Decomposition of Pyrimidines by *Nocardia corallina*

By R. D. BATT* AND D. D. WOODS

Microbiology Unit, Department of Biochemistry, University of Oxford

(Received 27 July 1960)

SUMMARY

A bacterial species which degrades the pyrimidines, uracil, thymine and cytosine by induced enzymes has been characterized as *Nocardia corallina* (strain S). All other strains of *N. corallina* investigated oxidized thymine, but varied in their abilities to oxidize uracil and cytosine.

Organisms adapted to pyrimidines converted uracil to barbituric acid and thymine to 5-methylbarbituric acid. Oxidation of uracil by thymine-grown organisms was almost entirely by a pathway in which barbituric acid was an intermediate. Oxidation of thymine by uracil-grown organisms was similarly almost entirely via 5-methylbarbituric acid.

Oxidation of uracil by uracil-grown organisms and of thymine by thymine-grown organisms occurred, at least in part, through the respective barbituric acids. Discrepancies between the theoretical and observed values for O₂ uptake suggested however that other pathways may also occur in these cases.

Pyrimidine-grown organisms oxidized 2-thiouracil to 2-thiobarbituric acid and 2-thiothymine to a compound which was probably 5-methyl-2-thiobarbituric acid. These products were not further degraded by the organism.

Barbituric acid was oxidized by uracil-grown organisms to CO₂, NH₃ and urea with concurrent oxidative assimilation. The oxidation of barbituric acid was inhibited by isobarbituric acid and sodium azide although barbiturase activity in cell-free extracts was not affected by these substances. Barbiturase preparations converted barbituric acid anaerobically to malonic acid, CO₂ and NH₃, but barbituric acid was not degraded by whole organisms under anaerobic conditions. Whole organisms, grown on uracil, degraded urea but did not oxidize malonic acid. Acetic and propionic but not malonic or barbituric acids were activated by cell-free extracts as judged by hydroxamate formation. From the evidence presented, it is unlikely that free malonic acid is an intermediate in the breakdown of barbituric acid.

INTRODUCTION

Pyrimidine catabolism may be initiated either by a reduction yielding a dihydropyrimidine or by an oxidation giving a barbituric acid. The degradation of pyrimidines through dihydropyrimidines has been described in (a) animal tissues (Fink, Fink & Henderson, 1958; Fink, Cline, Henderson & Fink, 1956; Canellakis, 1956; Fritzson, 1957; Fritzson & Pihl, 1957); (b) *Pseudomonas aeruginosa* for uracil and thymine (Fink, Cline & Koch, 1954); (c) *Clostridium uracilicum* for uracil

* Present address: Department of Biochemistry, University of Otago Medical School, Dunedin, New Zealand.

(Campbell, 1957*a-c*); (d) *Zymobacterium oroticum* and some species of *Corynebacterium* for orotic acid (Lieberman & Kornberg, 1953, 1954, 1955; Reynolds, Lieberman & Kornberg, 1955). A reductive pathway for uracil catabolism by *Torula utilis* was suggested from growth studies (Di Carlo, Schultz & Kent, 1952), but it is now known that dihydro-orotic acid is not attacked by this organism (Batt, Martin & Ploeser, 1953).

Pyrimidine catabolism with barbituric acids as intermediates has been demonstrated with certainty only in bacteria; species active in this respect have been isolated by the elective culture technique with pyrimidines as main source of carbon and nitrogen. The organisms obtained include a *Mycobacterium* sp. and a *Corynebacterium* (strain 161) (Hayaishi & Kornberg, 1952), a *Bacterium* sp. (Wang & Lampen, 1952*a*) and two strains of *Nocardia corallina* (Lara, 1952*a*; Batt & Woods, 1951). Results obtained, with both whole organisms and partially purified

Fig. 1. Pathways of oxidative catabolism of pyrimidines (after Hayaishi & Kornberg, 1952).

enzyme preparations, have led to the schemes for oxidative pyrimidine catabolism shown in Fig. 1. Barbituric acid was isolated as a product of uracil oxidation by whole organisms (Hayaishi & Kornberg, 1952), and this reaction has also been demonstrated with enzyme preparations from several different bacteria (Hayaishi & Kornberg, 1952; Wang & Lampen, 1952*b*; Lara, 1952*b*). The conversion of thymine to 5-methylbarbituric acid has been shown with enzyme preparations (Hayaishi & Kornberg, 1952), but the acid has not been clearly demonstrated to be an intermediate with intact organisms.

Barbituric acid was hydrolysed to malonic acid and urea by extracts of uracil-adapted organisms of a strain of *Mycobacterium* (Hayaishi, 1952) and a similar enzyme (barbiturase) was described in cell-free extracts of *Nocardia corallina*

grown on thymine (Lara, 1952*b*). Crude extracts of both these organisms showed urease activity and the products of the breakdown of barbituric acid were malonic acid, NH_3 and CO_2 . Intact organisms of both species oxidized barbituric acid.

Barbiturase had no action on 5-methylbarbituric acid, 2-thiobarbituric acid or isobarbituric acid (Hayaishi & Kornberg, 1952). Intact organisms of the *Mycobacterium* sp. were tested for barbiturase activity by Hayaishi & Kornberg (1952) who stated that 'the removal of barbituric acid, which is carried out by cell-free enzymes at the same rate in the presence or absence of oxygen, does not occur under anaerobic conditions when intact cells are used'. The possibility was considered that energy may be required for the entry of barbituric acid into the organisms. Malonic acid was poorly utilized by uracil-adapted *Mycobacterium* sp. and although the organism may be impermeable to malonic acid, the possibility was suggested by Hayaishi & Kornberg (1952) that barbiturase produced from barbituric acid a labile derivative of malonic acid which was degraded to malonic acid by the isolation procedures.

The present paper describes the isolation and characterization of a bacterium able to utilize pyrimidines as the main source of carbon and nitrogen for growth. The main object of the work done with it was to investigate the possible pathways of degradation of a given pyrimidine when the organism was grown on the same or a different pyrimidine.

METHODS

Organisms. The bacterium with which most of the work was done was isolated from a sample of medium A (0.5% KH_2PO_4 , 0.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.2% CaCl_2 ; pH 7.2) containing uracil (0.5%) which had been exposed to the atmosphere for 12 hr. at room temperature (Batt & Woods, 1951). Following successive single colony isolations on tryptic meat digest agar, the organism was identified by Professor H. L. Jensen as a strain of *Nocardia corallina*; it will be referred to subsequently as strain S of this species.

