METABOLISM OF CYTOSINE, THYMINE, URACIL, AND BARBITURIC ACID BY BACTERIAL ENZYMES*

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Early studies with pyrimidines administered to animals established the fact that these compounds are metabolized (3, 4). Present concepts of the pathways of pyrimidine metabolism stem largely from the work of Cerecedo (4), in which he measured the changes in the urinary excretion of urea after feeding pyrimidines and related substances. On the basis of these studies, he proposed an initial oxidation at carbon 5, which in the case of uracil yields isobarbituric acid and with thymine results in thymine glycol. It was further proposed that oxalic acid, formic acid, and urea are ultimately obtained from uracil and that acetol, carbon dioxide, and urea result from thymine. However, the indirect nature of the evidence leaves this scheme open to question.

Our approach to this problem has been first to obtain, through enrichment culture, bacterial strains which rapidly metabolize pyrimidines and then to investigate the detailed metabolic pathway with enzymes obtained from these bacteria. Results both with whole cells and partially purified enzymes have suggested the pathway of pyrimidine metabolism indicated in Fig. 1.

Methods

Materials—Pyrimidines were commercial products, the identity of which was verified spectroscopically.[1] Isobarbituric acid was prepared according to Behrend and Roosen (5) and possessed the extinction coefficients cited by Heyroth and Loofbourow (6). Urease and protamine sulfate were products of the Nutritional Biochemicals Corporation.

5-Methylbarbituric acid was synthesized by the method of Gerngross (7). The melting point (203–204°, uncorrected) indicated identity with the α form described by Nishikawa (8). The analysis[2] was as follows:

Calculated, C 42.29, H 4.26, N 19.23; found, C 42.50, H 4.29, N 19.46

* Preliminary reports of this work have been presented (1, 2).

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[1] A commercial sample of cytosine was found to contain less than 2 per cent of cytosine and over 95 per cent of uracil.

[2] Microanalyses were performed by the Microanalytical Laboratory of the National Institutes of Health under the supervision of Dr. W. C. Alford.

Spectroscopic examination showed fairly close agreement with the data of Stuckey (9), except that the absorption maximum at pH 7.0 is at 267 mμ and in 0.1 N NaOH is at 269 mμ (ϵ = 19,700 and 17,300, respectively); Stuckey reported an absorption maximum in 0.1 N NaOH at 262 mμ (ϵ = 19,200). As previously observed (8, 9), this compound is unstable at acid and neutral pH and is readily oxidized by air to yield 5-hydroxy-5-methylbarbituric acid, which has only negligible absorption in the ultra-

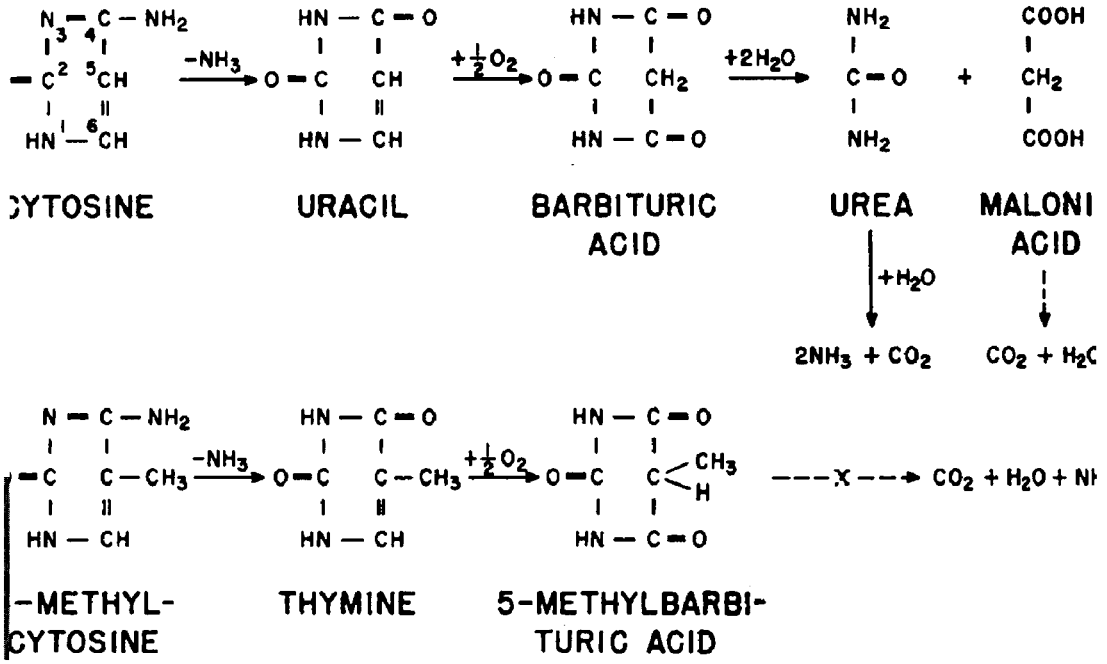

FIG. 1. Pathway of bacterial metabolism of pyrimidines. The numbering system for the pyrimidine ring conforms to current usage by *Chemical Abstracts*. The old system was employed previously (1, 2).

violet region. The stability at various temperatures and pH values is shown in Table I.

