciprocal plot of Lineweaver & Burk (1934) gave K_m for thymine of 1.08×10^{-4} M at 2.5×10^{-5} M-methylene blue and pH 8.85, 25°C. V was found to be 7.63×10^{-3} µmol of thymine/min/ml, which, with an enzyme concentration of 0.363 µg of enzyme/ml, gave the specific activity of 21.02 µmol of thymine/min/mg. Three separate determinations gave similar values for the K_m and the specific activity (differences within ± 3%).

b. Uracil

To determine the affinity of uracil dehydrogenase for uracil (methylene blue as the alternate substrate), standard

enzyme assay conditions (Section 2.3.5a) were used except that thymine was replaced by uracil at various concentrations and the reaction rates were followed at 255 nm to follow the formation of barbituric acid as described by Hayaishi & Kornberg (1952). The methylene blue concentration used (2.5×10^{-5} M) gave the maximum rate with 1.0×10^{-4} M uracil in air (see d. in this section). From an analysis of the data (Figure 6.14), K_m for uracil was calculated to be 4.0×10^{-4} M at 2.5×10^{-5} M-methylene blue and pH 8.85, 25°C. V was found to be 6.45×10^{-3} µmol of uracil/min/ml, which, with an enzyme concentration of 0.297 µg of enzyme/ml, gave the specific activity of 21.71 µmol of uracil/min/mg.

c. Thymine and uracil

Since thymine and uracil were both substrates of uracil dehydrogenase (Section 6.5) an attempt was made to determine whether the two compounds competed for the same binding site on the enzyme. The method for measuring the rate of oxidation of each pyrimidine in the presence of the other is described in Section 2.3.4.

Figure 6.15 shows the dependence of rate of both thymine and uracil oxidation on the thymine concentration when the latter was varied between 0 and 1.4×10^{-4}M at a fixed uracil concentration (1.2×10^{-4} M) with methylene blue (2.5×10^{-5} M) as the alternate substrate. The rate of uracil oxidation decreased with increasing thymine concentration, showing half maximum rate (or 50% inhibition) at approximately 1×10^{-4} M-thymine. An analysis of the data (not shown) by the double reciprocal of Lineweaver & Burk (1934) gave an apparent K_m for thymine of 0.86×10^{-4} M, which is less than the K_m determined in the absence of uracil. The sig-

nificance of this result will be considered in the Discussion section.

When the experiment was performed at various uracil concentrations (0 to 1.6×10^{-4} M) at fixed thymine concentration (0.8×10^{-4} M), it could be seen (Figure 6.16) that the rate of thymine oxidation was not appreciably affected within the range of uracil concentrations used. The scatter of points was too great to allow the determination of the K_m apparent for uracil.

The data were not sufficient to determine the type of inhibition in either case.

d. Methylene blue

The activity of uracil dehydrogenase was determined at various methylene blue concentrations with thymine or uracil as the alternate substrate.

The plot of the initial velocity against substrate concentration with thymine (0.1 mM) as the alternate substrate was found to be sigmoidal rather than hyperbolic (Figure 6.17). The optimum methylene blue concentration was in the range 1.7×10^{-5} to 3.0×10^{-5} M with the half maximum rate at approximately 0.5×10^{-5} M-methylene blue at 0.1 mM-thymine and pH 8.85, 25°C. V was found to be 2.6×10^{-3} µmol of thymine/min/ml, which, with an enzyme concentration of 0.248 µg of enzyme/ml, gave the specific activity of 10.48 µmol of thymine/min/mg. The double reciprocal plot (Figure 6.17, B) is non-linear, and no determination of K_m was possible.

With uracil (0.1 mM) as the alternate substrate, the results obtained were similar to those with thymine. The

curve is also sigmoidal (Figure 6.18), giving an optimum methylene blue concentration in the range 2.0×10^{-5} to 5.0×10^{-5} M, with the half maximum rate at approximately 0.5×10^{-5} M-methylene blue at 0.1 mM-uracil and pH 8.85, 25°C. V was found to be 2.1×10^{-3} µmol of uracil/min/ml, which, with an enzyme concentration of 0.478 µg of enzyme/ml, gave the specific activity of 4.39 µmol of uracil/min/mg.

e. Phenazine methosulphate

The activity of uracil dehydrogenase was determined at various phenazine methosulphate (PMS) concentrations with thymine as the alternate substrate. Standard enzyme assay conditions (Section 2.3.5a) were used, except that methylene blue was replaced by varying concentrations of PMS. Since reduced PMS is very rapidly oxidised by molecular oxygen (Jones, 1969), it was assumed that the absorbance change at 270 nm, during oxidation of thymine by the enzyme, was not affected by changes in the concentrations of PMS and reduced PMS (as shown in the analogous case with methylene blue, Section 6.1). Hence, the rate of reaction was determined by the rate of change in absorbance at 270 nm, as described for the standard enzyme assay (Section 2.3.5a).

The results are shown in Figure 6.19. Since, the rate curves were non-linear particularly at high concentrations of PMS (from 5×10^{-5} to 10×10^{-5} M), the initial velocities (v) were correspondingly inaccurate. At lower concentrations of PMS, 0.8×10^{-5} to 4.0×10^{-5} M, v was approximately constant. Over this range of substrate concentrations, therefore, the enzyme was operating at the maximum rate. V was found to be approximately 7.2×10^{-3} µmol of thymine/min/ml, which, with an

enzyme concentration of 0.32 µg of enzyme/ml, gave the specific activity of 22.5 µmol of thymine/min/mg. Since lower concentrations of substrate were not used, no estimate of K_m can be made, but the half maximum rate must be considerably less than 0.4×10^{-5} M-PMS at 0.1 mM-thymine and pH 8.85, 25°C.

The results from the data in Figures 6.13, 6.14, 6.17, 6.18 and 6.19 are summarised in Table 6.3. The enzyme is shown to have approximately the same V with either thymine or uracil as the substrate. V for methylene blue with thymine as the alternate substrate is greater than that for methylene blue with uracil. V for PMS with thymine as the alternate substrate is greater than that for methylene blue. The affinity of the enzyme for thymine is greater than that of the enzyme for uracil. The affinity of the enzyme for PMS is greater than that of the enzyme for methylene blue.

6.9 Effect of miscellaneous inhibitors and activators on enzyme activity

The effect of various compounds including metal ions, chelating agents, flavins, H_2O_2 and inhibitors on the activity of uracil dehydrogenase was investigated. Details of experimental conditions are given with the Tables 6.4, 6.5 and Figure 6.21.

Table 6.4 and Figure 6.20 show the effect of metal ions on uracil dehydrogenase activity. All metal salts tested were inhibitory to the enzyme. From the Table, the enzyme was only slightly inhibited by Ba^{2+}, Mg^{2+} and Ca^{2+} at 0.1 mM while Zn^{2+}, Fe^{2+}, Cu^{2+} and Cd^{2+} were potent inhibitors and

TABLE 6.3

KINETIC PARAMETERS FOR URACIL DEHYDROGENASE

Values are for reaction at 25°C, pH 8.85, ionic strength 0.043 as in the standard enzyme assay conditions (Section 2.3.5a), and are determined from the data in Figures 6.13, 6.14, 6.17, 6.18 and 6.19. The enzyme concentrations were calculated as in Section 6.7.

Substrate A	Substrate B	Enzyme concn. (μg/ml)	V (μmol/min/mg)	K_m ($\times 10^{-4}$ M)
Thymine	Methylene blue (2.5×10^{-5} M)	0.363	21.02	1.08
Uracil	Methylene blue (2.5×10^{-5} M)	0.297	21.71	4.00
Methylene blue	Thymine (1.0×10^{-4} M)	0.248	10.48	0.05[a]
Methylene blue	Uracil (1.0×10^{-4} M)	0.478	4.39	0.05[a]
PMS	Thymine (1.0×10^{-4} M)	0.320	22.50	$<$0.04[a]

[a] substrate concentration for the half maximum rate at 0.1 mM of the corresponding alternate substrate.

Figure 6.13 Dependence of the rate of reaction of uracil dehydrogenase on the concentration of thymine with methylene blue as the alternate substrate

Standard enzyme assay conditions were used with different thymine concentrations as shown on the graph. Purified uracil dehydrogenase was used in the assay (0.0116 units ≡ 1.09 μg enzyme in a total assay volume of 3 ml). Plotted points are the means of duplicate, which did not differ by more than approximately 5%.

A, initial reaction velocity as a function of thymine concentration; and

B, double reciprocal plot of data from A.

1/VELOCITY
(μmol thymine·min⁻¹·ml⁻¹)⁻¹ (x10⁻²)
6·0
5·0
4·0
3·0
2·0
1·0
0
-0·93
1·0
2·0
3·0
1/THYMINE (mol·l⁻¹)⁻¹ (x10⁻⁴)
v (μmol·min⁻¹·ml⁻¹)(x10⁴)
40
20
0
0·4
0·8
1·2
[S] (mol·l⁻¹)(x10⁴)

Figure 6.14 Dependence of the rate of reaction of uracil dehydrogenase on the concentration of uracil with methylene blue as the alternate substrate

The enzyme preparation (0.0094 units ≡ 0.89 μg enzyme in a total assay volume of 3 ml) was the same as that used in the experiment for which the results are shown in Figure 6.13. Standard enzyme assay conditions were used except that thymine was replaced by uracil at various concentrations and the reaction rates were followed at 255 nm. Plotted points are the means of duplicates, which did not differ by more than approximately 5%.

A, initial reaction velocity as a function of uracil concentration; and

B, double reciprocal plot of data from A.