The organism was aerobic, Gram-positive, non-motile, rod-shaped, non-sporulating, not acid-fast and it exhibited considerable pleomorphism when grown on different media. Neither acid nor gas was produced from glucose, sucrose, lactose, maltose, dulcitol or salicin, whereas acid but no gas was formed from sorbitol and mannitol. Gelatin was not liquefied and milk was not clotted; H_2S was produced by the organism growing in tryptic meat digest broth. By direct microscopy of young cultures (12–24 hr.) on agar small mycelia with granules were observed which were, possibly, the rudiments of aerial hyphae.

Four other strains of *Nocardia corallina* were made available by Professor Jensen and were designated strains P.F.M., 117, K_3 and Th_3 . The growth conditions established for strain S were used for these organisms.

Growth tests. Medium A (5 ml.) supplemented with the test substance (20 mm) and Difco yeast extract (0.01%) was distributed in 150 × 19 mm. tubes and autoclaved at 121° for 20 min. After inoculation with a dilute suspension of organisms (0.1 ml.; 0.05 mg. dry wt./ml.) harvested from a tryptic meat agar slope (30°; 24 hr.) the tubes were incubated at 30° in racks sloped at 5° above horizontal. Growth was estimated with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with a neutral density filter. The relationship between dry wt. of

organisms and instrument reading was linear up to a reading of 40; a reading of 20 was equivalent to 0.2 mg. dry wt. of organisms/ml.

Preparation of suspensions of organisms. For most experiments medium *A* (150 ml.) supplemented with a pyrimidine (1 g./l.) and yeast extract (Difco) (0.1 g./l.), was distributed in Roux bottles and autoclaved at 121° for 20 min. The inoculum was 0.75 mg. dry wt. of organisms harvested from a tryptic meat agar slope (24 hr.) The bottles were incubated horizontally in air at 30° for 65 hr. The organisms were harvested by centrifuging (2000 g for 20 min.), washed in the culture volume of 67 mM-phosphate buffer, pH 7.2, and resuspended in the same buffer at 10 mg. dry wt./ml.

For a few experiments the organisms were grown on tryptic digest of meat broth under the same conditions and with the same inoculum.

Preparation of cell-free enzyme preparations. A suspension of washed organisms (60 mg. dry wt. in 6 ml. of 67 mM-phosphate buffer, pH 7.2) was mixed with 4 g. ballotini beads (No. 13; Chance Bros., Smethwick, Staffs.), and 0.1 ml. of tributylcitrate, cooled in ice-water and shaken on a vibratory tissue disintegrator (H. Mickle, Gomshall, Surrey; Mickle, 1948) at maximum amplitude for 30 min. After centrifuging at 2500 g the straw-coloured supernatant fluid was stored at 0°.

Acetone powders were prepared by suspending 100 mg. dry wt. of organisms in 5 ml. of distilled water, chilling, and adding with vigorous stirring to acetone (50 ml.) previously cooled to -10°. The deposit after centrifuging was resuspended in acetone (20 ml.) and again centrifuged. After three further washings with acetone (10 ml.) the residue was freed from acetone by evacuating for 3 hr.; the yield of powder was 73 mg.

Estimations. Manometric estimations of O₂ consumption and CO₂ production were carried out by conventional methods (Umbreit, Burris & Stauffer, 1949). The manometer vessels contained 0.1 M-phosphate buffer (pH 7.2; 1.0 ml.), suspension of organisms (0.5 ml.) equivalent to 5 mg. dry wt. and distilled water (0.5 ml.) in the main compartment and 20 mM substrate solution (0.5 ml.) or distilled water (for the controls) in the sidebulb. The temperature was 30°.

For the estimation of non-gaseous products, the manometer vessels were chilled in ice-water, the contents poured into chilled tubes and centrifuged. The clear supernatant fluids were decanted and stored at 0°.

Pyrimidines were estimated spectrophotometrically. The samples were diluted 1 in 50 with either 0.1 N-HCl or 0.1 N-NaOH. The estimation of pyrimidines in binary mixtures followed the method described by Hotchkiss (1948) for determining purines in mixtures. The method of Markham & Smith (1949) was used for the separation and identification of the pyrimidines on paper chromatograms.

Ammonia was estimated in the presence of urea by distillation in the apparatus described by Markham (1942) after the addition of 0.35 M-borate buffer (pH 8.5). Urea was estimated by the difference in ammonia content of the solution before and after incubation with urease (Nimmo-Smith & Appleyard, 1956).

The method of Rose (1955) was used for the detection and estimation of hydroxamates formed from hydroxylamine. The enzyme preparation (0.5 ml.) was incubated in a total volume of 0.85 ml. with the substrate (340 μ mole), adenosine triphosphate (8 μ mole), Tris(2-amino-2-hydroxymethylpropane-1:3-diol; 100 μ mole), MgCl₂ (10 mole) and hydroxylamine (930 mole); pH 7.7. After 30 min. at 30°, 10% (w/v)

trichloroacetic acid (1 ml.) was added and the mixture centrifuged. Ferric chloride solution (4 ml.; 1.25 % (w/v) in N-HCl) was added to the supernatant fluid and the colour intensity estimated in a photoelectric colorimeter using a 540 m μ filter.

Chemicals. 5-Methylbarbituric acid was synthesized by the method of Holmberg (1945) from diethylmethylmalonate and urea, the product obtained having a m.p. of 197° (uncorrected). 5-Hydroxymethyluracil was prepared by reacting formaldehyde with uracil in an alkaline solution (R. E. Cline, personal communication). Thymine glycol was synthesized by the method of Baudisch & Davidson (1925), and melted with decomposition at 210°. All other compounds used were obtained commercially.

RESULTS

Growth of the organism on pyrimidines

Nocardia corallina (strain S) grew in medium *A* containing either uracil, thymine, cytosine, 5-hydroxymethyluracil, barbituric acid or 5-methylbarbituric acid as the main source of carbon and nitrogen. In each case the lag phase was shortened by the addition to the medium of a small amount of yeast extract (Difco; 0.01 %) which also supplied a growth requirement of the organism for thiamine (Martin & Batt, 1957). The growth response to various pyrimidines of the five available strains of *N. corallina* were compared (Table 1); all grew well on thymine but they differed in their ability to utilize uracil and cytosine.