Cell-free extracts of bacteria were prepared by grinding with alumina by the method of McIlwain (10). Washed cells were ground with twice their weight of alumina (Alcoa A-301) at 0° for 5 minutes. The paste was extracted with 10 parts of buffer (see below) and the mixture was centrifuged at 0° at 16,000 × *g* for 10 minutes. All subsequent manipulations in the purification of enzymes were conducted at 0–2°.

Assay of Uracil-Thymine Oxidase—Since the extinction of the oxidation products was much greater than that of the substrates, the rate of increase in optical density served as the basis for measurement. The test system (at 22–25°) contained 0.3 ml. of uracil or thymine (0.001 M), 0.3 ml. of methylene blue (2.67×10^{-3} M), 2.3 ml. of tris(hydroxymethyl)aminomethane buffer (0.02 M, pH 8.5), and 0.1 ml. of enzyme. Readings were

taken at 2 minute intervals (at 255 mμ in the case of uracil and at 270 mμ in the case of thymine) in a model DU Beckman spectrophotometer. A unit of enzyme was defined as the amount producing a density increase of 0.100 during the first 10 minutes and specific activity was defined as units per mg. of protein. With about 1 unit of enzyme in this test system, the reaction rate is linear for about 30 minutes and proportionality is observed between rate and the amount of enzyme added (0 to 5 units).

Assay of Barbiturase—The test system (22–25°) contained 0.1 ml. of barbituric acid (0.02 M, sodium salt), 0.3 ml. of glycylglycine buffer (0.2 M, pH 8.25), 0.4 ml. of water, 2.5 mg. of crystalline bovine serum albumin, and 0.2 ml. of enzyme. At 10 minute intervals, 0.1 ml. aliquots were removed and the reaction was stopped by dilution to 3.0 ml. with phosphate buffer (0.02 M, pH 7.0). Readings were taken at 255 mμ. A unit of enzyme was defined as the amount producing a density decrease of 0.100 in a 10 minute interval.

TABLE I

Stability of 5-Methylbarbituric Acid As Influenced by Time, Temperature, and pH

Extinction at	pH 1.9		pH 6.9		pH 9.7	
	0°	25°	0°	25°	0°	25°
hrs.						
0	16,430	16,430	16,820	16,820	16,820	16,820
2.5	13,720	5,000	16,200	13,750	16,500	17,000
16	2,650	470	15,500	4,000	16,800	16,000

The concentration of 5-methylbarbituric acid was 0.001 M. The values are the optical density units at 270 mμ at a final pH of 7.0 calculated for a 1 M solution in a cell of 1 cm. light path.

Protein was determined by the method of Lowry *et al.* (11). Ammonia was distilled by the microdiffusion method of Conway (12) and determined by nesslerization (13). Ion exchange chromatography was performed according to Cohn (14). Dowex 1 anion exchange resin (200 to 400 mesh) was prepared by washing the resin first with 3 N HCl until the washings were free of material absorbing at 260 mμ and then with distilled water until the chloride ion test was faint. Paper chromatography was carried out with Whatman No. 1 filter paper by the ascending method. After 16 hours at room temperature, the paper was dried and the spots visualized with a model V41 Mineralight fluorescent lamp.

Results

Experiments with Intact Cells

Isolation and Properties of Organism—Samples of soil were suspended in 0.8 per cent NaCl solution (1 part of soil to 9 parts of saline). 1 ml. of

soil suspension was incubated in 9 ml. of a medium containing K_2HPO_4 (0.15 per cent), KH_2PO_4 (0.05 per cent), $MgSO_4 \cdot 7H_2O$ (0.02 per cent), and thymine and uracil (0.1 per cent each) in distilled water. After growth of the organisms became visible, several transfers were made successively in the same medium at daily intervals and finally the culture was plated out on this medium containing 2 per cent agar. The strain which grew most abundantly was isolated and designated as strain 161.

Strain 161 is an aerobic, gram-positive rod, non-motile and non-acid-fast, and does not form spores. It shows considerable pleomorphism; 16 hour cultures are made up exclusively of rod-shaped organisms, but after 36 hours (at 30°) there are irregular forms and granular staining. On the basis of these morphological characteristics, strain 161 has been tentatively assigned to the genus *Corynebacterium*.[3]

A strain of *Mycobacterium*[4] was subsequently found to metabolize uracil and thymine in a similar way and, because of its rapid growth, was used for enzymatic studies. It shows similar morphological properties to strain 161 except that it retains Ziehl's fuchsin after treatment with 5 per cent sulfuric acid for several seconds.

Strain 161 was cultured in a medium containing thymine or uracil (0.1 per cent) as the sole source of carbon and nitrogen; the salts were as described above. The organisms were grown in 20 liter glass carboys containing 10 liters of the medium at about 26° for 40 hours, with constant mechanical shaking. Cells were harvested by centrifugation in a Sharples supercentrifuge, washed once with a 0.5 per cent NaCl-0.5 per cent KCl solution, and suspended in phosphate buffer (0.02 M, pH 7.0) at a concentration of approximately 1 mg. of dry weight per ml. The yield of cells was approximately 0.5 gm. (wet weight) per liter of medium.