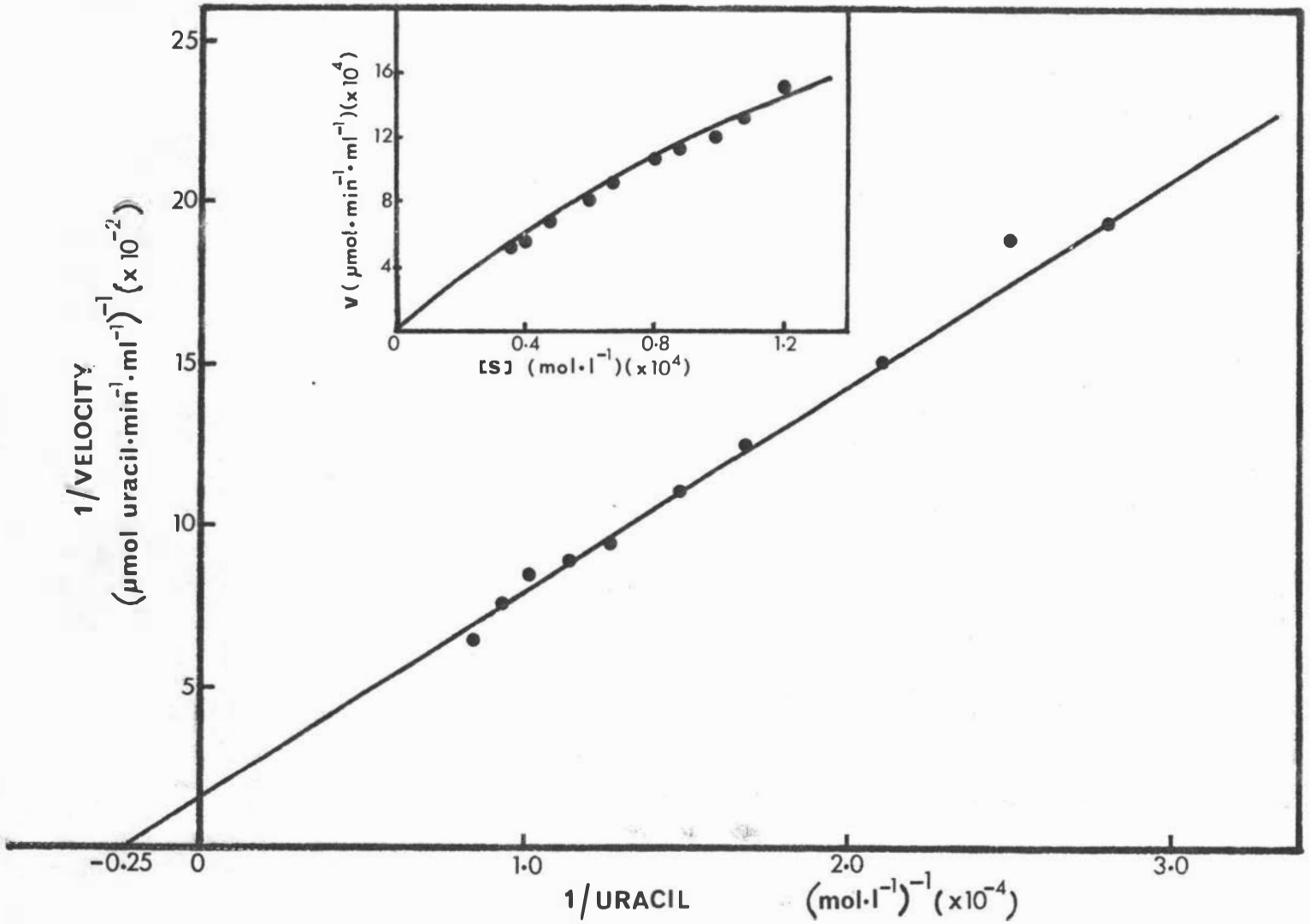

25
20
15
10
5
-0.25
0
1·0
2·0
3·0
1/VELOCITY
(μmol uracil·min⁻¹·ml⁻¹)⁻¹ (x 10⁻²)
1/URACIL
(mol·l⁻¹)⁻¹ (x10⁻⁴)
v (μmol·min⁻¹·ml⁻¹)(x10⁴)
16
12
8
4
0
0·4
0·8
1·2
[S] (mol·l⁻¹)(x10⁴)

Figure 6.15 Rates of oxidation of thymine and uracil at fixed initial uracil concentration but varying thymine concentrations with methylene blue as the alternate substrate

The enzyme preparation (0.01 units ≡ 0.94 μg enzyme in a total assay volume of 3 ml) was the same as that described in the legend to Figure 6.13. Standard enzyme assay conditions were used with different concentrations of thymine between 0 and 1.4×10^{-4} M at a fixed uracil concentration of (1.2×10^{-4} M). The reaction rates were followed at 270 nm and 250 nm wavelengths (see Section 2.3.4).

A, the initial reaction velocities for thymine oxidation were plotted against the concentrations of thymine; and

B, the initial reaction velocities for uracil oxidation were plotted against the concentrations of thymine.

VELOCITY
(μmol thymine or uracil·min⁻¹·ml⁻¹) (x10⁴)
60
40
20
A
B
0.4
0.8
1.2
1.6
THYMINE (mol·l⁻¹) (x10⁴)

Figure 6.16 Rates of oxidation of thymine and uracil at fixed initial thymine concentration but varying uracil concentrations with methylene blue as the alternate substrate

The enzyme preparation (0.01 units ≡ 0.94 μg enzyme in a total assay volume of 3 ml) was the same as that described with Figure 6.13. Standard enzyme assay conditions were used with uracil concentrations between 0 and 1.6×10^{-4} M and at a fixed thymine concentration of 0.8×10^{-4} M. The reaction rates were followed at 270 nm and 250 nm wavelengths (see Section 2.3.4).

A, the initial reaction velocities for uracil oxidation were plotted against the concentrations of uracil; and

B, the initial reaction velocities for thymine oxidation were plotted against the concentrations of uracil.

VELOCITY
(μmol thymine or uracil·min⁻¹·ml⁻¹)(×10⁵)
0.6
0.4
0.2
A
B
0.4
0.8
1.2
1.6
uracil (mol·l⁻¹)(×10⁴)

Figure 6.17 Dependence of the rate of reaction of uracil dehydrogenase on the concentration of methylene blue with thymine as the alternate substrate

The enzyme preparation was the same as that used for the experiment described with Figure 6.13. Standard enzyme assay conditions were used with different concentrations of methylene blue and thymine at an initial concentration of 0.1 mM. The enzyme preparation used contained 0.0078 units (0.74 µg of enzyme) in a total assay volume of 3 ml. Plotted points are the means of duplicates, which did not differ by more than approximately 5%.

A, initial reaction velocity as a function of methylene blue concentration; and

B, double reciprocal plot of data from A.

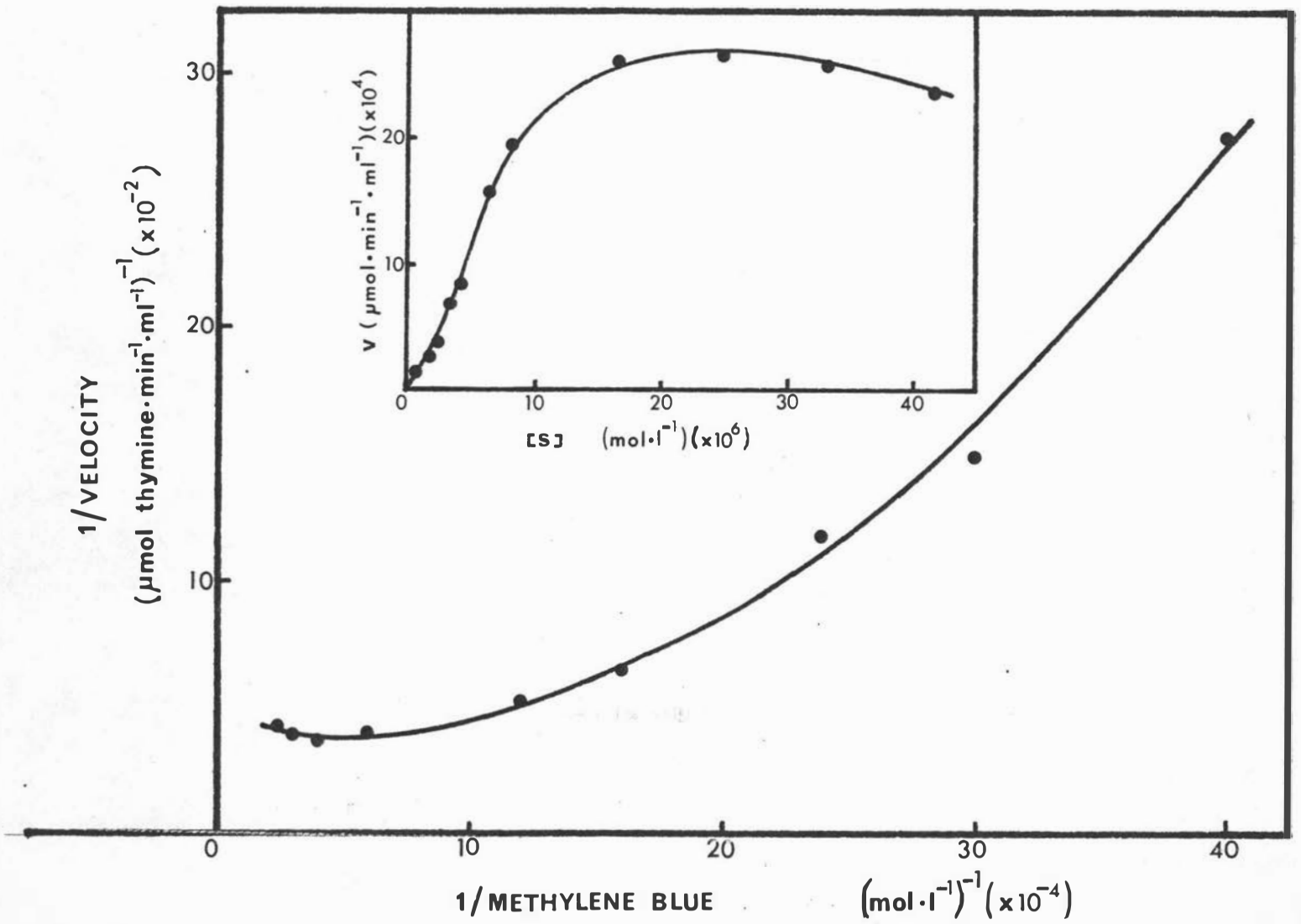

1/VELOCITY
(μmol thymine·min⁻¹·ml⁻¹)⁻¹(x10⁻²)
30
20
10
0
10
20
30
40
1/METHYLENE BLUE
(mol·l⁻¹)⁻¹(x10⁻⁴)
v (μmol·min⁻¹·ml⁻¹)(x10⁴)
20
10
0
10
20
30
40
[S] (mol·l⁻¹)(x10⁶)

Figure 6.18 Dependence of the rate of reaction of uracil dehydrogenase on the concentration of methylene blue with uracil as the alternate substrate

The enzyme preparation was the same as that described in the legend to Figure 6.13. Standard enzyme assay conditions were used except with different concentrations of methylene blue and uracil (0.1 mM) instead of thymine. The reaction rates were followed at 255 nm. The enzyme preparation used contained 0.0152 units (1.43 μg of enzyme) in a total assay volume of 3 ml. The initial reaction velocities were plotted against the concentrations of methylene blue. Plotted points are the means of duplicates, which did not differ by more than approximately 5%.

VELOCITY
(μmol uracil·min⁻¹·ml⁻¹)(x10⁴)
20
10
10
20
30
40
50
60
METHYLENE BLUE (mol·l⁻¹)(x10⁶)

Figure 6.19 Dependence of the rate of uracil dehydrogenase on the concentration of phenazine methosulphate with thymine as the alternate substrate

The enzyme preparation (0.0102 units ≡ 0.96 μg enzyme in 3 ml assay volume) was the same as that used for the experiment described with Figure 6.13. Standard assay conditions were used with different concentrations of phenazine methosulphate in place of methylene blue and with thymine at a concentration of 0.1 mM. The initial reaction velocities were plotted against the concentrations of phenazine methosulphate.

VELOCITY
(μmol thymine·min⁻¹·ml⁻¹)(x10⁵)
2
4
6
8
PHENAZINE METHOSULPHATE (mol·l⁻¹)(x10⁵)
2
4
6
8
10

Fe^{2+} showed a greater inhibitory effect than Fe^{3+}. A wide range of concentrations was tested for each metal ion and it was found that the percentage of inhibition was proportional to the logarithm of the inhibitor concentrations over the major part of the range of concentrations tested (Figure 6.20).

The effect of various chelating agents on uracil dehydrogenase activity are shown in Figure 6.21. Murexide and cupron had small effect on the enzyme while 8-hydroxyquinoline inhibited it to approximately 40%. No inhibition was observed with any of the other chelating agents tested. Treatment with diethyldithiocarbamate; ethylene diaminetetra-acetic acid; α,α' dipyridyl and 1,10-phenanthroline could have produced a slight stabilization of the enzyme (compared to control).