Table 1. *Growth of strains of Nocardia corallina on pyrimidines*

The medium (see Methods section) contained the pyrimidine stated (20 mM); incubated for 72 hr.

Strain of <i>N. corallina</i>	Growth (EEL reading) with		
	Uracil	Thymine	Cytosine
S	10	10	7
P.F.M.	7	11	3
227	3	11	4
K ₃	1	15	2
Th ₃	0	10	0

The substances thought by Cerecedo (1927, 1931) to be intermediates in the catabolism of uracil and thymine in dogs (dialuric acid, isodialuric acid, isobarbituric acid and thymine glycol) did not support the growth of strain S. The following substances also failed to support the growth of this organism on medium *A* supplemented with 0.01 % yeast extract (Difco): 5:6-dihydrouracil, 5:6-dihydrothymine, orotic acid, 2-thiothymine, 2-thiouracil, 2-thiobarbituric acid, 6-methyluracil, isocytosine, 2-aminopyrimidine, 2-amino-5-methyl-4-hydroxypyrimidine, 5-amino-2:4-dihydroxypyrimidine, 4:6-dihydroxypyrimidine.

General action of suspensions of strain S on pyrimidines

Suspensions of organisms harvested after growth on either uracil or thymine rapidly oxidized uracil, thymine, barbituric acid and 5-methylbarbituric acid (Figs. 2, 3). Organisms grown on tryptic digest of meat broth oxidized all four pyrimidines

only after a lag of at least 4 hr.; the enzyme systems are therefore induced by the presence of the substrate.

Organisms grown on the pyrimidines and tested on uracil, thymine and the barbituric acids completely removed the substrates, i.e. the rate of O_2 uptake decreased to values equal to those for the endogenous respiration and no pyrimidine remained.

Fig. 2

Fig. 3

Fig. 2. Uptake of oxygen by uracil-grown organisms (strain S) acting on thymine (○), 5-methylbarbituric acid (●), uracil (◐) and barbituric acid (◑). Conditions as described in Methods section with $10 \mu\text{mole}$ of substrate. All values corrected for endogenous O_2 uptake.

Fig. 3. Uptake of oxygen by thymine-grown organisms (strain S) acting on thymine (○), 5-methylbarbituric acid (●), uracil (◐) and barbituric acid (◑). Conditions as for Fig. 2.

The endogenous respiration of suspensions was high and, under the test conditions, amounted to about one-third of the O_2 uptake resulting from the degradation of the pyrimidine. Justification for subtracting the endogenous O_2 uptake from the experimental values in the presence of pyrimidines was obtained by determining the O_2 uptake with a constant amount of organism and different amounts of uracil; the corrected O_2 uptake values were directly proportional to the amount of substrate (Table 2).

Table 2. *Total oxygen uptake with varying amounts of uracil*

Uracil-grown organisms (strain S) incubated (as in Methods section) with the amount of uracil stated. The reaction was continued until the rate of O_2 uptake with uracil decreased to that with the organism alone. Corrected values are those in which the endogenous O_2 uptake value has been deducted.

Amount of uracil (μmole)	O_2 uptake			
	Corrected		Uncorrected	
	μmole	$\mu\text{mole}/\mu\text{mole}$ uracil	μmole	$\mu\text{mole}/\mu\text{mole}$ uracil
17.90	18.6	1.04	24.2	1.35
8.95	9.3	1.04	18.1	1.46
4.47	4.5	1.01	7.3	1.63

Urea, NH_3 and CO_2 were produced from uracil, thymine, barbituric acid and 5-methylbarbituric acid by organisms grown on either uracil or thymine. However, the values for the final O_2 uptake were much lower than the theoretical values calculated for the complete degradation of the pyrimidines to CO_2 and NH_3 (Table 3). No products other than urea, NH_3 and CO_2 were detected at the completion of the experiments of Table 3. The possibility that some of the substrate was undergoing oxidative assimilation was tested by carrying out the oxidations in the presence of sodium azide. With organisms grown on a given pyrimidine and

Table 3. *Total oxygen consumption during the oxidation of various pyrimidines*

Suspensions of organisms (strain S) grown on either uracil or thymine were incubated as described in the Methods section with 10 μmole of the stated pyrimidine. The O_2 uptake values have been corrected for the endogenous uptake. The theoretical O_2 uptake values were calculated for the complete conversion of the pyrimidines to CO_2 and NH_3 .

Substrate	O ₂ uptake ($\mu\text{mole}/\mu\text{mole}$ pyrimidine) for organisms grown on		Theoretical O ₂ uptake ($\mu\text{mole}/\mu\text{mole}$ pyrimidine)
	Uracil	Thymine	
Uracil	1.01	1.48	2.5
Barbituric acid	0.87	1.08	2.0
Thymine	2.07	2.05	4.0
5-Methylbarbituric acid	1.47	0.88	3.5

tested on the same pyrimidine there was a marked increase in O_2 consumption although the theoretical value for complete oxidation was not reached (Table 4). With organisms grown on uracil and tested on thymine (and vice versa) even the lower concentration of azide used inhibited O_2 uptake (Table 4).

5:6-Dihydrouracil and 5:6-dihydrothymine were not oxidized by suspensions of organisms grown on uracil and thymine respectively.

Table 4. *Effect of sodium azide on the oxygen uptake with uracil and thymine*

Organisms grown as stated on either uracil or thymine were incubated as described in the Methods section with either uracil or thymine in the presence of the stated concentration of sodium azide. The reaction was normally continued until the O_2 uptake decreased to the endogenous rate; * however indicates that the results were calculated after 5 hr. incubation even though the rate was still above the endogenous value.