Manometric Studies—Oxygen uptake by a suspension of resting cells cultured on thymine was determined with thymine, uracil, or barbituric acid as the substrate. As shown in Fig. 2, each of these substrates brings about an immediate consumption of oxygen, the total uptake corresponding approximately to 4, 3, and 2 atoms per mole of thymine, uracil, and barbituric acid, respectively. These values represent about 50 to 60 per cent of the theoretical calculated for complete combustion and suggest that the remainder of the carbon has been assimilated into cell material. When the organism was grown on an ordinary broth medium, there was a long lag period before oxygen uptake was observed. That this lag was due to the adaptive formation of thymine-oxidizing enzymes was verified by a

[3] We are indebted to Dr. C. B. van Niel and Dr. R. Y. Stanier for advice concerning this classification.

[4] This strain, originally isolated from rabbit feces by Dr. Schatz, Dr. Savard, and Dr. Pintner, was classified and kindly furnished to us by Dr. T. Stadtman.

determination of thymine oxidase activity of cell-free extracts of cells before and some hours after exposure to thymine.

Manometric experiments with cells grown on uracil gave essentially the same results as those obtained with cells grown on thymine. Growth on uracil was less rapid than on thymine and the latter substance was usually employed for large scale cultivations.

Spectrophotometric Studies; Isolation of Barbituric Acid—Ultraviolet spectrophotometry carried out during the course of uracil or thymine oxidation revealed a transitory increase in density indicating the formation of intermediates with higher extinction coefficients than the substrates.[5] A large scale experiment on uracil oxidation designed to permit isolation

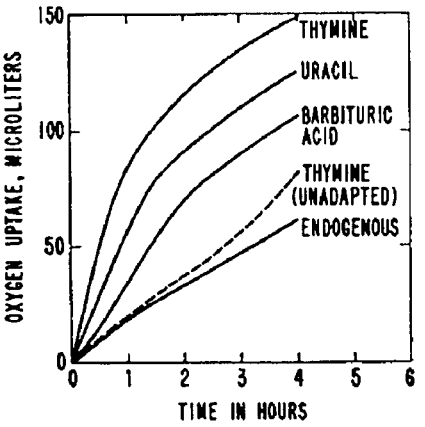

FIG. 2. Oxygen uptake by cells grown in the presence of thymine (solid line) and in the absence of thymine (dotted line). In the absence of thymine, 0.1 per cent of peptone was substituted. Each Warburg vessel contained 2 μM of substrate and about 1 mg. (dry weight) of cell material in a total volume of 2.0 ml. of 0.02 M phosphate buffer, pH 7.0. 0.2 ml. of 10 per cent KOH was in the center well. Temperature, 31.5°.

of the product was carried out as follows: Uracil (112.1 mg., 1.0 mM) was incubated at 37° with constant stirring with 50 ml. of a resting cell suspension and 50 ml. of phosphate buffer (0.02 M, pH 7.0). Aliquots (0.1 ml.) diluted to 25 ml. with phosphate buffer (0.02 M, pH 7.0) were examined at 260 mμ at hourly intervals. There was a steady increase in absorption, which at 4 hours reached a value almost 3 times that of the original; thereafter, the absorption decreased. At 4.25 hours, 90 ml. of the reaction mixture were centrifuged to remove the cells. The absorp-

[5] The extent and duration of this increase varied with different batches of cells related possibly to the age of the culture. For example, with thymine-grown cells (48 hours old) with thymine as substrate, the density (at 270 mμ) decreased, while with uracil as substrate, the density (at 255 mμ) increased initially. On the other hand, uracil-grown cells (48 hours old) usually brought about an initial density increase with thymine and a decrease with uracil.

tion spectrum of the supernatant at acid, neutral, and alkaline pH was essentially identical with that of the recrystallized reaction product and an authentic sample of barbituric acid. The supernatant solution was concentrated *in vacuo* to about 5 ml. and acidified to pH 1.0 with HCl. White rhombic crystals appeared on standing at 0° overnight. A sample recrystallized from dilute HCl (12.1 mg.) melted at 240–243°; mixed with authentic barbituric acid (242–243°), it melted at 240–243° (uncorrected). The nitrogen content was 21.41 per cent; calculated value 21.90 per cent. Paper chromatography of this product in three different solvent systems provided additional support of its identity with barbituric acid (Table II).

As indicated by the results of manometric studies described above (Fig.

TABLE II

Paper Chromatography of Uracil Oxidation Product

	Solvent A	Solvent B	Solvent C
	R_F values		
Thymine	0.47	0.86	0.72
Uracil	0.33	0.65	0.59
Barbituric acid	0.62	0.33	0.20
Reaction product	0.62	0.32	0.20
Isobarbituric acid			0.47

Solvent A, butanol saturated with 10 per cent aqueous urea solution (Carter, C. E., *J. Am. Chem. Soc.*, **72**, 1466 (1950)). Solvent B, butanol, ethylene glycol, and water, 4:1:1 (Vischer, E., and Chargaff, E., *J. Biol. Chem.*, **168**, 781 (1947)). Solvent C, propanol and water, 10:3.