The effect of other compounds on the activity of uracil dehydrogenase are shown in Table 6.5. The enzyme was very sensitive to the sulfhydryl reagents p-chloromercuribenzoate and iodoacetate but unaffected by iodoacetamide. The enzyme was inhibited to some extent by metal binding agents such as potassium thiocyanate and it was very sensitive to potassium cyanide. The enzyme was also very sensitive to H_2O_2 and 2-mercaptoethanol. Among the flavins, FAD did not affect the enzyme but FMN and riboflavin showed some inhibiting effect. Riboflavin was a more powerful inhibitor than FMN. Potassium ferricyanide appeared to be an activator for the enzyme; it did not oxidise thymine in the absence of enzyme under the assay

TABLE 6.4

EFFECTS OF METAL IONS ON THE ACTIVITY OF URACIL DEHYDROGENASE

Metal ions were added to the standard enzyme assay mixture prior to the addition of enzyme. Standard assay (Section 2.3.5a) was used except that metal salts were added at the indicated concentration. The pH and volume of the assay mixture were kept constant; the assay system (3 ml) contained 1.1 μg of enzyme (specific activity 9.2 units/mg). The results are expressed as percentage inhibition at two representative inhibitor concentrations.

Compounds tested	% Inhibition at inhibitor concn. of 0.1 mM	1.0 mM
$BaCl_2$	<1	65
$MgCl_2$	8	50
$CaCl_2$	13	50
$MnCl_2$	40	57
$NiCl_2$	52	84
$CoCl_2$	57	
$FeSO_4$	85	
$NH_4Fe(SO_4)_2$	41	
$FeCl_3$	67	
$CuCl_2$	71	
$ZnCl_2$	86	
$AlCl_3$	48	
$CdCl_2$	76	

Figure 6.20 Effects of metal ions on the activity of uracil dehydrogenase

Semilogarithmic plot of the percentage inhibition of the enzyme activity against the concentration of the metal salts. The experimental conditions were described as in the legend to Table 6.4.

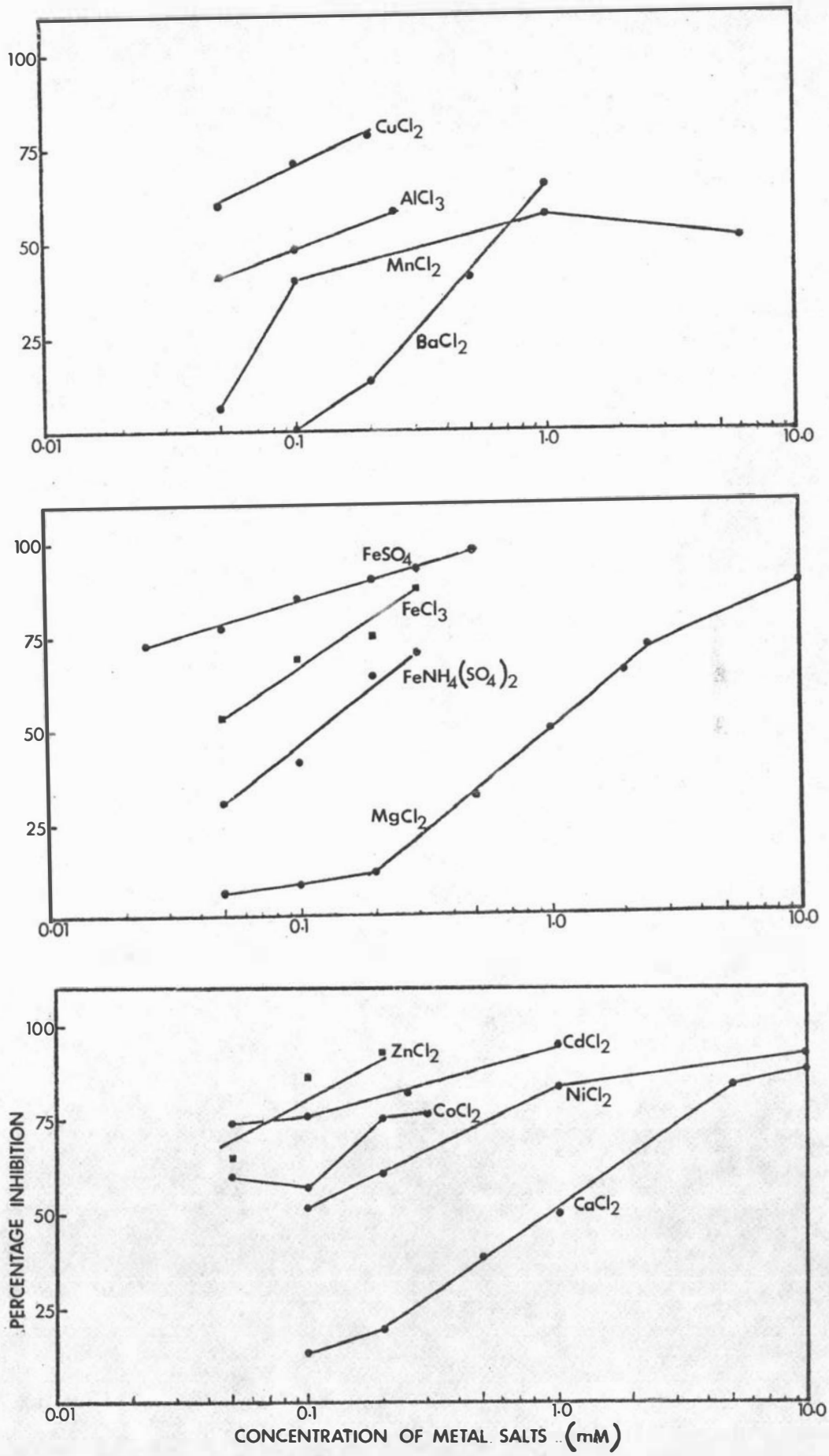

100
75
50
25
0·01
0·1
1·0
10·0
CuCl2
AlCl3
MnCl2
BaCl2
FeSO4
FeCl3
FeNH4(SO4)2
MgCl2
ZnCl2
CdCl2
NiCl2
CoCl2
CaCl2
PERCENTAGE INHIBITION
CONCENTRATION OF METAL SALTS (mM)

Figure 6.21 Effects of chelating agents on the activity of uracil dehydrogenase

The enzyme solution (specific activity 8.5 units/mg) was preincubated with each of chelating agents at pH 8.85 and 0-4°C (23 μg of enzyme in a final volume of 1 ml in 0.05 M-Tris-HCl, pH 8.85). The concentration of each chelating agent in the preincubation mixture is shown on the figure or given below. Aliquots (0.05 ml) of the mixtures were taken for the assay of enzyme at the time indicated and the standard enzyme assay (Section 2.3.5a) was used. Other chelating agents tested (not shown on Figure) were: ethylene diaminetetra-acetic acid (1 mM); α,α' -dipyridyl (1 mM); 1,10-phenanthroline (1 mM); toluene 3,4-dithiol (0.2 mM) and dimethylglyoxime (0.1 mM). These all gave results similar to those of diethyldithiocarbamate or the control, the first three following more closely to the curve for diethyldithiocarbamate.

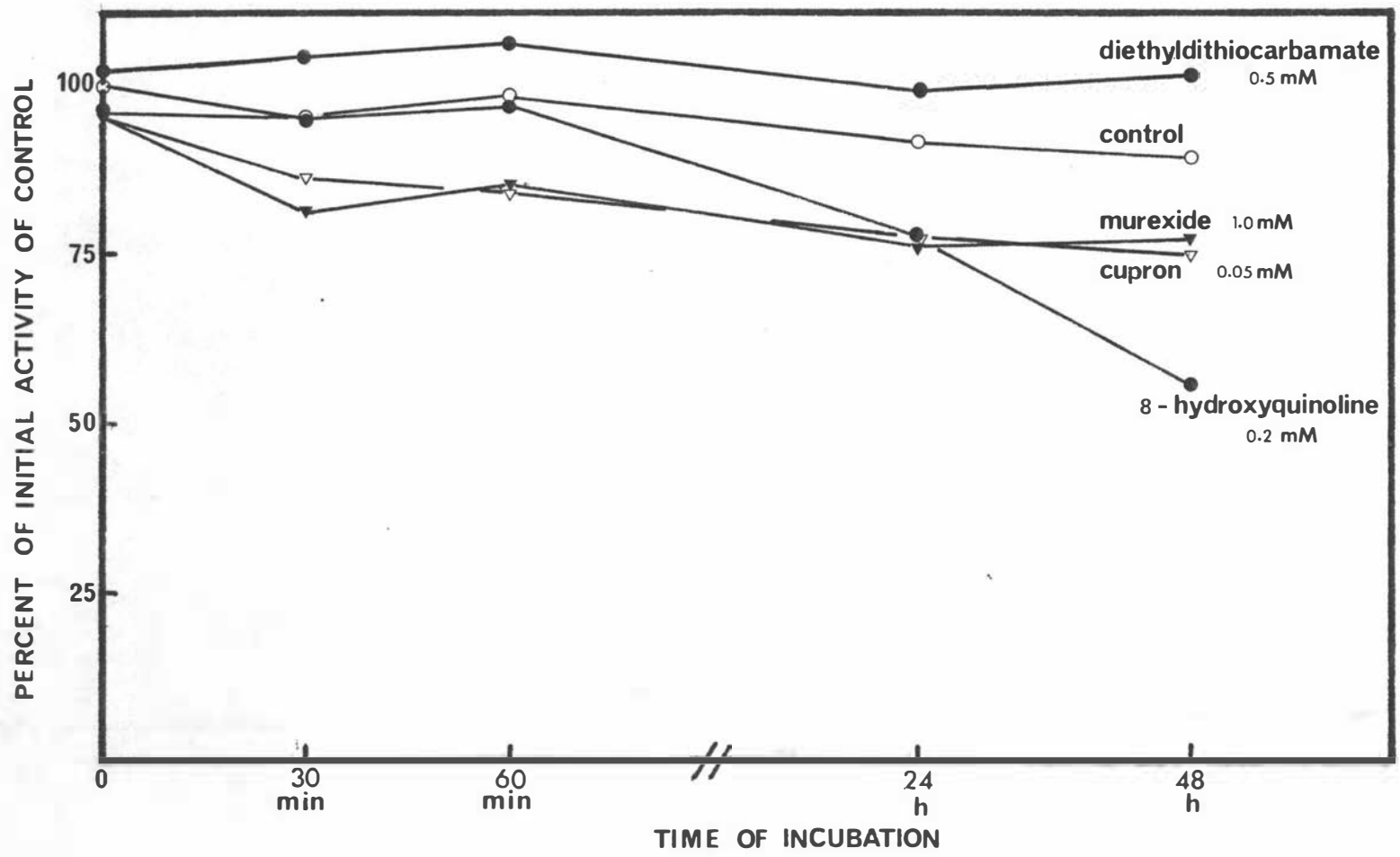

PERCENT OF INITIAL ACTIVITY OF CONTROL
100
75
50
25
0
30 min
60 min
24 h
48 h
TIME OF INCUBATION
diethyldithiocarbamate 0.5 mM
control
murexide 1.0 mM
cupron 0.05 mM
8 - hydroxyquinoline 0.2 mM

TABLE 6.5

EFFECTS OF MISCELLANEOUS COMPOUNDS ON THE ACTIVITY OF URACIL DEHYDROGENASE

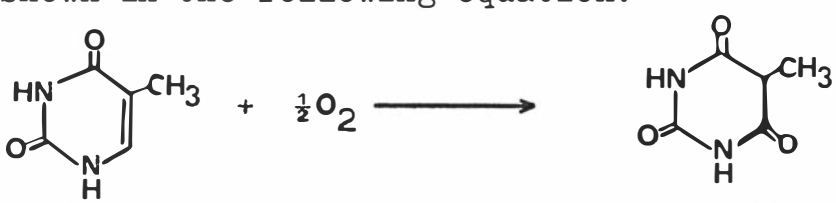
The concentration of each compound in the reaction mixture is shown in the table. The experimental conditions were the same as described in the legend to Table 6.4. The results are expressed as percentage activity of the control (in normal standard enzyme assay without inhibitors added) at the indicated concentrations.