Conc. of azide (mm)	O ₂ uptake ($\mu\text{mole}/\mu\text{mole}$ pyrimidine) with			
	Uracil-grown organisms oxidizing		Thymine grown organisms oxidizing	
	Uracil	Thymine	Uracil	Thymine
0	1.07	2.12	1.43	1.90
5	1.92	0.62	1.06	2.82
20	1.5*	0.49	0.47	2.5*
Theoretical value for complete oxidation	2.5	4.0	2.5	4.0

Oxidation of uracil and thymine to the corresponding barbituric acids

Thymine oxidation by organisms grown on uracil. The rate of uptake of O_2 showed a sudden change at a value of approximately 0.5 mole O_2/mole thymine (Figs. 2, 4). After this time no thymine remained but a compound was present which absorbed

ultraviolet radiation. At the end of the first phase of thymine oxidation (about 60 min.) 0.12 mole CO_2 and 0.09 mole NH_3 /mole thymine were present but only traces of urea were detected (Fig. 4). The oxidation product of thymine had identical λ_{max} values in 0.1N-HCl (262 $\text{m}\mu$) and 0.1N-NaOH (268 $\text{m}\mu$) to those for synthetic 5-methylbarbituric acid. On the assumption that the oxidation product is 5-methylbarbituric acid, the amount present at the point of inflexion of the O_2 uptake curve is equivalent to 95–100% conversion of the added thymine to the acid. Both disappearance of thymine and production of 5-methylbarbituric acid were complete at the time O_2 consumption reached 0.5 mole/mole thymine originally present (Fig. 4).

Fig. 4

Fig. 5

Fig. 4. Metabolism of thymine (28 μmole) by organisms grown on uracil. Thymine (○), 5-methylbarbituric acid (●), O_2 uptake (◐), NH_3 (◑) and urea (◒). All values corrected for control values without substrate.

Fig. 5. Metabolism of thymine (28 μmole) by organisms grown on thymine. Symbols as for Fig. 4.

The primary oxidation product of thymine was isolated and characterized as 5-methylbarbituric acid as follows. Uracil-grown organisms were incubated with thymine in a number of manometer vessels until the change in rate of O_2 uptake occurred. After removing the organisms by centrifuging, the supernatant fluid was evaporated to dryness under vacuum. The residue was dissolved in a minimum volume of water and 30% (w/v) CaCl_2 added until no further precipitation occurred. The filtrate obtained after removal of the calcium phosphate was evaporated to dryness and the residue extracted with β -ethoxyethanol; the extract was again evaporated. Crystallization from ethanol yielded a white solid (30 mg., m.p. 193°) which gave a mixed melting-point with authentic 5-methylbarbituric acid (m.p. 197°) of 195° . The absorption maximum (262 $\text{m}\mu$ in 0.1N-HCl and 268 $\text{m}\mu$ in 0.1N-NaOH) and R_f values (Table 5) were identical with the values for 5-methylbarbituric acid.

The curve for O_2 uptake with 5-methylbarbituric acid was almost identical to the curve for the second phase of thymine oxidation (Fig. 2). 5-Methylbarbituric acid is spontaneously and rapidly oxidized in air, under both acid and neutral conditions, to 5-hydroxy-5-methylbarbituric acid (Nishikawa, 1931; Stuckey, 1942). When 5-methylbarbituric acid (10 μ mole) in phosphate buffer (0.08 M; pH 7.2) was shaken aerobically in a manometer vessel, the theoretical O_2 uptake for conversion to 5-hydroxy-5-methylbarbituric acid was reached in 30 min. However, when 5-methylbarbituric acid was incubated with a suspension of organisms (Fig. 1) in a solution of the same phosphate concentration and pH, the rapid conversion to 5-hydroxy-5-methylbarbituric acid did not occur; the products of the oxidation in the presence of the organism were CO_2 , NH_3 and urea. 5-Methylbarbituric acid was detected in the reaction mixture throughout the period of oxidation (5 hr.). The presence of the actively metabolizing suspension of organisms effectively suppressed the formation of 5-hydroxy-5-methylbarbituric acid; the latter compound was not itself oxidized by the organism.

Table 5. *Chromatography of pyrimidines and products formed from them by strain S*

Solvent systems: (A) *n*-butanol saturated with water, (B) isopropanol + water (100:30), (C) isopropanol + water ammonia; the same solvent as (B), a beaker containing ammonia (sp.gr., 0.88) being placed in the chromatography tank, (D) isopropanol + water + HCl (10N) (680:156:164).

All of the oxidation products showed as yellow spots on chromatograms sprayed with 0.1% methyl red in borate buffer pH 8.5 indicating that they were all acidic compounds.

	<i>R_F</i> with (solvent system)			
	(A) Butanol + water	(B) Isopropanol + water	(C) Isopropanol + water + ammonia	(D) Isopropanol + water + HCl
Pyrimidine				
Uracil	0.85	0.60	0.54	0.67
Thymine	0.51	0.69	0.64	0.76
2-Thiouracil	0.57	0.66	0.58	0.70
2-Thiothymine	0.69	0.75	0.67	0.79
Barbituric acid	0.05	0.20	0.43	0.49†
5-Methylbarbituric acid	0.05	0.23	0.49	—*
2-Thiobarbituric acid	0.05	0.26	0.48	0.66†
Oxidation product formed from				
Uracil	0.05	0.21	0.44	0.49†
Thymine	0.05	0.24	0.50	—*
2-Thiouracil	0.05	0.25	0.47	0.66†
2-Thiothymine	0.08	0.30	0.58	—*

* 5-Methylbarbituric acid spots were obtained with acid solvents only when relatively large quantities were chromatographed. The compound formed from thiothymine resembled 5-methylbarbituric acid in being unstable in acid solvents.

† The barbituric acids showed as yellow spots on chromatograms run with isopropanol + water + HCl. The oxidation product from thiouracil also showed as a yellow spot on the chromatograms.

Thymine oxidation by organisms grown on thymine. In this case O_2 consumption was more rapid and no sudden change in rate occurred (Figs. 3, 5). Only a small amount of 5-methylbarbituric acid accumulated transiently during the incubation (Fig. 5); the same was true of urea, but NH_3 was formed rapidly from the outset.

Uracil oxidation by organisms grown on thymine. A change in the rate of O_2 uptake was observed at a point corresponding to 0.5 mole O_2 /mole uracil (Fig. 8). After this point, no uracil remained but a compound had accumulated which absorbed ultraviolet radiation. This compound disappeared slowly on further incubation (Fig. 6). The absorption characteristics of the compound were identical in 0.1N-HCl (λ_{max} , 257 m μ) and 0.1N-NaOH (λ_{max} , 259 m μ) to the values for barbituric acid; the R_F values for the oxidation product determined on paper chromatograms with several solvent systems were the same as the values for barbituric acid (Table 5).

Fig. 6

Fig. 6. Metabolism of uracil (28 μ mole) by organisms grown on thymine. Uracil (○), barbituric acid (●), O_2 uptake (◐), NH_3 (●) and urea (○). All values corrected for control values without substrate.