2), cells grown on either thymine or uracil were adapted to the oxidation of barbituric acid. They were not able to oxidize a large number of other pyrimidines tested including cytosine, 5-methylcytosine, isobarbituric acid, 6-methyluracil, dihydrouracil, 2-thiouracil, and 2-thio-5-methyluracil. With regard to 5-methylbarbituric acid, the anticipated product of thymine oxidation, manometric experiments were complicated by its instability, but spectrophotometric measurements revealed essentially the same rate of decomposition as that of barbituric acid.

Spectrophotometric Studies with Cells Grown on Cytosine—When cytosine was substituted for thymine or uracil in the culture media, the growth was much less favorable unless an additional carbon source, such as glucose, was added. Suspensions of cells grown on a cytosine-glucose (0.1 per cent of each) medium under similar conditions metabolized either cytosine or 5-methylcytosine immediately as judged by a decrease in optical density at 265 mμ (Table III). Cells grown on either thymine or uracil (with or

without glucose) did not metabolize cytosine or 5-methylcytosine under the same test conditions. However, the cytosine-adapted cells produced changes in light absorption with uracil or thymine as substrate which resemble those produced by cells grown on thymine or uracil.

It may be noted that the growth substrate influences the nature of the initial changes in optical density produced by intact cells. In some instances, there is an increase in density as an indication of the accumulation of an intermediate, while in others no such accumulation is observed.

TABLE III

Influence of Growth Substrate in Spectrophotometric Studies with Intact Cells

Growth substrate	Test substrate			
	Thymine	Uracil	Cytosine	5-Methyl-cytosine
	Δ, optical density (0–30 min.)			
Thymine (glucose)	−0.090	+0.045	0.000	−0.005
Uracil (glucose)	+0.213	−0.150	+0.005	−0.005
Cytosine (glucose)	+0.205	+0.213	−0.095	−0.100

Pyrimidines (0.1 per cent) and glucose (0.2 per cent) in the basal salt mixture. After 48 hours, the cells were harvested, washed, and weighed. Yields of 0.80, 0.42, and 0.38 gm. per 500 ml. of culture medium were obtained with thymine, uracil, and cytosine, respectively. The cell suspension was made with 0.8 per cent KCl solution and the concentration was standardized to give a density of 0.46 at 650 mμ in the Coleman model 6B junior spectrophotometer. 0.2 ml. of cell suspension, 0.3 ml. of 0.001 M pyrimidine solution, and 2.5 ml. of 0.02 M phosphate buffer (pH 7.0) were incubated in a Beckman cuvette and the reaction was followed at 270, 255, 260, and 265 mμ for thymine, uracil, cytosine, and 5-methylcytosine, respectively.

Uracil-Thymine Oxidase

Preparation of Enzyme—Large scale cultivation of *Mycobacteria* was carried out as described above for *Corynebacteria* (strain 161). About 0.5 to 1.0 gm. of wet cells was obtained per liter of culture medium and could be stored at −10° without loss in activity for a period of at least 6 months. Cell-free extracts prepared by grinding with alumina and extracting with tris(hydroxymethyl)aminomethane buffer (0.02 M, pH 9.0) were treated with ammonium sulfate (24.5 gm. per 100 ml. of extract). The precipitate was removed by centrifugation and more ammonium sulfate was added to the supernatant (10.5 gm. per 100 ml. of extract). The resulting precipitate, collected by centrifugation, was dissolved in tris(hydroxymethyl)aminomethane buffer (0.02 M, pH 9.0) to a volume corresponding to one-twentieth that of the extract. This fraction contained

35.6 units per ml. and 1.2 mg. of protein per ml. The specific activity of cell-free extracts (uracil as a substrate) was 10.9 when the cells were grown on a cytosine-glucose medium and 10.2 when uracil was substituted for cytosine. This fact coupled with the behavior of intact cells (Table III) indicates that the initial step of deamination of cytosine or 5-methylcytosine is an adaptive process and these compounds are metabolized by way of uracil or thymine, as previously reported with other microorganisms (15–17).

Isolation and Identification of Reaction Products—Uracil or thymine (1.0 ml. of 0.02 M) was incubated at 30° (with constant shaking) with enzyme (2.0 ml.), methylene blue (2.0 ml. of 2.67×10^{-3} M), and tris(hydroxymethyl)aminomethane buffer (15 ml. of 0.2 M, pH 8.7). The course of reaction was followed by the increase in optical density (at 255 mμ for uracil and 270 mμ for thymine). When the reaction was complete, a small aliquot was removed for determination of the absorption spectrum and the remainder was adsorbed at 2° on a Dowex 1 chloride column and eluted with ammonium chloride-ammonium hydroxide buffer (0.1 M, pH 9.9). All operations with the thymine oxidation product were performed in the cold to minimize the autoxidation of 5-methylbarbituric acid, the presumed product.

The absorption spectra of the final uracil and thymine incubation mixtures determined at acid, neutral, and alkaline pH are identical with the spectra of barbituric acid and 5-methylbarbituric acid respectively (Fig. 3). Additional evidence supporting identity of the uracil oxidation product with barbituric acid and of the thymine oxidation product with 5-methylbarbituric acid was provided by ion exchange analysis (Fig. 4).