Compounds tested	Concn. (mM)	% Activity relative to the control
Control	none added	100
Iodoacetamide	1.00	102
Iodoacetate	0.17	60
	0.34	0
p-Chloromercuribenzoate	0.17	0
2-Mercaptoethanol	0.50	6
	1.00	4
KSCN	1.00	90
	2.00	78
KCN	0.01	20
	0.02	0
H_2O_2	0.002	6
	0.004	0
FAD	0.01	99
FMN	0.01	90
	0.02	86
	0.03	73
Riboflavin	0.01	30
	0.02	0
$K_3Fe(CN)_6$	0.003	116
	0.010	121
	0.033	156
	0.166	156

conditions used.

6.10 Stoichiometry of oxygen uptake for thymine oxidation

The uptake of oxygen for thymine oxidation had been studied, *in vivo* (Batt & Woods, 1961), using a cell suspension of *Nocardia corallina*. The results showed that approximately half a mole of molecular oxygen was taken up for each mole of thymine oxidised. The stoichiometry of the reaction is shown in the following equation:



thymine — 5-methylbarbituric acid (6.1)

In the present studies, the stoichiometry of oxygen uptake for thymine oxidation with the purified uracil dehydrogenase was determined using an oxygen electrode. It was calibrated by the absolute calibration method of Robinson & Cooper, 1970 (as described in Section 2.3.5b) immediately before use.

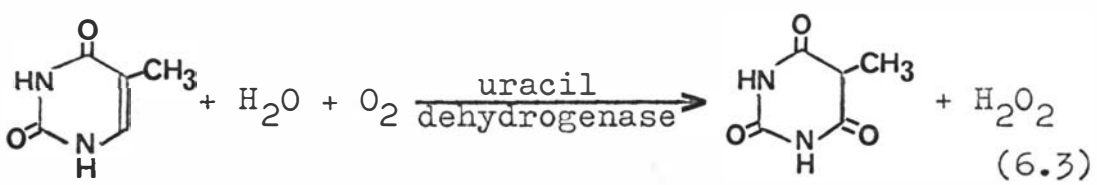
Figure 6.22 shows the recorder response (pen deflection) on the addition of various amounts of NADH. Knowing the amount of NADH added and the pen deflection (scale divisions), the µmol of oxygen per scale division could be calculated (Table 6.6) by using the stoichiometry of the following reaction:

$$NADH + H^+ + \tfrac{1}{2}O_2 \longrightarrow NAD^+ + H_2O \qquad (6.2)$$

The recorder response in term of µmol of oxygen/scale division (calibration constant) varied with the amount of oxygen present in the reaction mixture (Table 6.6). Changes

were reproducible showing a consistent decrease in the calibration constant with decreasing oxygen concentration, and it was possible to draw a calibration curve relating μmol of oxygen/scale division to the pen position on the chart (Figure 6.23). The uptake of oxygen for thymine oxidation could be estimated by the use of this calibration curve.

The uptake of oxygen by thymine in the presence of the purified uracil dehydrogenase is shown in Figure 6.24. The results were calculated in terms of moles of oxygen/mole of thymine and are shown in Table 6.7, and it was found that for each mole of thymine oxidised, 1.08 ± 0.04 (8) moles of oxygen was consumed. This result is in agreement with the following reaction:



(6.3)

Addition of catalase after the final thymine oxidation (Figure 6.24) caused the production of 0.36 μmol of oxygen, which was equivalent to 0.43 mole of oxygen per mole of thymine. The theoretical value for the two reactions (6.3) and (6.4),

$$H_2O_2 \longrightarrow H_2O + \tfrac{1}{2} O_2 \qquad (6.4)$$

is 0.5 mole of oxygen per mole of thymine.

6.11 Anaerobic assay of uracil dehydrogenase activity

During the oxidation of thymine in air, catalysed by uracil dehydrogenase and with methylene blue as an electron acceptor, methylene blue was reduced to leucomethylene blue which then reoxidised at a rate which, in the steady state

Figure 6.22 Calibration of the oxygen electrode by serial additions of different amounts of NADH to the reaction vessel

The reaction mixture contained 20 μg of PMS, 800 Sigma units (26.7 μg) of catalase and 285 μmol of phosphate buffer, pH 7.4 in a total volume of 3 ml, at 25°C. Additions of NADH are shown by arrows.

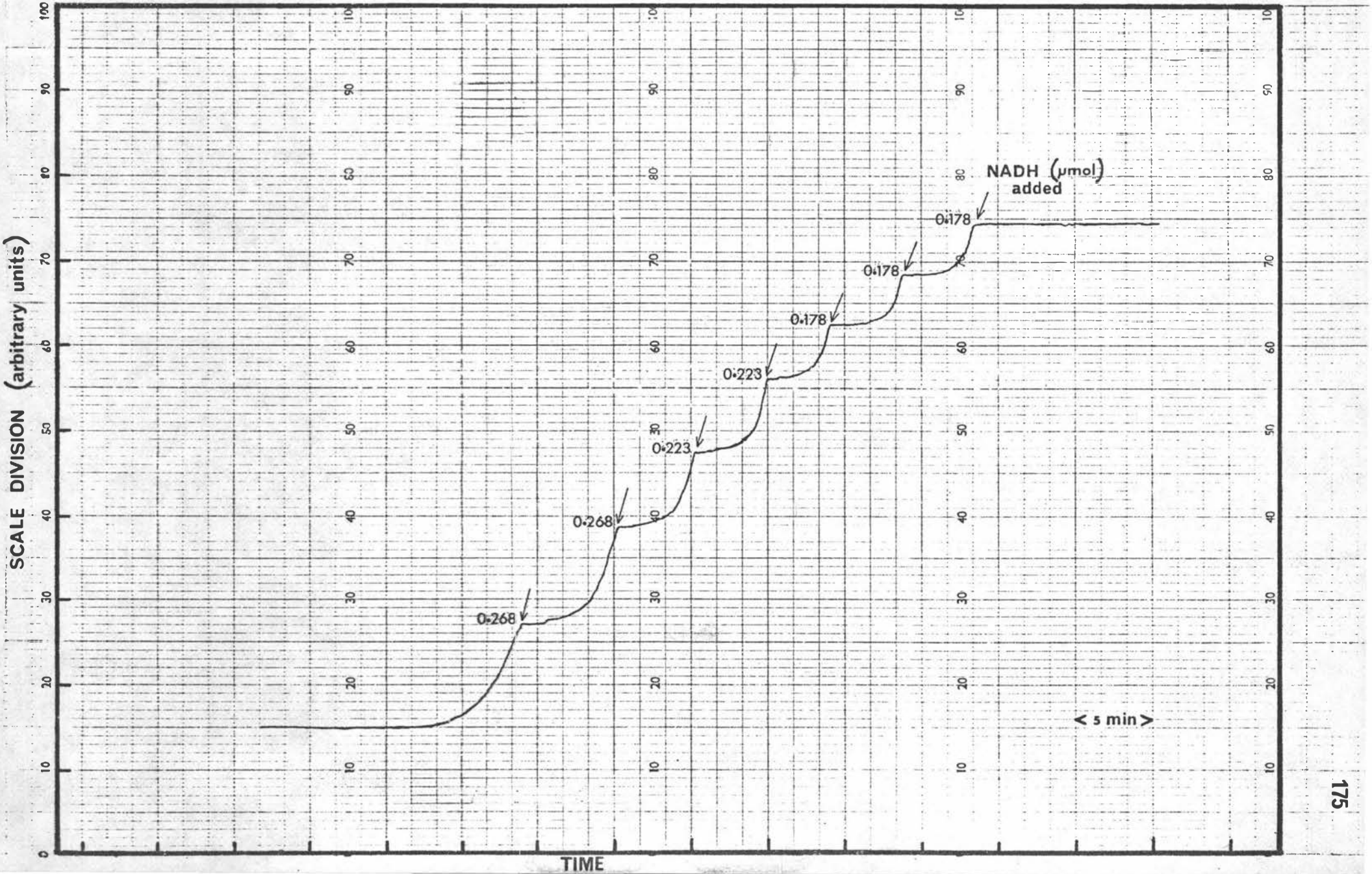

SCALE DIVISION (arbitrary units)
TIME
NADH (μmol) added
0·178
0·178
0·178
0·223
0·223
0·268
0·268
< 5 min >
0
10
20
30
40
50
60
70
80
90
100

TABLE 6.6

CALIBRATION OF THE OXYGEN ELECTRODE USING PHENAZINE METHOSULPHATE AND LIMITING CONCENTRATIONS OF NADH

(Data from Figure 6.22)

NADH added[a] (μmol)	Pen deflection (scale divisions)	Pen position on the chart at mid-point of oxidation (scale divisions)	μmol of oxygen/ scale division
0.178	5.9	3.0	0.0151
	6.0	8.9	0.0148
	6.5	15.2	0.0137
0.223	8.5	22.7	0.0131
	8.8	31.3	0.0126
0.268	11.4	41.4	0.0117
	12.2	53.2	0.0109

[a] the exact concentration was determined spectrophotometrically using ϵ at 340 nm of 5270 litre$\cdot$mol$^{-1}\cdot$cm^{-1} in glycine-NaOH, pH 10.0 (as specified by the supplier of NADH: BDH).

TABLE 6.7

STOICHIOMETRY OF OXYGEN UPTAKE FOR THYMINE OXIDATION

(Data from Figure 6.24)

Thymine added[a] (μmol)	Pen deflection (scale divisions)	μmol of oxygen/[b] scale division	mole of oxygen/ mole of thymine
0.049	3.6	0.0153	1.124
	3.4	0.0149	1.034
	3.6	0.0145	1.065
0.098	7.2	0.0140	1.029
	7.9	0.0133	1.072
0.147	13.5	0.0124	1.139
	14.3	0.0114	1.109
0.196	19.7	0.0104	1.045
Mean			1.080
Standard deviation			0.044

[a] the concentration was determined spectrophotometrically using ϵ at 270 nm of 6620 litre·mol^{-1}·cm^{-1} (Section 2.3.4)

[b] values were obtained for the corresponded pen position on the chart (from the calibration curve, Figure 6.23).

Figure 6.23 Calibration curve of the oxygen electrode

Experimental data are from Table 6.6. The micromoles of oxygen/scale division are plotted against the pen positions on the chart.