Fig. 7

Fig. 7. Metabolism of uracil (28 μ mole) by organisms grown on uracil. Symbols as for Fig. 6.

At its maximum the amount of barbituric acid formed was equivalent to about 90 % of the uracil added (Fig. 6). Formation of NH_3 was linear from the outset, but urea was only detected after 2–3 hr. The rate of O_2 uptake in Fig. 6 is for 28 μ mole of uracil added initially; when the ratio of amount of organisms to pyrimidine concentration was reduced, as in this experiment, the change in rate of O_2 uptake was usually less pronounced than that shown in Fig. 3.

Uracil oxidation by organisms grown on uracil. As with the oxidation of thymine by thymine-grown organisms no abrupt changes in the rate of O_2 consumption were observed during the incubation (Figs. 2, 7). Accumulation of barbituric acid was small and transitory (Fig. 7). Urea and NH_3 production were both linear but the latter was the more rapid.

Oxidation of 2-thiouracil and 2-thiothymine

During a search for compounds capable of inhibiting pyrimidine catabolism it was found that strain S, grown in the presence of either uracil or thymine, oxidized 2-thiouracil and 2-thiothymine with an overall O_2 uptake of 0.5 mole O_2 /mole of 2-thiopyrimidine removed. The products of thiopyrimidine oxidation absorbed ultraviolet radiation. During the oxidation of 2-thiothymine there was a change in the $\lambda_{\max}$ value (pH 7.2) from 275 to 272 $m\mu$, while with 2-thiouracil as substrate the change was from 270 to 265 $m\mu$; the $\lambda_{\max}$ for 2-thiobarbituric acid at pH 7.2 is 265 $m\mu$. The rates at which 2-thiouracil and 2-thiothymine were oxidized were approximately equal to the rates of oxidation of uracil or thymine by the same bacterial suspension.

The oxidation products of 2-thiouracil and 2-thiothymine were chromatographed in various solvents and the R_f values compared (Table 5) with different reference compounds including the products formed when the organism oxidized uracil and thymine. The acidic nature of the compounds formed from 2-thiouracil and 2-thiothymine was demonstrated by spraying the chromatograms with a buffered solution of methyl red. When the oxidation of 2-thiouracil or 2-thiothymine by thymine-grown organisms was carried out in unbuffered medium the pH fell rapidly (for 2-thiothymine from 6.8 to 4.0) and only a small O_2 uptake was observed. The results indicate that the product formed from 2-thiothymine was 2-thiobarbituric acid and that 2-thiothymine, by analogy, was probably converted to 2-thio-5-methylbarbituric acid; an authentic sample of the last-named substance was not available. As these reactions were only observed with pyrimidine-adapted organisms, it was concluded that the oxidations were catalysed by the enzyme systems induced by uracil and thymine. 2-Thiobarbituric acid (and presumably 2-thio-5-methylbarbituric acid) was not attacked by pyrimidine-adapted suspensions of the organism.

Catabolism of barbituric acid

Effect of growth medium. Barbituric acid was oxidized immediately and rapidly to CO_2 , NH_3 and urea by suspensions of strain S which had been grown on uracil, thymine or barbituric acid, whereas when grown in media containing no added pyrimidines no oxidation occurred until after a lag of about 8 hr. It appears that barbituric acid, uracil and thymine can all induce the enzymes required for the degradation of barbituric acid. The O_2 uptake curves obtained when organisms grown on barbituric acid were tested with uracil, thymine, barbituric acid and 5-methyl-barbituric acid (Fig. 8) were similar to those obtained when uracil-grown organisms oxidized these four pyrimidines (fig. 2) suggesting that barbituric acid can also induce enzymes for the initial attack on uracil and thymine.

Requirement for oxygen. Suspensions of the organism (grown on uracil) had no action on barbituric acid in an atmosphere of N_2 .

Products of barbituric acid catabolism. The equations for the oxidation of barbituric acid by suspensions of strain S (1) and for the complete oxidation of barbituric acid to CO_2 and NH_3 (2) are:

The rates of production of ammonia and urea from barbituric acid by uracil-grown organisms were compared with the rate of removal of the acid and the rate of O_2 uptake (Fig. 9). The total O_2 uptake values were reproducible to within 10%; for six experiments the values (corrected for the endogenous O_2 uptake) were 0.89, 0.85, 0.89, 0.81, 0.90 and 0.92 $\mu\text{mole } O_2/\mu\text{mole barbituric acid removed}$. The incomplete recovery of carbon and nitrogen (as CO_2 , NH_3 and urea) suggested the possibility that concurrent oxidative assimilation was occurring during the oxidation of barbituric acid.

Effect of sodium azide. An increase of 54% in the total O_2 uptake was obtained when barbituric acid was oxidized by suspensions of the organism in the presence of 2 mM-sodium azide; this was the largest increase observed with the range of concentrations (0.1 mM–0.2M) of azide tested.

Fig. 8

Fig. 8. Uptake of oxygen by barbituric acid-grown organisms acting on thymine ($\circ$), 5-methylbarbituric acid ($\bullet$), uracil ($\odot$) and barbituric acid ($\ominus$). The amount of each substrate was 10 μmole . All values corrected for endogenous O_2 uptake.

Fig. 9

Fig. 9. Metabolism of barbituric acid by organisms grown on uracil. Barbituric acid ($\circ$), O_2 uptake ($\odot$), NH_3 ($\bullet$) and urea ($\bullet$). All values corrected for control values without substrate.

High concentrations of azide (0.2M) completely inhibited the oxidation of barbituric acid by uracil-grown organisms and also caused barbituric acid to accumulate in the reaction mixture when similar organisms were oxidizing uracil. With thymine-grown organisms acting on uracil in the presence of 0.2M-azide, the accumulation of barbituric acid was equivalent to the amount of uracil which had disappeared. The activity of the enzymes catalysing the initial attack on uracil and thymine was not affected by azide at this concentration.

Effect of isobarbituric acid. During a search for compounds which might be either intermediates in barbituric acid catabolism or inhibitors of its utilization, iso-

barbituric acid was found almost completely to suppress the oxidation of an equimolar concentration of barbituric acid (Table 6).