Specificity, pH Optimum, and Substrate Affinity—Under the spectrophotometric test conditions described for uracil and thymine oxidation, the enzyme did not act upon the following pyrimidines: barbituric acid, isobarbituric acid, 5-methylbarbituric acid, 6-methyluracil, dihydrothymine, dihydrouracil, 2-thiouracil, 2-thio-5-methyluracil, and cytosine.

The dependence of reaction rate on substrate concentration is shown in Fig. 5, *A*. The Michaelis constants (18) calculated from these data are 0.35×10^{-4} and 1.31×10^{-4} mole per liter for thymine and uracil, respectively.

The optimum pH of the reaction is about 8.5; the activity at neutral pH is only one-tenth as great (Fig. 5, *B*). The enzyme is most stable at an alkaline pH. There is no appreciable loss of activity for at least several months on storage at pH 9.0 at −10°.

The fact that the ratio of the rate of thymine to uracil oxidation is almost identical in cell-free extracts from either uracil- or thymine-adapted cells or in the partially purified enzyme preparations indicates the identity

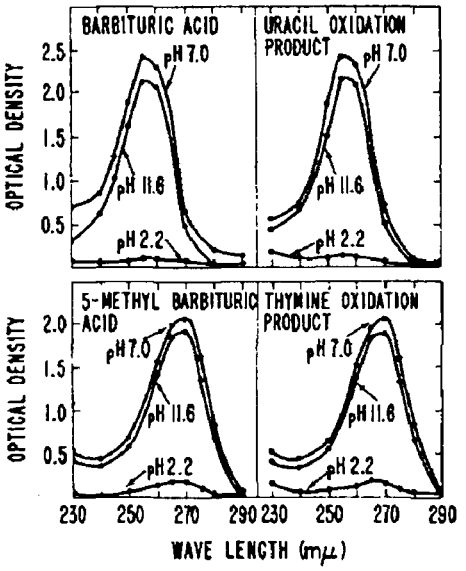

FIG. 3

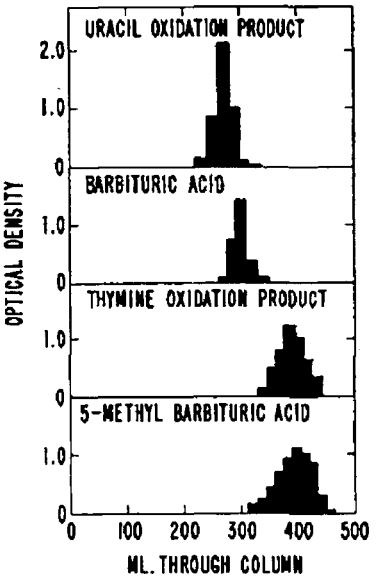

FIG. 4

FIG. 3. Absorption spectra of reaction products and authentic barbituric acid and 5-methylbarbituric acid. The thymine and uracil oxidation products were chromatographed on an ion exchange resin column (see Fig. 4). The eluates were pooled and used for determination.

FIG. 4. Ion exchange chromatogram. Conditions as described in the text. A 9.5 cm. × 1.0 sq. cm. column was used. The rate of flow was about 19 ml. per 30 minutes. Recoveries based on the absorption at 260 mμ were 88.0, 90.5, 95.0, and 82.0 per cent, respectively, reading from top to bottom.

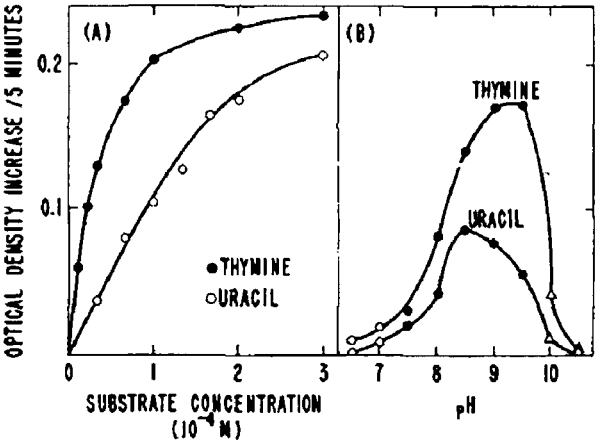

FIG. 5. Rate of thymine and uracil oxidation as a function of substrate concentration (*A*) and pH (*B*). 0.03 ml. of enzyme (0.063 mg. of protein), 0.1 ml. of 0.001 M thymine or uracil, 0.3 ml. of 2.67×10^{-4} M methylene blue, and 0.1 M of buffer in a total volume of 3.0 ml. Glycylglycine buffer, pH 8.8, was used in *A*. Thymine oxidase activity was measured at 270 mμ and uracil oxidase activity at 255 mμ. In *B*, the values were corrected for the change of absorption coefficient of the substrates at different pH values. ○, phosphate; ●, glycylglycine; △, glycine.

of the two oxidase activities. Further proof was provided by a kinetic analysis of the competitive inhibitory action of uracil and thymine. As shown in Table IV, the affinity of uracil for the enzyme was the same whether it was determined with uracil as a substrate or as a competitive inhibitor of thymine oxidation; similar results were obtained with thymine.