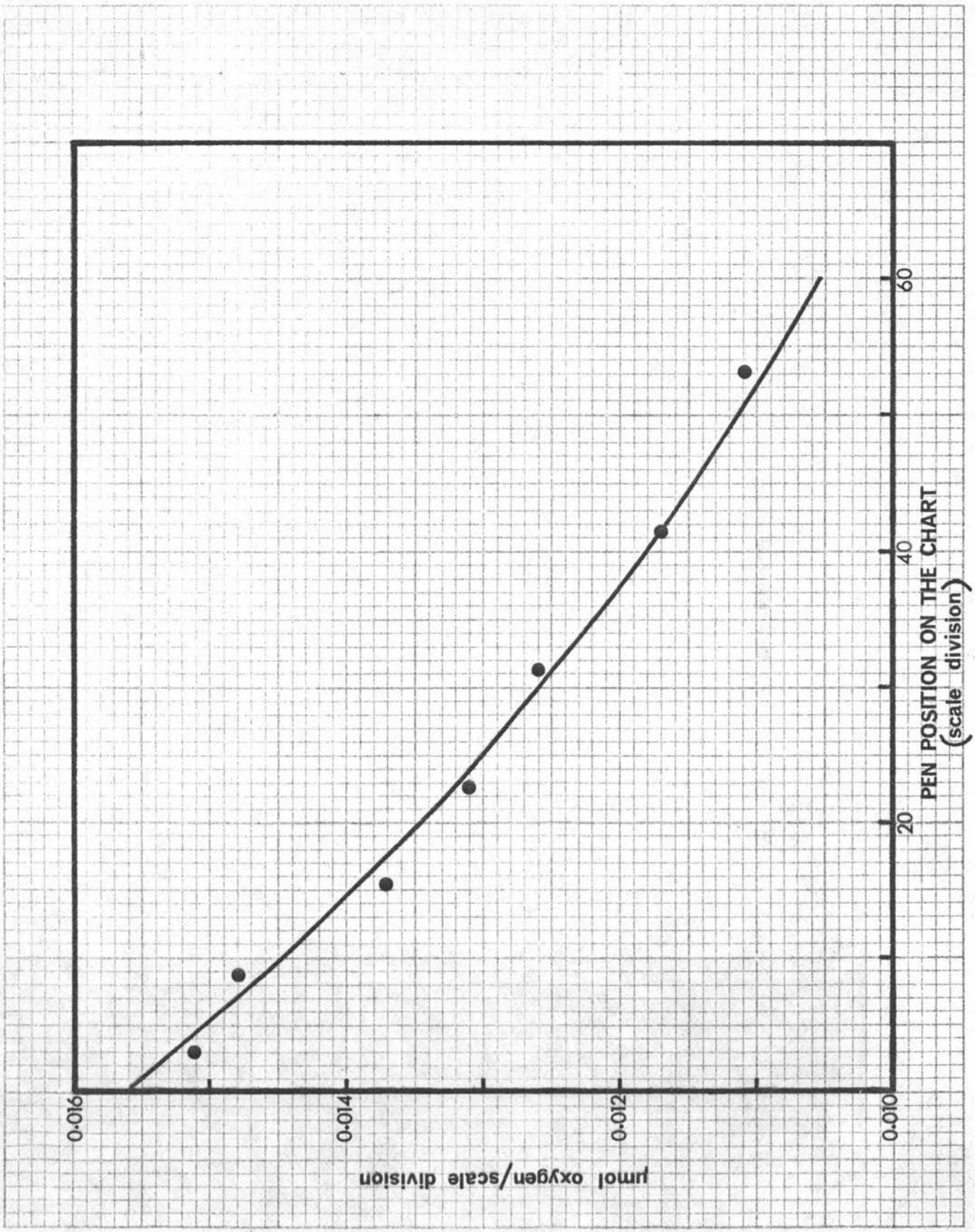
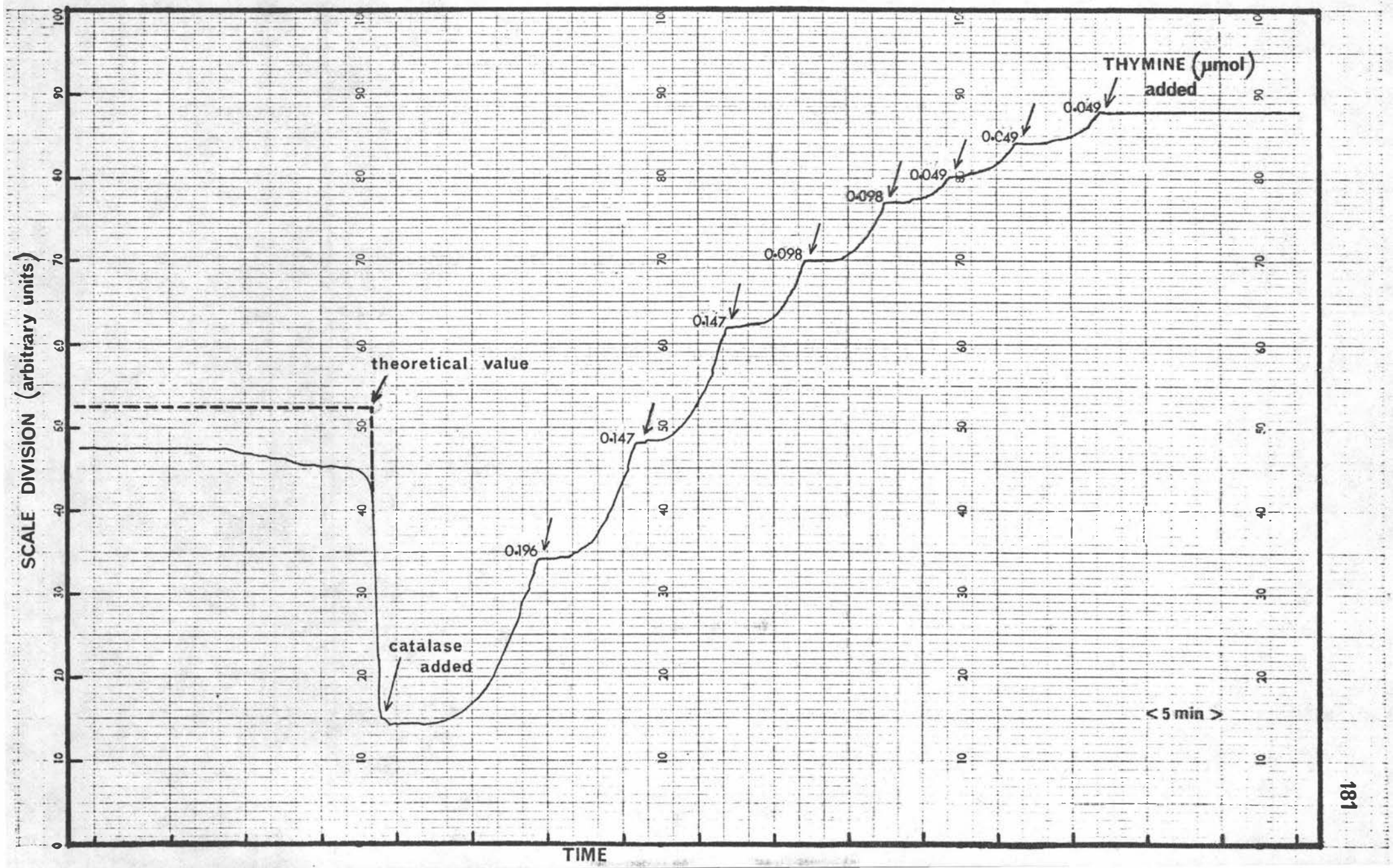

0·016
0·014
0·012
0·010
μmol oxygen/scale division
20
40
60
PEN POSITION ON THE CHART
(scale division)

Figure 6.24 Determination of the stoichiometry of oxygen uptake for thymine oxidation

The reaction mixture contained 0.125 μmol of methylene blue, 150 μmol of Tris-HCl, pH 8.85 and 15 μg of enzyme (specific activity 10.2 units/mg) in a total assay volume of 3 ml at 25°C. Additions of thymine indicated by arrows. After the reaction for the last addition of thymine was completed, 800 Sigma units (26.7 μg) of catalase were added as shown in the figure.



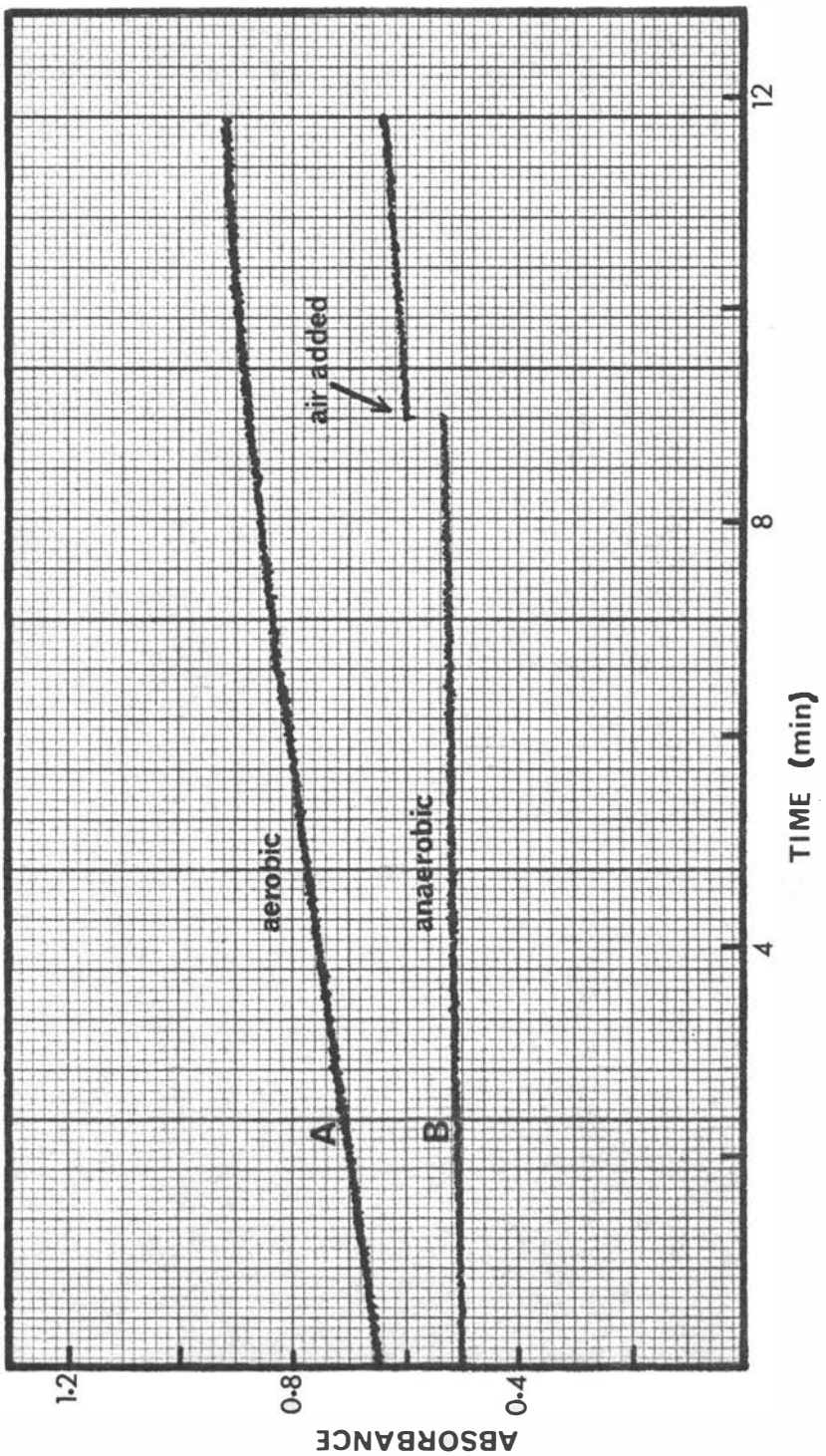
(Section 6.1), was equal to the rate of methylene blue reduction. In the absence of air, however, leucomethylene blue can not be reoxidised, and the oxidation of thymine continues only until the methylene blue is all reduced.