Degradation by cell-free extracts. Extracts made from uracil-grown organisms with the Mickle tissue disintegrator decomposed barbituric acid anaerobically to NH_3 , CO_2 and malonic acid; 9 μmole of barbituric acid was removed by 1 ml. of enzymic extract in 1 hr. A similar extract from thymine-grown organisms had

Table 6. *Effect of isobarbituric acid on pyrimidine catabolism*

Organisms grown on either uracil or thymine as indicated were incubated for 3 hr. with uracil or barbituric acid (each 10 μmole) in the absence or presence of isobarbituric acid (10 μmole). After removing the organisms by centrifuging, the supernatant fluids were analysed for pyrimidines.

	Pyrimidines (μmole) formed or removed by			
	Uracil-grown organisms with isobarbituric acid		Thymine-grown organisms with isobarbituric acid	
	Absent	Present	Absent	Present
Uracil removed	10	6.6	10	10
Barbituric acid removed	10	1	10	1
Barbituric acid formed from uracil	0	1	0	5

only one-quarter of the activity. Malonic acid (50 mg.) was isolated by the method of Hayaishi (1952) from the products of a large-scale experiment in which enzymic extract (8 ml.) was incubated with barbituric acid (200 mg.) in 12 ml. of 0.2M-phosphate buffer (pH 7.2) in the presence of toluene (0.5 ml.) at 30° until the barbituric acid had been decomposed (28 hr.). The enzyme catalysing the anaerobic breakdown of barbituric acid will be referred to as barbiturase; the crude preparation used above also contained urease.

Sodium azide and isobarbituric acid, which both inhibited the oxidation of barbituric acid by whole organisms, were without action on the activity of barbiturase in the cell-free extracts.

Urea was degraded by intact organisms, acetone powders and extracts made with the Mickle tissue disintegrator. It is likely that the primary products of barbiturase action are malonic acid and urea, and that the latter is then decomposed to NH_3 and CO_2 by the urease present in the preparations.

Metabolism of dicarboxylic acids

Several dicarboxylic acids were tested as possible sources of carbon for aerobic growth in medium *A* supplemented with phosphate buffer (0.1M; pH 7.2), thiamine (3 mM) and $(\text{NH}_4)_2\text{SO}_4$ (25 mM); the carbon sources were added, as the sodium salts, at a final concentration of 0.1 mM. Succinic, malic, fumaric and methylmalonic acids supported the growth of the organism but no growth was obtained with malonic, ketomalonic and tartronic acids.

Suspensions of uracil-grown organisms did not oxidize malonic acid. In addition malonic acid had no effect on the oxidation of uracil or barbituric acid by such organisms.

Formation of hydroxamic acids by cell-free extracts. Hayaishi (1955) showed that

malonic acid was degraded to acetic acid by cell-free enzyme preparations of *Pseudomonas fluorescens* with the intermediate formation of coenzyme A derivatives of malonic acid and acetic acid. If these two acids are intermediates in the aerobic catabolism of barbituric acid by strain S it is possible that the organism would contain enzyme systems for their activation and conversion to coenzyme A derivatives. Activation was tested by the method of Rose (1955) by studying the formation of hydroxamates by cell-free extracts in the presence of hydroxylamine and adenosine triphosphate; a number of other carboxylic acids were also tested (Table 7). The enzymic extract contained activation systems for acetic and propionic acids but not for malonic acid or the other substances tested.

Table 7. *Formation of hydroxamates by cell-free extracts of strain S*

Cell-free extracts were prepared with the Mickle tissue disintegrator and incubated with the test substrates as described in the Methods section.

Substrate	Colorimeter reading
None	0
Acetate	12
Propionate	22
Malonate	0
Barbiturate	0
Uracil	0
Lactate	1
Succinate	2
β -Hydroxypropionate	0

Oxidation of acetic acid. Since acetic acid or a derivative (acetyl coenzyme A) was considered as a possible intermediate in barbituric acid catabolism its oxidation by suspensions of strain S was studied in the presence and absence of sodium azide. It was oxidized rapidly and without lag, irrespective of the nature of the growth medium from which the organism had been harvested. With organisms grown on uracil, the final O_2 uptake was 1.04 mole O_2 /mole acetate and concurrently, 1.1 mole CO_2 /mole acetate was produced. The oxidation of acetic acid was partially uncoupled by azide (2 mM) which gave an increase from 1.04 to 1.66 mole O_2 /mole acetate removed; the theoretical value for complete oxidation is 2 mole O_2 /mole acetate.

Analysis of medium after growth

It was considered possible that strain S metabolized barbituric acid by two pathways, and that one of these, catalysed by barbiturase, yielded malonic acid which was not further utilized by the organism. The possibility was tested by determining whether malonic acid accumulated during growth on uracil. Organisms were grown on uracil as described for the preparation of washed suspensions except that a sample of sterile medium was removed before inoculation for pyrimidine analysis. After culture for 65 hr. the organisms were removed by centrifugation and the supernatant fluid analysed for pyrimidines, corrections being made for evaporation of water during the incubation period. The amount of uracil removed was 54.5% of that initially present and barbituric acid was formed in amounts equivalent to 5.5% of the uracil added.

The supernatant fluid was evaporated to a small volume, acidified with 3N-H₂SO₄ and continuously extracted with ether for 48 hr. The very small amount of material extracted by the ether contained no malonic acid as indicated by chromatography on paper. No substances could be sublimed from the ether extract.

Pyrimidine oxidation by strain K₃

Nocardia corallina strain K₃ grew much better on thymine than on uracil or cytosine (Table 1). Suspensions of thymine-grown organisms rapidly oxidized both thymine and 5-methylbarbituric acid and uracil was converted to barbituric acid (Table 8). Barbituric acid was metabolized only slowly.

Table 8. *Pyrimidine catabolism by suspensions of strain K₃*

Organisms grown on thymine and incubated aerobically in manometer vessels (as described in Methods section) with 10 μ mole of the substrate indicated.
Substrate

	Uracil	Thymine	Barbituric acid	5-Methylbarbituric acid
Substrates removed (μ mole)	8.0	8.8	1.5	10.0
Barbituric acid formed (μ mole)	7.0	—	—	—
5-Methylbarbituric acid formed (μ mole)	—	3.0	—	—

DISCUSSION

The long lag period which elapsed before pyrimidines were attacked by suspensions of *Nocardia corallina* from medium containing no added pyrimidine makes it clear that the enzyme systems required for the overall oxidative metabolism of pyrimidines by strain S were induced by the presence of the substrate. Either uracil or thymine can induce the enzyme(s) for an initial attack on both pyrimidines, yielding the corresponding barbituric acids, and this enzyme will be referred to as the uracil-thymine oxidase (Hayaishi & Kornberg, 1952). Growth on either uracil or thymine also induced in the organism ability to oxidize (probably to the corresponding barbituric acids) both 2-thiouracil and 2-thiothymine and it is probable that uracil-thymine oxidase also catalyses these reactions. A cell-free preparation of a similar enzyme from a *Mycobacterium* sp. was found by Hayaishi & Kornberg (1952) not to oxidize 2-thiouracil.