Electron Transport—When cell-free extracts were incubated with the

TABLE IV

K_I of Uracil and Thymine for Uracil-Thymine Oxidase

Thymine, M	10^{-4}	10^{-4}	10^{-4}	10^{-4}
Uracil, M	0	10^{-4}	2×10^{-4}	3×10^{-4}
Thymine oxidized*	0.356	0.292	0.240	0.210
K_I of uracil		1.19×10^{-4}	1.08×10^{-4}	1.10×10^{-4}
Uracil, M	10^{-4}	10^{-4}	10^{-4}	10^{-4}
Thymine, M	0	10^{-4}	2×10^{-4}	3×10^{-4}
Uracil oxidized†	0.177	0.066	0.042	0.035
K_I of thymine		0.38×10^{-4}	0.35×10^{-4}	0.42×10^{-4}

The incubation mixture contained 0.03 ml. of enzyme (protein 0.063 mg.), 0.15 ml. of 2.07×10^{-4} M methylene blue, uracil, and thymine, as indicated, and 0.1 M glycylglycine buffer (pH 8.0) to make a total volume of 1.5 ml. Depth of cells, 0.5 cm. After 20 minutes at 26°, readings were taken at both 250 and 270 mμ and the amount of each substrate oxidized was determined. K_S for uracil = 1.31×10^{-4} M; K_S for thymine = 0.35×10^{-4} M. K_I, dissociation constant of inhibitor-enzyme complex; K_S, dissociation constant of substrate-enzyme complex; I, concentration of inhibitor; S, concentration of substrate; v, velocity; v_I, velocity in the presence of inhibitor.

$$K_I = K_S \left[\frac{I}{K_S + S}\right] \Big/ \left[\frac{v - v_I}{v_I}\right]$$

* Increase in optical density at 270 mμ corrected for the change due to the oxidation of uracil.

† Increase in optical density at 250 mμ corrected for the change due to the oxidation of thymine.

substrate, neither oxygen consumption nor substrate removal was observed. It was noted, however, that under anaerobic conditions methylene blue was decolorized by these extracts in the presence of substrate and that the reaction also proceeded aerobically if methylene blue was present. The physiologic mechanism of electron transport has not been determined. Xanthine oxidase from milk and reduced diphosphopyridine nucleotide oxidase from *Clostridium kluyveri*[6] did not serve as mediators in place of methylene blue. Reduction of added di- or triphosphopyridine nucleotide was not detectable under the conditions employed nor was there any

[6] Kindly furnished by Dr. Leon A. Heppel.

stimulation of the reaction by added adenyl coenzymes, metals, boiled enzyme, or boiled yeast extract.

Barbiturase

Purification of Enzyme—Mycobacteria were cultured under essentially the same conditions as for the preparation of uracil and thymine oxidase with the exception that uracil (0.1 per cent) and glucose (0.2 per cent) provided the sole nitrogen and carbon sources. The inclusion of glucose increased the yield of cells to about 1.5 gm. per liter of culture medium and also increased the yield and specific activity of the enzyme 3- to 4-fold. Cell-free extracts prepared by grinding with alumina and extracting with phosphate buffer (0.02 M, pH 6.65) were lyophilized and stored at $-10°$. 500 mg. of lyophilized powder (obtained from 5.3 liters of culture medium) were dissolved in 20 ml. of distilled water and insoluble material was centrifuged off and discarded. To the supernatant (cell extract, Table V) were added 20 ml. of phosphate buffer (0.02 M, pH 7.0) and 4 ml. of protamine sulfate (10 mg. per ml.). After 3 minutes, the precipitate was collected by centrifugation and extracted with 20 ml. of 0.5 M K_2HPO_4. The opalescent extract was diluted with 60 ml. of water. Removal of the resulting precipitate by centrifugation yielded a clear, colorless solution (protamine fraction). While this step yielded little or no purification on a protein basis, it succeeded in removing all the nucleic acid which was present in the cell-free extract. 10 ml. of the protamine fraction were adsorbed on a Dowex 1 formate column (8 cm. × 1 sq. cm.) and eluted with 0.1 M K_2HPO_4 at a rate of 0.3 ml. per minute. The eluate was tested for both urease and barbiturase activity and the fraction between 18 and 22 ml. was observed to possess the highest specific activity of barbiturase and practically no urease activity; the urease activity appeared in a later eluate.

Isolation and Identification of Reaction Products—With the crude cell-free extract, the disappearance of barbituric acid was matched by a total release of ammonia in amounts approximating the theoretical nitrogen content. However, a significant lag in NH_3 production was observed (Fig. 6, *A*) which could be eliminated by the addition of crystalline urease (Fig. 6, *B*). In the presence of urease 1 mole of CO_2 was evolved per mole of barbituric acid destroyed. With the purified enzyme only 0.16 mole of ammonia was released per mole of barbituric acid removed.[7] In the pres-

[7] Similar results were obtained by inhibiting urease with silver ions. With crude cell-free extracts, 0.5×10^{-3} M AgCl inhibited barbiturase 27 per cent and urease 100 per cent. After the reaction was completed, accumulation of urea could be demonstrated by diluting the reaction mixture and observing the effect of added urease.

ence of added urease the value was 1.97 (Table VI), and similar values were observed during the entire course of the reaction. The reaction proceeded at the same rate and to the same extent under anaerobic conditions.