In studies performed with the standard enzyme assay mixture under anaerobic conditions (Section 2.3.5a), the absorbance change was linear with time and the rate of thymine oxidation in the absence of oxygen was much lower (approximately 10%) than the rate in the presence of oxygen (Figure 6.25). When air was admitted to the reaction cuvette while the anaerobic oxidation of thymine was still proceeding, the rate of thymine oxidation increased immediately (Figure 6.25). The results indicate that oxidation of thymine by methylene blue - uracil dehydrogenase does not require oxygen but that oxygen increases the rate of reaction. The slow rate of thymine oxidation observed under anaerobic conditions, is not significantly enhanced by the presence of methylene blue at concentrations in excess of those present in the standard enzyme assay mixture (result not shown).

Figure 6.25 Absorbance change (at 270 nm) during aerobic and anaerobic oxidation of thymine

A. The standard enzyme assay conditions were used (Section 2.3.5a). The assay reaction mixture (3 ml) contained 0.74 µg of enzyme (specific activity 8.6 units/mg). The reactions were carried out aerobically.

B. Using the identical conditions as in A except that the reactions were carried anaerobically in a Thunberg cuvette (Section 2.3.5a). Air was admitted to the reaction cuvette as shown in the figure.


ABSORBANCE
1·2
0·8
0·4
TIME (min)
4
8
12
aerobic
anaerobic
air added
A
B

6.12 The oxidase activity of uracil dehydrogenase

The activity of uracil dehydrogenase has been routinely determined with methylene blue as the electron carrier. The result shown in Figure 6.26 demonstrates that purified uracil dehydrogenase is capable of catalyzing the oxidation of thymine with oxygen in the absence of any added electron carrier. In this experiment, an enzyme concentration 10 times higher than in the routine enzyme assay mixture was used. The activity of the enzyme apparently results in the formation of 5-methylbarbituric acid as indicated by the spectral changes which are similar to those seen in the presence of methylene blue (Figure 6.27). The activity with oxygen is calculated to be approximately 1/2000th of the rate with methylene blue. No spectral changes were observed in the absence of enzyme (result not shown).

Figure 6.26 Ultraviolet spectra of the reaction mixture during the aerobic oxidation of thymine by uracil dehydrogenase

The assay mixture (3.0 ml) contained 0.3 μmol of thymine, 150 μmol of Tris-HCl, pH 8.85 and 0.164 units of uracil dehydrogenase (specific activity 10.2 units/mg) and was incubated at 20°C. The spectrum of the reaction mixture was recorded as indicated in the figure; a, zero time; b, 1½ h; and c, 5½ h.

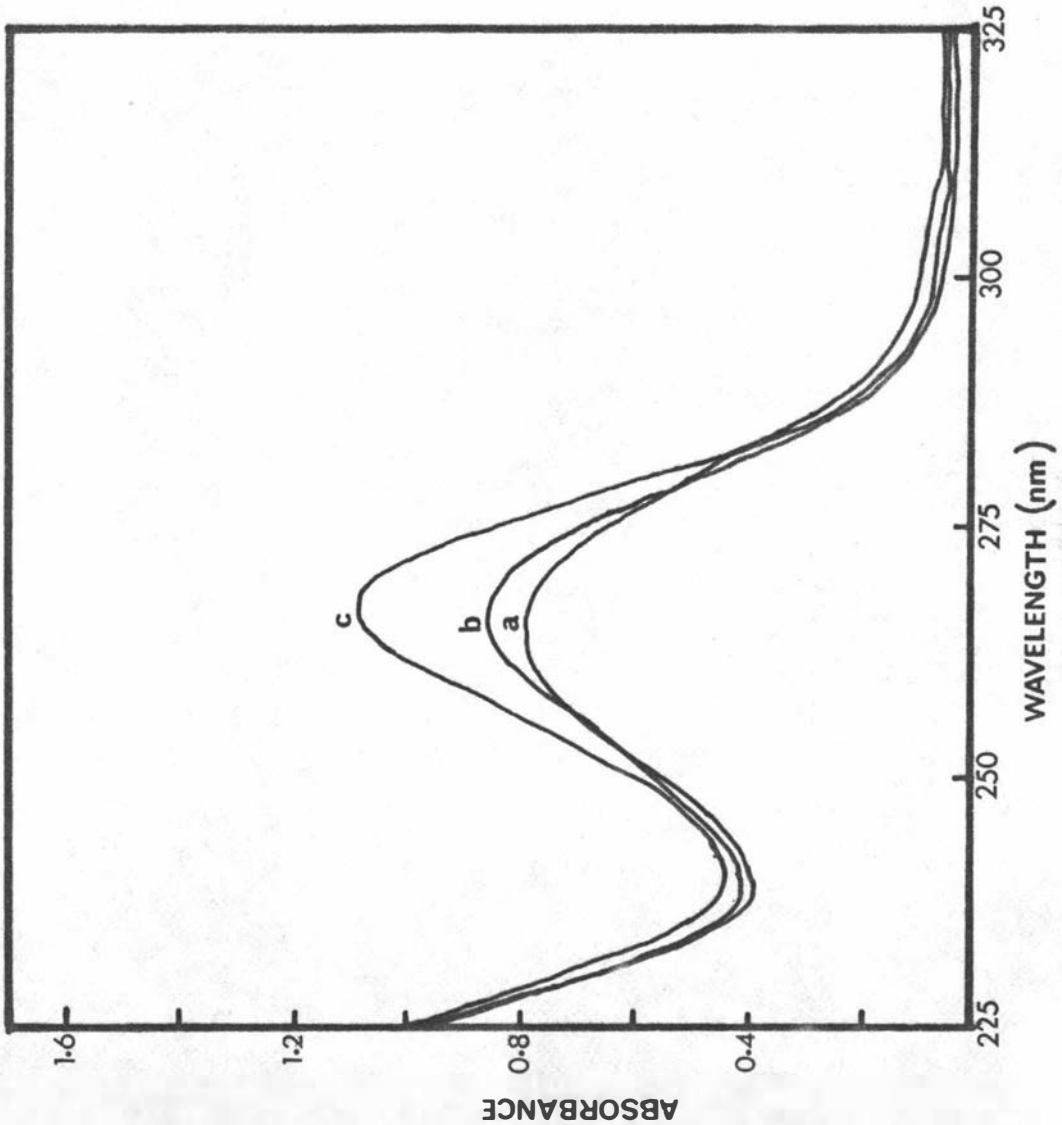
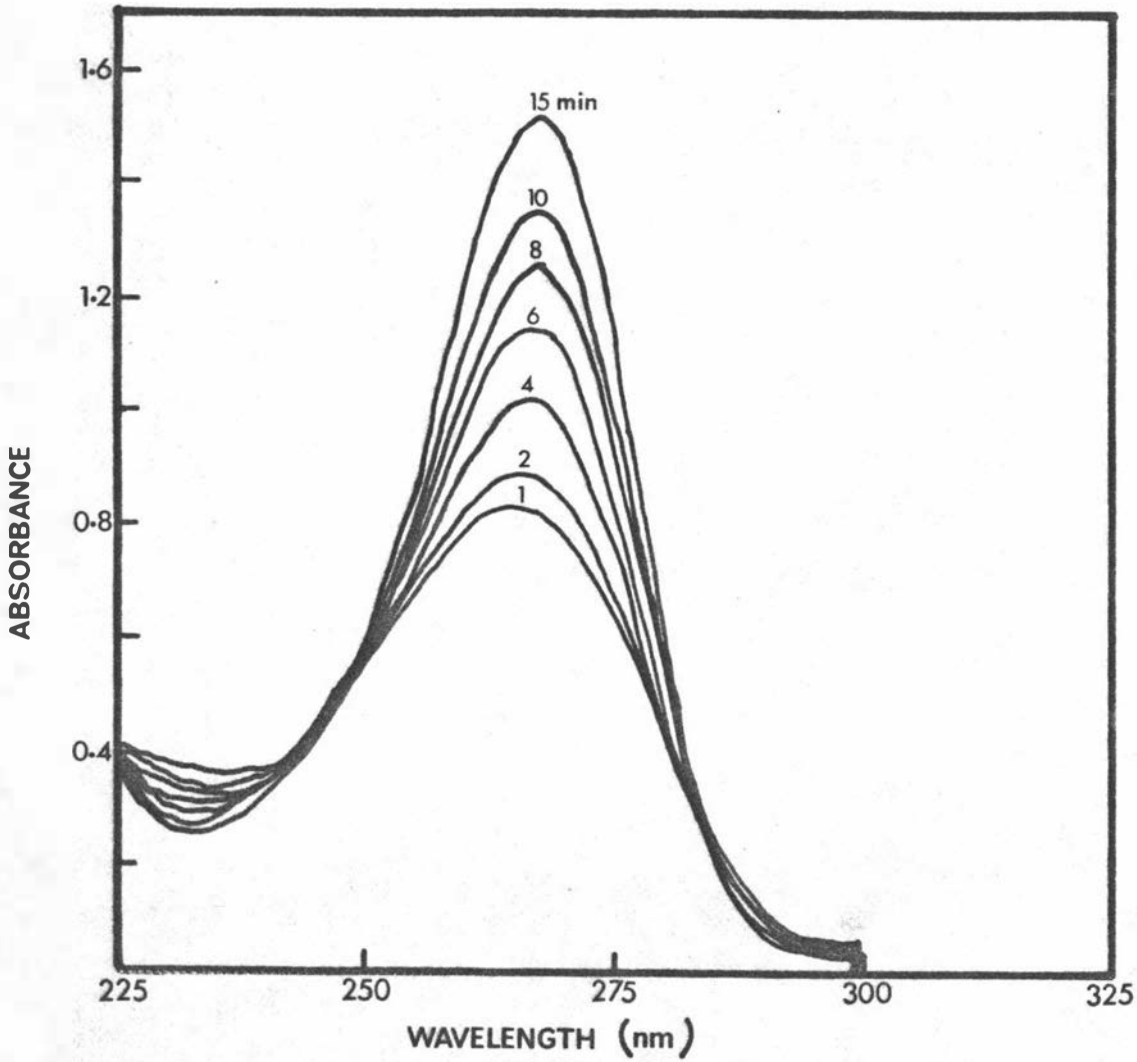

ABSORBANCE
1·6
1·2
0·8
0·4
225
250
275
300
325
WAVELENGTH (nm)
a
b
c

Figure 6.27 Ultraviolet spectra of the reaction mixture during the aerobic oxidation of thymine by uracil dehydrogenase in the presence of methylene blue

The standard assay mixture (3.0 ml) contained 0.3 µmol of thymine, 0.075 µmol of methylene blue, 150 µmol of Tris-HCl, pH 8.85 and 0.0164 units of uracil dehydrogenase (specific activity 10.2 units/mg) and was incubated at 25°C. The reference tube contained Tris-HCl and methylene blue at the same concentrations as the sample tube. The spectrum of the reaction mixture was recorded at the times indicated in the figure.