Since neither 5:6-dihydrouracil nor 5:6-dihydrothymine supported growth of strain S and were not oxidized by washed suspensions it is unlikely, unless these substances do not enter the organism, that a preliminary reduction (see Introduction) is an initial step in the overall oxidation of uracil and thymine.

The O₂ uptake with thymine and uracil by organisms grown with either pyrimidine was considerably less than the values required for complete oxidation to CO₂ and NH₃, but no other product apart from urea (whose conversion to CO₂ and NH₃ requires no O₂) was detected. It is probable therefore that extensive oxidative assimilation of the substrate into cell material was taking place. However, O₂ uptake was increased by an uncoupling agent (sodium azide) only when organisms were oxidizing the pyrimidine on which they had been grown and not,

for example, when thymine-grown organisms were oxidizing uracil (Table 4). The failure to demonstrate oxidative assimilation by the use of azide in the latter case may be explained as follows. It may be assumed that organisms grown on uracil contain fully developed all the enzymes required for the complete oxidation of uracil. On the other hand, organisms grown on thymine are adapted for the conversion of uracil to barbituric acid, but the enzymes for the degradation of the latter are not fully induced and barbituric acid at first accumulates almost quantitatively (Fig. 6). It is suggested that azide inhibits the formation of the enzymes which metabolize barbituric acid and give rise to the intermediates used in oxidative assimilation. This view is supported by the observation that barbituric acid itself was oxidized by uracil-grown organisms and that the oxidation was partly uncoupled from assimilation by azide; barbituric acid was not however oxidized by thymine-grown organisms in the presence of azide.

Similar arguments apply to the converse case in which oxidative assimilation could not be demonstrated with uracil-grown organisms metabolizing thymine. It is concluded that failure to demonstrate oxidative assimilation with azide in these two cases does not mean that such assimilation does not occur.

When uracil was oxidized by organisms grown on thymine there was an initial and almost theoretical accumulation of barbituric acid (Fig. 6) which was later further oxidized (Fig. 3); the bulk of uracil metabolism therefore occurred via barbituric acid. With uracil-grown organisms, however, barbituric acid was present in only small amounts at any stage of the reaction. The results might be explained on the assumption that the enzymes for further metabolism of barbituric acid are not fully developed in thymine-grown organisms; the initial rate of O_2 uptake with barbituric acid was only one-third that with 5-methylbarbituric acid (Fig. 3). However, if the whole of the uracil was also metabolized by uracil-grown organisms through the intermediate formation of barbituric acid it would be expected that the O_2 consumption with barbituric acid should be 0.5 mole less than that with uracil, i.e. by the amount of O_2 required for the conversion of uracil to barbituric acid. Experimentally the difference was only 0.15 mole O_2 (Table 3).

The position with regard to oxidation of thymine by organisms grown on uracil and thymine, respectively, was analogous. 5-Methylbarbituric acid was established as an intermediate with certainty only with uracil-grown organisms. With thymine-grown organisms a little of the acid accumulated, but the O_2 uptake values for thymine and 5-methylbarbituric acid differed by almost 1.2 mole instead of the expected 0.5 mole. It is concluded therefore that in the case of the oxidation of uracil and thymine by organisms grown on uracil and thymine, respectively, there is a possibility that a second pathway exists in which the corresponding barbituric acids are not intermediates.

There is evidence from work with cell-free extracts of various bacteria (Hayaishi & Kornberg, 1952; Lara, 1952*b*) that barbituric acid is initially attacked by an enzyme, barbiturase, which degrades it anaerobically to malonic acid and urea. The same enzyme is present in cell-free extracts of the present organism but intact organisms attacked barbituric acid only when O_2 was present. Furthermore isobarbituric acid and sodium azide inhibited completely the metabolism of barbituric acid by whole organisms, but did not affect the barbiturase activity of cell-free extracts. The possible role of barbiturase in the aerobic metabolism of barbituric acid

by whole organisms is therefore not clear; some of the difficulties have been considered by Hayaishi & Kornberg (1952). Suspensions of strain S harvested from growth on either barbituric acid or uracil rapidly oxidized barbituric acid to CO_2 , NH_3 or urea with considerable concurrent oxidative assimilation.

Hayaishi (1955) showed the presence in *Pseudomonas fluorescens* of enzymes for the conversion of malonic acid to a coenzyme A derivative and for the conversion of the latter to acetic acid through the intermediate formation of acetyl coenzyme A. Evidence for the activation of malonic acid by cell-free extracts of strain S was sought by a study of hydroxamate formation but none was found; acetate was activated under the same conditions. Malonate was not oxidized by pyrimidine-adapted suspensions of strain S nor could it be detected in the medium after growth of the organism on uracil. The possibility exists that an active derivative of malonate is formed directly from barbituric acid and that this is immediately metabolized without intermediate conversion to free malonate. It is also possible that malonate does not permeate the organism, though this would not explain the lack of activation by cell-free extracts.

One of the authors (R. D. B.) is deeply indebted to the Wellcome Foundation for a personal grant during part of the time of this work. He was a Nuffield Dominions Demonstrator in the University of Oxford for the remainder of the period. The research was aided by a grant to the Department from the Rockefeller Foundation.