When urea had been established as a product, it was presumed that malonic acid might be the other product. Accordingly, a large scale ex-

TABLE V

Purification of Barbiturase

	Total activity	Specific activity	280:260*
	units	*units per mg. protein*	
Uracil-glucose cell extract	234	12.85	0.55
Protamine treatment	173	13.9	1.45
Dowex 1 column, Fraction *a*†	168	77.0	1.47
" 1 " " *b*	56	94.0	1.48

* Ratio of optical density at 280 mμ compared with that at 260 mμ.

† Fraction *a* is the eluate between 13.5 and 36.0 ml.; Fraction *b* is the eluate between 18 and 22.5 ml.

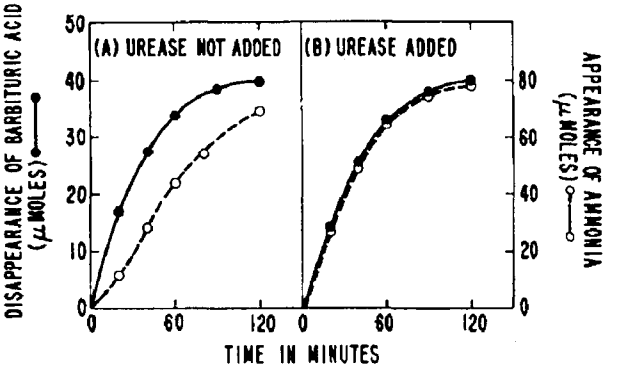

FIG. 6. Disappearance of barbituric acid and formation of ammonia. 40 μM of barbituric acid (sodium salt), 0.6 ml. of crude extract (3.36 mg. of protein), 1.2 ml. of water, and 0.2 ml. of 0.2 M glycylglycine buffer, pH 8.2. Barbituric acid was measured spectrophotometrically at 270 mμ.

periment designed to isolate the product was performed; barbituric acid (25 ml., 0.04 M) was incubated with 20 ml. of cell-free extract (60 mg. of protein) and 5 ml. of glycylglycine buffer (0.2 M, pH 8.25). After 100 minutes at 26° (when the ultraviolet absorption of barbituric acid was completely removed) 2 N sulfuric acid was added to bring the pH to 2.2 and the resulting precipitate was removed by centrifugation. The supernatant was extracted with ether continuously in a Kutcher-Steudel extraction apparatus for 6 hours. The ether extract was taken to dryness and the white solid sublimed at 60° under reduced pressure (0.05 mm. of Hg). The white crystalline product (86 mg.) gave the following analysis:

found C 34.94 per cent, H 4.01 per cent, m.p. 134–135° (uncorrected); calculated (for malonic acid), C 34.60 per cent, H 3.84 per cent (melting point of authentic sample 134–135° (uncorrected)). The identity of the product with malonic acid was further verified by the characteristic yellow

TABLE VI

Accumulation of Urea with Cell-Free Extract and Purified Enzyme

		Without urease	With urease
		μM	μM
Cell-free extract	Barbituric acid	−8.5	−8.5
	Ammonia	+11.8	+16.0
Purified enzyme	Barbituric acid	−6.8	−7.0
	Ammonia	+1.1	+13.8

The experiment with cell-free extract was carried out with 0.6 ml. of extract (0.84 mg. of protein), 0.25 ml. of 0.04 M barbituric acid (sodium salt), 0.2 ml. of 0.2 M glycylglycine buffer (pH 8.2), and water to a final volume of 4.0 ml. The experiment with purified enzyme was carried out with 1.1 ml. of enzyme (Fraction *b*, Table V), 0.5 ml. of 0.04 M barbituric acid (sodium salt), 0.2 ml. of 0.2 M glycylglycine buffer (pH 8.5), 0.2 ml. of water, and 5 mg. of crystalline bovine albumin. 4 mg. of urease were added as indicated. Incubation, 1 hour at 24°.

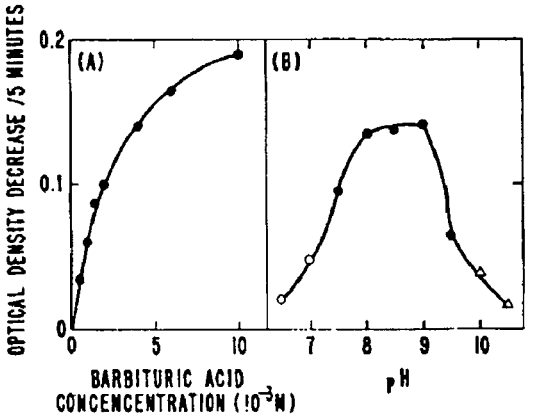

FIG. 7. Rate of barbiturase reaction as a function of substrate concentration (*A*) and pH (*B*). 0.1 ml. of enzyme (0.21 mg. of protein), 0.1 ml. of 0.02 M barbituric acid (sodium salt), and 0.8 ml. of 0.1 M buffer. Glycylglycine buffer (pH 8.0) was used in *A*. ○, phosphate; ●, glycylglycine; △, glycine.

fluorescence under ultraviolet light upon heating with acetic anhydride (19) and by the characteristic appearance of its barium salt in dilute ethanol solution (19).