1·6
1·2
0·8
0·4
ABSORBANCE
15 min
10
8
6
4
2
1
225
250
275
300
325
WAVELENGTH (nm)

CHAPTER 7

DISCUSSION AND CONCLUSIONS

7.1 Purification of uracil dehydrogenase

Growth of the organism:

The growth yield of Nocardia corallina is low for two main reasons, namely (a) the slow growth rate of the organism and (b) the low solubility of thymine in the growth medium (Section 3.1). In the routine cultivation of N. corallina, cells were grown for 20 h in 15 flasks (1 litre) and approximately 10 g of wet cells were obtained from 5 litres of culture medium. When the organism was required for large-scale enzyme isolations, the batch culture procedure was repeated daily, over a period of 10 days, to yield about 100 g of cells. Scaling up growth of the organism in a Fermacell fermenter (New Brunswick Scientific Co. Inc.), under forced aeration and stirring at 30°C, in 20 litres of the culture medium was not very successful since the organism adhered to the walls of the vessel above the level of the medium, even when antifoam was added.

Preparation of cell-free extracts:

The organism is difficult to disintegrate. Disruption of cells by passage through a French pressure apparatus was selected as the routine method (Section 3.2). The maximum yield of uracil dehydrogenase was obtained with a cell suspension at the optimum dilution of 1 : 15 (w/v), and it required two passages of each cell suspension through a manual operated pressure apparatus, to achieve this maximum yield.

Purification of uracil dehydrogenase:

Uracil dehydrogenase in most preparations of cell-free extracts was not very stable, losing half of its initial activity when stored at 0°C for 30 h. Neither 2-mercapto-ethanol (1mM) nor ethylene diamine tetraacetate (1mM) had any appreciable stabilizing effect on the enzyme during storage. The cause of the instability of the enzyme is not well understood. Treatment of the extracts with calcium phosphate gel results in purification and an improvement in the stability of the enzyme and was therefore employed as the first step in the purification procedure. The enzyme extracted from calcium phosphate gel was rapidly concentrated by chromatography on a DEAE-cellulose column. Alternatively, ultrafiltration could be used for concentration resulting in a recovery of approximately 95% of the enzyme. A further 4-fold purification was achieved by ammonium sulphate fractionation and this was followed by gel filtration through a column of Sepharose-4B which both desalted the enzyme and yielded a small degree of purification. The specific activity of most enzyme preparations from DEAE-Sephadex A-50 chromatography did not increase. However, if this step was omitted, the enzyme preparation obtained from the final step, Sephadex G-200 gel filtration, showed additional, minor, enzymatically-inactive bands, on polyacrylamide gel electrophoresis.

The purification procedure (Section 4.2, Table 4.1) has been very reproducible yielding approximately 85-fold purification in the specific activity with an 18% recovery of the initial activity present in the cell-free extracts. It is a rather more complex purification procedure than

required for most enzymes, and is comparable with the purification scheme for thymine 7-hydroxylase from Neurospora crassa reported by Liu et al. (1973).

Homogeneity:

The purity of the enzyme preparation from the final step, Sephadex G-200 gel filtration, was judged by several criteria. The elution pattern of the enzyme (Figure 4.5) from the Sephadex G-200 column gave a single protein peak which could be superimposed on the activity peak. The purified enzyme preparation when submitted to polyacrylamide gel electrophoresis (Section 4.3) yielded only one major band and two slow-moving minor protein bands which were all enzymatically active. The minor protein bands account for no more than 2 to 5% of the total protein. Furthermore, a high degree of purity was indicated by the absence of leucine, an amino acid found in most proteins. Calculations based on the recovery of activity and the 85-fold increase in specific activity indicated that the enzyme may represent as much as 1.2% of the total protein in the initial cell-free extracts.

7.2 Structure of uracil dehydrogenase

Molecular weight:

The molecular weight of uracil dehydrogenase was determined by two independent methods (Section 5.1). Using the method of gel filtration on Sephadex G-200, an approximate molecular weight of 300 000 was obtained. As shown in Figure 5.1, the enzyme was eluted from the column at the lower part of the calibration curve extending beyond the linear calibration range. A value of 10% for the uncertainty in the molecular weight determination would seem to approp-

riate. A more accurate result might have been obtained by including appropriate reference proteins ranging between 250 000 and 500 000. One of the most accurate procedures for determining the molecular weight of proteins is by sedimentation equilibrium ultracentrifugation, although this method does require a value of the partial specific volume, $\bar{v}$, of the protein. In the present work the apparent specific volume of uracil dehydrogenase has been calculated from its amino acid composition (Section 5.3), and this has been used in the calculations for the sedimentation equilibrium experiments. Using this approach, a molecular weight of 298 000 has been determined for uracil dehydrogenase, and this value was used where required throughout the thesis.

Subunit structure:

Polyacrylamide gel electrophoresis of uracil dehydrogenase in the presence of the powerful denaturing agent SDS, indicated that the enzyme is composed of three distinct subunits (Section 5.2). The mobility of polypeptides in SDS-polyacrylamide gels is proportional to the logarithm of their molecular weights and by including appropriate standards it was determined that the uracil dehydrogenase subunits possess molecular weights of 92 000, 36 000 and 21 000. From a scan of the stained gel it seemed likely that these polypeptides were present in the native molecule in an equimolar ratio. By comparison with the molecular weight of the enzyme, as determined by the sedimentation equilibrium analysis, it is suggested that the native molecule contains two each of the three dissimilar polypeptides.

When uracil dehydrogenase is fractionated by poly-

acrylamide gel electrophoresis in the absence of detergents, some enzymatically active minor bands are observed and these are thought to correspond to oligomers of the enzyme. Consistent with this interpretation is the observation that the protein corresponding to these minor bands possess identical subunit structure, as determined by SDS-polyacrylamide gel electrophoresis, to the major protein band. One puzzling aspect of this finding was that both the major and minor bands gave rise to a polypeptide of molecular weight 71 000, in addition to the expected polypeptides of molecular weights 92 000, 36 000 and 21 000. The most likely explanation of this result is that the additional polypeptide is the result of proteolytic cleavage of the polypeptide of molecular weight 92 000. The other additional polypeptide, with a molecular weight of 21 000, which would be expected from this explanation, (a) may have been removed during the initial acrylamide gel fractionation of the native molecule, (b) it may be present in the SDS-polyacrylamide gel, but undetected (it has a predicted molecular weight identical to the smallest subunit of uracil dehydrogenase), or (c) it may have been further degraded to smaller polypeptides. Certainly, the problems associated with proteases, which may be present as minor contaminants, or may represent an inherent property of the enzyme being studied, can complicate analyses based on SDS-polyacrylamide gel electrophoresis (Weber et al., 1972).

Amino acid composition:

No previous study of the amino acid composition of uracil dehydrogenase has been published. The present result (Table 5.1) shows that the amino acid composition of the

purified enzyme is characterized by a high content of cystine (half) residue (16.6% of total residue) and an absence of leucine. By contrast, Bray et al. (1965) reported that a highly purified milk xanthine oxidase, which is a nonheme iron flavoprotein, contained 1.5% of cystine (half) and 8.7% of leucine. The apparent specific volume, $\bar{v}$, of uracil dehydrogenase has been calculated from the amino acid composition (Section 5.3) giving a value of 0.70 ml/g. This value is lower than $\bar{v}$ values for the majority of proteins, which fall in the range 0.72 to 0.74 ml/g (Edelstein & Schachman, 1973), and reflects, in addition to the high content of cystine, large amounts of glutamic acid (10.1% of total residue), aspartic acid (8.4%) and glycine (8.7%), all of which have low specific volumes.

Metal content:

Metal analysis by atomic absorption spectrophotometry showed that the enzyme is an iron metalloenzyme, and the iron content based on the molecular weight of the enzyme of 298 000 was calculated to be four atoms of iron per mole of protein (Table 5.5). Since exhaustive dialysis of the enzyme against iron chelating agents did not cause any loss of iron or inhibition of enzyme activity, the iron must be bound very tightly to the protein and is presumably essential for enzyme activity. Since only one determination could be made for molybdenum, because of the limited supply of the purified enzyme, the presence of molybdenum is still doubtful. In addition to the presence of iron; zinc, magnesium and copper were present in all three enzyme preparations which were tested. However, after dialysis against chelating agents, these latter ions were present at

considerably less than molar amounts and apparently are unrelated to enzyme activity.

Rajagopalan & Handler (1964) demonstrated with three iron containing flavoproteins (rabbit liver aldehyde oxidase, milk xanthine oxidase, and Zymobacterium oroticum dihydro-orotic acid dehydrogenase), a direct proportionality between the absorbance at 550 nm, where flavins exhibit no absorbance, and the nonheme iron content. From the absorbance at 550 nm (Figure 5.7) of uracil dehydrogenase, and the molar extinction coefficient of 2900 litre·mol^{-1}·cm^{-1} for iron flavoproteins (Rajagopalan & Handler, 1968), it was calculated that 3.9 atoms of iron were present per mole of enzyme (molecular weight 298 000). This value is in excellent agreement with the value obtained from the atomic absorption spectrophotometric analyses.

Labile sulphide content :

The results in Section 5.8 showed that uracil dehydrogenase contains approximately 4 atoms of labile sulphide per mole of the enzyme. These results show that uracil dehydrogenase contains equimolar amounts of nonheme iron and labile sulphide. Similar observations have been made for other nonheme iron flavoproteins, including pig liver xanthine oxidase, bovine milk xanthine oxidase and dihydro-orotic dehydrogenase from Z. oroticum (Brumby et al., 1965), chicken liver xanthine dehydrogenase (Rajagopalan & Handler, 1968), and glutamate synthase from Escherichia coli (Miller & Stadtman, 1972).

Spectral characteristics:

Spectral analyses of uracil dehydrogenase indicated that the enzyme is a nonheme iron flavoprotein (Section 5.4). The flavin prosthetic group was identified as FAD by three different methods: namely, paper chromatography, increase in fluorescence by snake venom phosphodiesterase, and activation of the D-amino-acid oxidase apoenzyme (Section 5.5).

The ultraviolet absorption spectrum of the enzyme (Figure 5.6) gave an A_{280}/A_{260} ratio of 1.16, which reflects the strong absorption of FAD at 260 nm, with a millimolar extinction coefficient of 37.0 litre$\cdot$mol$^{-1}\cdot$cm^{-1} (Whitby, 1953). For a typical protein, crystalline enolase, the ratio A_{280}/A_{260} equals 1.75 (Warburg & Christian, 1941).