REFERENCES

- BATT, R. D., MARTIN, J. K. & PLOESER, J. M. (1953). The alkaline decomposition of the dihydropyrimidines *Proc. Univ. Otago med. Sch.* **31**, 40.
- BATT, R. D. & WOODS, D. D. (1951). The oxidation of thymine by an unidentified bacterium. *Biochem. J.* **49**, lxx.
- BAUDISCH, O. & DAVIDSON, D. (1925). The mechanism of oxidation of thymine. 4,5-Dihydroxyhydrothymine (thymine glycol). *J. biol. Chem.* **64**, 233.
- CAMPBELL, L. L. (1957*a*). Reductive degradation of pyrimidines. I. The isolation and characterization of a uracil fermenting bacterium, *Clostridium uracilicum* nov. spec. *J. Bact.* **73**, 220.
- CAMPBELL, L. L. (1957*b*). Reductive degradation of pyrimidines. II. Mechanism of uracil degradation by *Clostridium uracilicum*. *J. Bact.* **73**, 225.
- CAMPBELL, L. L. (1957*c*). Reductive degradation of pyrimidines. III. Purification and properties of dihydrouracil dehydrogenase. *J. biol. Chem.* **227**, 693.
- CANELLAKIS, E. S. (1956). Pyrimidine metabolism. I. Enzymatic pathways of uracil and thymine degradation. *J. biol. Chem.* **221**, 315.
- CERECEDO, L. R. (1927). Studies on the physiology of pyrimidines. *J. biol. Chem.* **75**, 661.
- CERECEDO, L. R. (1931). Studies on the physiology of pyrimidines. IV. Further experiments on the intermediary metabolism of uracil. *J. biol. Chem.* **93**, 269.
- DI CARLO, F. J., SCHULTZ, A. S. & KENT, A. M. (1952). On the mechanism of pyrimidine metabolism by yeasts. *J. biol. Chem.* **199**, 333.
- FINK, K., CLINE, R. E., HENDERSON, R. B. & FINK, R. M. (1956). Metabolism of thymine (methyl- C^{14} or -2- C^{14}) by rat liver in vitro. *J. biol. Chem.* **221**, 425.
- FINK, R. M., CLINE, R. E. & KOCH, H. M. G. (1954). Chromatographic determination of pyrimidine reduction products: microbiological application. *Fed. Proc.* **13**, 207.
- FINK, R. M., FINK, K. & HENDERSON, R. B. (1953). β -Amino acid formation by tissue slices incubated with pyrimidines. *J. biol. Chem.* **201**, 349.

- FRITZSON, P. (1957). The catabolism of C¹⁴-labelled uracil, dihydrouracil and β -ureidopropionic acid in rat liver slices. *J. biol. Chem.* **226**, 223.
- FRITZSON, P. & PHIL, A. (1957). The catabolism of C¹⁴-labelled uracil, dihydrouracil and β -ureidopropionic acid in the intact rat. *J. biol. Chem.* **226**, 229.
- HAYAISHI, O. (1952). Enzymatic conversion of barbituric acid to urea and malonic acid. *Fed. Proc.* **11**, 227.
- HAYAISHI, O. (1955). Enzymatic decarboxylation of malonic acid. *J. biol. Chem.* **215**, 125.
- HAYAISHI, O. & KORNBERG, A. (1952). Metabolism of cytosine, thymine, uracil and barbituric acid by bacterial enzymes. *J. biol. Chem.* **197**, 717.
- HOLMBERG, G. A. (1945). Experiments with triphenylmethyl chloride and different barbituric acids. *Svensk kem. Tidskr.* **57**, 198.
- HOTCHKISS, R. D. (1948). The quantitative separation of purines, pyrimidines and nucleosides by paper chromatography. *J. biol. Chem.* **175**, 315.
- LARA, F. J. S. (1952a). On the decomposition of pyrimidines by bacteria. I. Studies by means of the technique of simultaneous adaptation. *J. Bact.* **64**, 271.
- LARA, F. J. S. (1952b). On the decomposition of pyrimidines by bacteria. II. Studies with cell-free enzyme preparations. *J. Bact.* **64**, 279.
- LIEBERMAN, I. & KORNBERG, A. (1953). Enzymatic synthesis and breakdown of a pyrimidine, orotic acid. I. Dihydro-orotic dehydrogenase. *Biochim. biophys. acta*, **12**, 223.
- LIEBERMAN, I. & KORNBERG, A. (1954). Enzymatic synthesis and breakdown of a pyrimidine, orotic acid. II. Dihydro-orotic acid, ureidosuccinic acid and 5-carboxymethylhydantoin. *J. biol. Chem.* **207**, 911.
- LIEBERMAN, I. & KORNBERG, A. (1955). Enzymatic synthesis and breakdown of a pyrimidine, orotic acid. III. Ureidosuccinase. *J. biol. Chem.* **212**, 909.
- MARKHAM, R. (1942). A steam distillation apparatus suitable for micro-Kjeldahl analysis. *Biochem. J.* **36**, 790.
- MARKHAM, R. & SMITH, J. D. (1949). Chromatographic studies of nucleic acids. I. A technique for the identification and estimation of purine and pyrimidine bases, nucleosides and related substances. *Biochem. J.* **45**, 294.
- MARTIN, J. K. & BATT, R. D. (1957). Studies on the nutrition of *Nocardia corallina*. *J. Bact.* **74**, 225.
- MICKLE, H. (1948). Tissue disintegrator. *J. R. micr. Soc.* **68**, 10.
- NIMMO-SMITH, R. H. & APPLEYARD, G. (1956). Studies with a Pseudomonad able to grow with creatine as main source of carbon and nitrogen. *J. gen. Microbiol.* **14**, 386.
- NISHIKAWA, T. (1931). Über die Stereoisomerie der C-methylbarbitursäure. I. Mitteilung. *Mem. Ryoj. Coll. Engng*, **3**, 277.
- REYNOLDS, E. S., LIEBERMAN, I. & KORNBERG, A. (1955). The metabolism of orotic acid in aerobic bacteria. *J. Bact.* **69**, 250.
- ROSE, I. A. (1955). Acetate kinase of bacteria (acetokinase). In *Methods in Enzymology*, **1**, 591. Ed. S. P. Colowick and N. O. Kaplan. New York: Academic Press Inc.
- STUCKEY, R. E. (1942). The ultraviolet absorption spectra of barbituric acid derivatives. III. Ionisation and 5-monosubstituted barbituric acid derivatives. *Quart. J. Pharm.* **15**, 370.
- UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, 2nd ed. Minneapolis: Burgess Publishing Co.
- WANG, T. P. & LAMPEN, J. O. (1952a). Metabolism of pyrimidines by a soil bacterium. *J. biol. Chem.* **194**, 775.
- WANG, T. P. & LAMPEN, J. O. (1952b). Uracil oxidase and the isolation of barbituric acid from uracil oxidation. *J. biol. Chem.* **194**, 785.