Specificity, pH Optimum, and Substrate Affinity—There was no action, as judged spectrophotometrically, on the following compounds: 5-methylbarbituric acid, orotic acid, barbital, pentobarbital, 2-thiobarbituric acid, and isobarbituric acid.

The optimal pH is between 8 and 9 (Fig. 7, *B*) and the Michaelis constant (K_m) calculated from the data shown in Fig. 7, *A* is approximately 3.37×10^{-3} mole per liter.

DISCUSSION

It may be concluded from these and previous studies (15–17) that in bacteria aminopyrimidines such as cytosine and 5-methylcytosine are first deaminated to produce uracil and thymine, respectively. This reaction appears to be due to a single enzyme which is adaptive in character. Uracil and thymine are both oxidized at carbon 6 by a single enzyme, which is also adaptively produced, to form barbituric acid and 5-methylbarbituric acid, respectively. While this sequence of reactions is based on data derived from bacterial systems, it is a reasonable conjecture that the same pathway may be valid in mammalian systems.

Concurrent with our preliminary report (1), Wang and Lampen (20) described the metabolism by certain bacteria of uracil and thymine to a product which was later identified as barbituric acid (21). Batt and Woods (22) have also reported the accumulation of a compound when thymine is oxidized by resting bacterial cells. They consider this compound to be a phosphorylated uracil-5-carbinol (2,6-dihydroxy-5-hydroxymethylpyrimidine). However, their evidence for this formulation is not complete and the description of the spectroscopic characteristics of the compound do not preclude its identity with 5-methylbarbituric acid. Lara (23) has investigated the metabolism of pyrimidines by several bacterial strains and has obtained findings similar to those reported by us. He found that barbituric acid is the only substance among many listed which is oxidized without lag by cells adapted to uracil or thymine. He also found malonic acid to be a product of the enzymatic degradation of barbituric acid.

The precise way in which barbituric acid is converted to urea and malonic acid has not been established. The initial hydrolytic step can be assumed to yield a compound such as the half ureide of malonic acid with a subsequent hydrolytic cleavage to produce urea and malonic acid. Even with the most purified enzyme preparations there has been no indication of the accumulation of such an intermediate. In the presence of excess urease the liberation of ammonia exactly equals the removal of barbituric acid. The removal of the intermediate may be the result of the more effective action at this step by the same enzyme which carries out the first hydrolytic step or it may be due to the action of an additional enzyme or could even be a spontaneous reaction.

The fate of 5-methylbarbituric acid is still unknown. As stated previously, it is readily metabolized by the whole cell under aerobic conditions at the same rate as barbituric acid, but, after alumina grinding or

sonic treatment, bacterial homogenates[8] were uniformly inactive. It may be assumed, therefore, that the enzyme is extremely labile or that some rather complex reaction is necessary to initiate this degradation.

On the assumption that the initial attack on 5-methylbarbituric acid is non-oxidative, as in the case of barbiturase reaction, *anaerobic* experiments were conducted with 5-methylbarbituric acid and suspensions of thymine-grown cells. No change in optical density was observed, even after prolonged incubation. However, control experiments with barbituric acid under similar conditions revealed that there was no action on barbituric acid either. Thus, 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. This could be due to an energy requirement for absorption of the substrate into the cell which is supplied under aerobic conditions.

The ability of malonic acid to act as a competitive inhibitor of succinic acid in the succinic dehydrogenase reaction has created the common impression that malonic acid is an unnatural, toxic substance. This attitude can be seriously questioned in view of the current findings that malonic acid is a product of uracil metabolism and indications that it is a readily metabolizable substrate. The utilization of malonic acid by microorganisms (24–26) as well as by mice (27) and rats (28) has been reported. The formation of malonic acid from oxalacetic acid by pig heart preparations has also been observed (29).

We have isolated several strains from soil by growth on media in which malonic acid served as a sole carbon source. The rather poor utilization of malonic acid by uracil-adapted cells, noted in the present work, may be related to an inability of malonic acid to enter the cell readily. Another possibility is that a labile compound which yields malonic acid in the isolation procedure is the true and rapidly metabolized intermediate.

SUMMARY

1. The metabolism of pyrimidines by strains of *Corynebacterium* and *Mycobacterium* isolated by enrichment culture has been studied with cell suspensions and enzymes purified from cell-free extracts.

2. The results suggest the following metabolic pathways:

(*a*) Cytosine → uracil → barbituric acid → urea + malonic acid
↓ ↓
$CO_2 + NH_3$ $CO_2 + H_2O$

(*b*) 5-Methylcytosine → thymine → 5-methylbarbituric acid →
$NH_3 + CO_2 + H_2O$

[8] Bacterial homogenate designates the turbid suspension containing broken cell material, membranes, soluble components, and a negligible number of intact cells. Cells treated with alumina or disrupted by sonic oscillation were centrifuged at low speeds, just adequate to remove intact cells.

3. The oxidation of uracil and thymine at carbon 6 to yield barbituric acid and 5-methylbarbituric acid, respectively, was shown to be due to the action of a single enzyme, "uracil-thymine oxidase."

4. An enzyme, "barbiturase," catalyzing the hydrolysis of barbituric acid to urea and malonic acid was partially purified and freed of urease.

5. The oxidation of 5-methylbarbituric acid has been observed only with intact cells and the detailed mechanism has not been established.

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