The visible and near ultraviolet absorption spectrum of uracil dehydrogenase (Figure 5.7) is typical of an iron flavoprotein. FAD and FMN exhibit an A_{375}/A_{450} ratio of 0.82 and 0.85, respectively (Whitby, 1953), and, in general, these absorption characteristics are indicative of flavoproteins. However, uracil dehydrogenase has an A_{375}/A_{450} ratio of 1.18, which is significantly different from the absorption characteristics of most flavoproteins and reflects a contribution from the iron chromophore. The absorbance of simple flavoproteins which appear to include no metal component, such as D-amino-acid oxidase (Massey *et al.*, 1961), and L-lactate oxidase (Sullivan, 1968), in the region of 450 nm, is directly proportional to the flavin content. By contrast, nonheme iron flavoproteins such as xanthine oxidases from calf liver and milk (Kielley, 1955; Rajagopalan & Handler, 1964), rabbit liver aldehyde oxidase

(Rajagopalan *et al.*, 1962), and xanthine dehydrogenases from chicken liver and *Clostridium cylindrosporum* (Remy *et al.*, 1955; Bradshaw & Barker, 1960) give similar spectra showing significant absorption in the 550 nm region and a greater absorbance in the 450 nm region than that expected from their flavin contents. Rajagopalan & Handler (1968) reported the expected ratios of the absorbance at 450 nm and 550 nm for different iron-to-flavin ratios, based on the molar extinction coefficient of 11300 litre·mol^{-1}·cm^{-1} for flavin at 450 nm, and approximately 5900 and 2900 litre·mol^{-1}·cm^{-1} at 450 nm and 550 nm, respectively, for iron. Uracil dehydrogenase has an A_{450}/A_{550} ratio of 3, and is therefore comparable to that of bovine milk xanthine oxidase, rabbit aldehyde oxidase (Rajagopalan & Handler, 1964) and xanthine dehydrogenases from chicken liver and *Micrococcus lactilyticus* (Aleman *et al.*, 1965) which have nonheme iron to flavin ratios of 4 to 1. Spectral analyses were made to obtain the calculated difference spectrum (enzyme minus FAD) of uracil dehydrogenase (Section 5.4). There is a similarity between the uracil dehydrogenase difference spectrum, characterized by peaks at approximately 470 nm and 420 nm with a shoulder at 320 nm (Figure 5.9), and the difference spectra of rabbit liver aldehyde oxidase, and milk xanthine oxidase (Rajagopalan & Handler, 1964). These characteristic absorbance peaks reflect the amount and nature of the protein-bound iron (Rajagopalan & Handler, 1964). However, the difference spectrum of uracil dehydrogenase shows a three banded-peak in the region 400 nm to 500 nm with the additional peak at approximately 435 nm. The latter peak is thought to be due to the difference in the absorption spectrum of protein-

bound flavin of uracil dehydrogenase compared with free flavin. Free FAD in aqueous solution at pH 7.0 has a visible spectrum with peaks at 375 nm and 450 nm (Whitby, 1953), whereas for most flavoproteins, these peaks are shifted by a small, but varying degree (Palmer & Massey, 1968).

FAD content:

From spectral analysis it was estimated that there was one mole of FAD per mole of uracil dehydrogenase (Section 5.6). By contrast, fluorometric analysis gave a value of 0.25 mole of FAD per mole of the enzyme. The lower value obtained from fluorometric analysis was possibly due to FAD being not quantitatively released from the apoenzyme under the conditions of extraction used (10% trichloroacetic acid, 4° C, 15 min). In most flavoproteins, e.g., milk xanthine oxidase and rabbit liver aldehyde oxidase (Rajagopalan & Handler, 1964) and glutamate synthase from Escherichia coli (Miller & Stadtman, 1972), FAD or FMN are held by noncovalent linkages and are completely released by heating or denaturation with acids. By contrast, the flavin prosthetic group of enzymes, such as succinic dehydrogenase from aerobic yeast or mammalian mitochondria (Singer et al., 1957; Kearney, 1960), sarcosine dehydrogenase and dimethylglycine dehydrogenase from mitochondria of rat liver (Frisell & Mackenzie, 1962) and monoamine oxidase of bovine kidney mitochondria (Erwin & Hellerman, 1967) are held by covalent bonds to the peptide chain and only liberated after prolonged proteolytic digestion. The covalently bound-FAD of succinic dehydrogenase was first isolated as a pure flavin

peptide in 1960 by Kearney. Details concerning this tightly bound flavin can be found in reviews by Singer et al. (1957) and Beinert (1960) and recent studies have been described by Singer et al. (1971), Singer & Edmonson (1974), and Singer & Kenney (1974).

Free FAD is released from the enzyme after denaturation of the protein by heating since the flavin from uracil dehydrogenase was shown to be capable of activating the D-amino-acid oxidase apoenzyme (Section 5.5.3). Unfortunately the rate of release of FAD from uracil dehydrogenase has not been examined. Therefore it is not possible to distinguish between the enzyme containing one or two types of bound FAD. If one type of bound FAD is present then it is released very slowly. If two types of FAD are present, in the ratio of 1 : 3, then the latter type is very tightly bound and resistant to release by acid or heat treatment. Irrespective of the exact nature of the bound FAD, it is concluded from the spectral analysis that there is one mole of FAD per mole of uracil dehydrogenase.

7.3 Properties of uracil dehydrogenase

The enzyme appears to have a high substrate specificity with only thymine, uracil and 5-substituted uracils serving as substrates (Section 6.5). All compounds tested which differed from uracil in substitutions at position 1, 2, 4, or 6 were inactive, as also were the 5,6-dihydro-pyrimidines (Table 6.1).

The K_m values for thymine and uracil have been shown to be 1.08×10^{-4} M and 4.0×10^{-4} M, respectively (Table 6.3).

These values are approximately three times greater than the corresponding values of 0.35×10^{-4} M and 1.31×10^{-4} M reported by Hayaishi & Kornberg (1952) for a 2.5-fold purified uracil-thymine oxidase from a Mycobacterium sp. If thymine and uracil bind to identical sites on the enzyme then competition between the two substrates should result in an apparent increase in the K_m for both pyrimidines. When the apparent K_m for thymine was determined in the presence of uracil (Section 6.8) a value of 0.86×10^{-4} M was obtained, which is less than the K_m determined in the absence of the competitor. One interpretation of this result is that uracil and thymine bind to non-identical sites and that the binding of uracil enhances the binding of thymine. However, because of the problems associated with obtaining spectral analyses when both substrates are present (Section 2.3.4), further studies are required to clarify this point.

The effect of a variety of compounds on the activity of uracil dehydrogenase was investigated (Section 6.9). The enzyme was inhibited to various degrees by all the metal ions tested (Table 6.4) and chelating agents generally stabilized the enzyme during storage (Figure 6.21). An exception was 8-hydroxyquinoline, its presence during enzyme storage resulting in a significant loss in enzyme activity. The thiol reagents, p-chloromercuribenzoate and iodoacetate, inhibited the enzyme (Table 6.5) suggesting that an essential reactive thiol group is present. A similar sensitivity to p-chloromercuribenzoate is exhibited by xanthine oxidase from milk and chicken liver (Mackler et al., 1954; and Doisy et al., 1955).

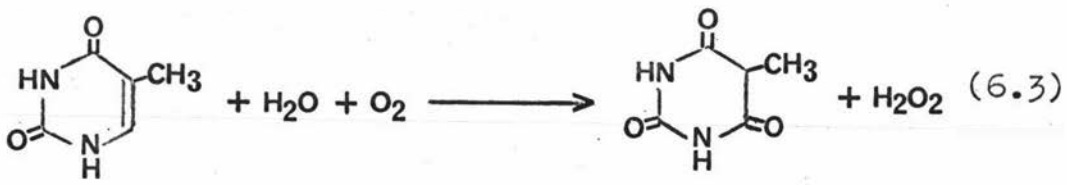
Uracil dehydrogenase, like many other metalloflavo-

proteins (Rajagopalan & Handler, 1968), can utilize a wide variety of electron acceptors (Table 6.2). Among the natural electron acceptors, cytochrome C, but not NAD or NADP, was found to act satisfactorily. The purified enzyme is also capable of reacting with oxygen in the absence of any added electron acceptor, although the rate is only 1/2000th that obtained with methylene blue under aerobic conditions (Section 6.12). This is the first demonstration of such an aerobic dehydrogenase activity for uracil dehydrogenase and contrasts with previous accounts for 'uracil oxidase' from a Bacterium sp. (Wang & Lampen, 1952b), 'uracil-thymine oxidase' from a Mycobacterium sp. (Hayaishi & Kornberg, 1952), and enzyme extracts from Nocardia corallina (Lara, 1952b). In these cases neither the uptake of oxygen nor the removal of substrate was reported to occur when thymine or uracil was incubated with enzyme in the presence of oxygen alone. Remy et al. (1951 and 1955) have reported that xanthine dehydrogenase from chicken tissues, which catalyses the oxidation of xanthine or hypoxanthine, exhibits aerobic activity of only about 1% of that in the presence of methylene blue. By convention such oxidoreductases, like uracil dehydrogenase, are known as dehydrogenases and not oxidases.

The rate of thymine oxidation, by uracil dehydrogenase, in the presence of methylene blue is markedly decreased by the absence of oxygen (Figure 6.25). This decrease in the rate does not appear to be simply a reflection of the depletion of methylene blue. It has been reported by Handler et al. (1964) for a group of nonheme iron flavoproteins (bovine milk xanthine oxidase, rabbit liver aldehyde oxidase, and dihydroorotic acid dehydrogenase from Zymobacterium oroticum), that reduction of oxygen occurs resulting in the

formation of the superoxide radical, $O_2^{\cdot -}$. McCord & Fridovich (1969) reported that in the aerobic oxidation of xanthine by milk xanthine oxidase, $O_2^{\cdot -}$ is generated and is the actual reductant of cytochrome C. In their later studies (1970) using chicken xanthine dehydrogenase, these authors demonstrated the production of $O_2^{\cdot -}$ in the presence of methylene blue. Massey *et al.* (1969) demonstrated many flavoprotein oxidases do not produce significant amounts of $O_2^{\cdot -}$, whereas the flavoprotein dehydrogenases do. The likely possibility that $O_2^{\cdot -}$ is produced in the aerobic oxidation of thymine by methylene blue-uracil dehydrogenase has not been examined in the present investigation.

It has been demonstrated with cell-free extracts (Wang & Lampen, 1952b; and Lara, 1952b) and *in vivo* (Batt & Woods, 1961) that the oxidation of thymine or uracil results in the consumption of 0.5 mole of oxygen per mole of pyrimidine. By contrast, purified uracil dehydrogenase consumes 1 mole of oxygen per mole of thymine (Section 6.10). It is suggested that this discrepancy is due to the presence of catalase in cell-free extracts or *in vivo*, which releases half of the consumed oxygen by the decomposition of hydrogen peroxide produced during the reaction. By analogy to the oxidation of xanthine by xanthine oxidase (Aleman *et al.*, 1965), it may be assumed that during the oxidation of thymine catalysed by uracil dehydrogenase, the oxygen atom introduced into the substrate molecule arises from water. Possibly through the intermediate formation of a superoxide radical, oxygen is reduced by uracil dehydrogenase to hydrogen peroxide. The stoichiometry of the reaction is given in equation 6.3



(6.3)

7.4 Future studies

Metalloflavoproteins include some of the most complex of all known enzymes. In this investigation, such a protein, uracil dehydrogenase, has been purified from Nocardia corallina and its basic structural features have been characterised. Some features of the enzymic reaction have been studied, but clearly much remains to be done. Fields for future study include the nature of active site, a detailed analysis of the role played by iron-sulphur, FAD, and oxygen in the electron transfer scheme, and the identification of the in vivo electron transport system.

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