THE METABOLISM OF OROTIC ACID IN AEROBIC BACTERIA[1]

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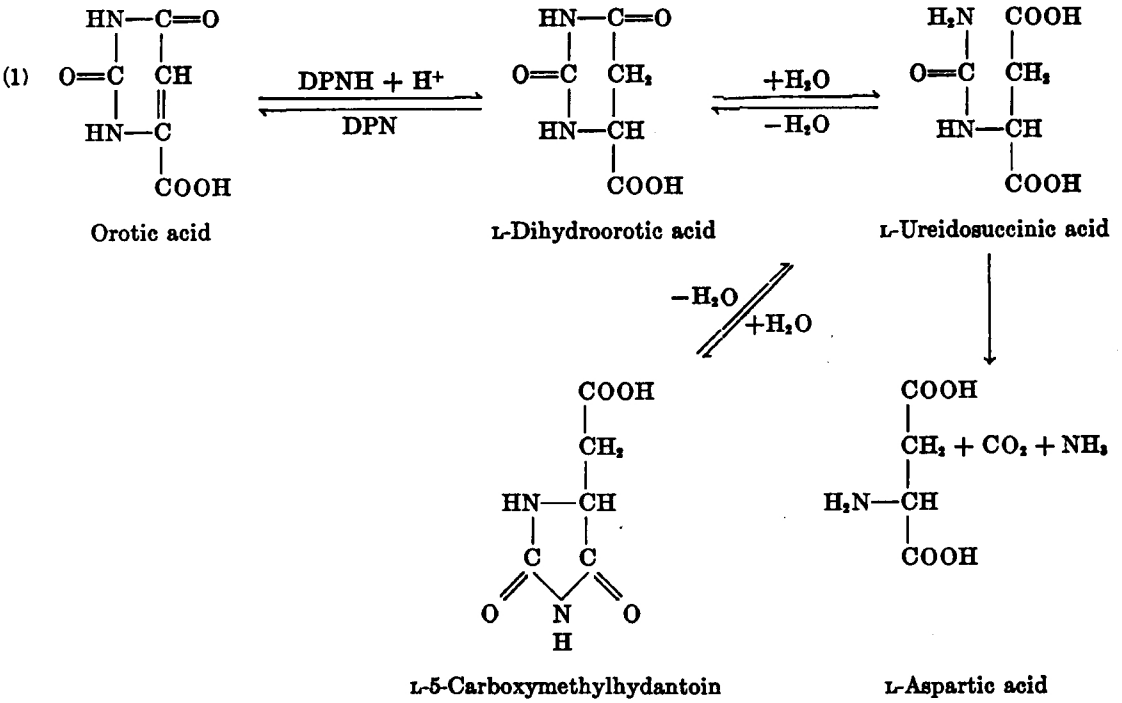
Orotic acid (4-carboxyuracil) was shown to be a growth factor for one of the group C streptococci (Rogers, 1944), *Lactobacillus casei* (Chattaway, 1944), and *Lactobacillus bulgaricus*, strain 09 (Wieland *et al.*, 1950; Huff *et al.*, 1950). Further studies with *L. bulgaricus*, strain 09 have revealed that ureidosuccinic acid partially replaces that orotic acid is also a nucleic acid pyrimidine precursor in mammalian tissues.

Studies of orotic acid synthesis and breakdown with enzyme preparations from an anaerobic bacterium, *Zymobacterium oroticum*, have indicated the following pathway (Lieberman and Kornberg, 1953*a*; 1954, *in preparation*):

(1)



Orotic acid — L-Dihydroorotic acid — L-Ureidosuccinic acid

L-5-Carboxymethylhydantoin — L-Aspartic acid

the orotic acid requirement (Wright *et al.*, 1950), and that both orotic and ureidosuccinic acids are precursors of nucleic acid pyrimidines (Wright *et al.*, 1953). Experiments with intact rats (Arvidson *et al.*, 1949; Reichard, 1949; Hurlbert and Potter, 1952) and rat liver slices (Weed *et al.*, 1950; Weed and Wilson, 1951) have demonstrated

A distinctive feature of this pathway is that it involves an initial, reversible reduction of orotic acid in contrast to the irreversible, oxidative metabolism of uracil and thymine observed with enzyme preparations from corynebacteria and mycobacteria (Hayaishi and Kornberg, 1951, 1952; Lara, 1952). It was of interest, therefore, to determine whether the metabolism of orotic acid by obligate aerobes involved a primary reduction to dihydroorotic acid, or some other pathway of metabolism.

With partially purified enzyme preparations

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from two aerobic bacteria isolated from soil, evidence has been found for a metabolic scheme similar to that observed in the anaerobic organism, *Z. oroticum*. One difference noted is that in the aerobic bacteria TPN rather than DPN is the coenzyme[3] for the reductive step.

MATERIALS AND METHODS

The organisms used in this study were isolated by enrichment culture from soil. Approximately 5 g of top soil were suspended in 26 ml of a medium consisting of $MgCl_2$, 0.001 per cent; KH_2PO_4, 0.25 per cent; K_2HPO_4, 0.54 per cent; and orotic acid, 0.2 per cent (previously adjusted to pH 7.0 with NaOH). Pure cultures were isolated from the liquid medium by repeated subculturing on a solid medium of the same composition (1.5 per cent agar).

Several different bacteria capable of metabolizing orotic acid were found. The two organisms studied are considered to be members of the genus *Corynebacterium* on the basis of the following characteristics: gram positive or gram variable, nonacid-fast, pleomorphic rods, nonsporeforming, nonmotile, metachromatic granulation, aerobic. They will be referred to as corynebacterium I and corynebacterium II.

Corynebacterium I was grown on a medium containing yeast extract (Difco), 0.1 per cent; orotic acid, 0.2 per cent (adjusted to pH 7.0); $MgCl_2$, 0.002 per cent; KH_2PO_4, 0.05 per cent; and K_2HPO_4, 0.11 per cent. The medium (1 or 2 liters) was prepared in 6 liter Erlenmeyer flasks and sterilized by autoclaving (15 lb, 15 minutes). After cooling, it was inoculated with a 48 hour old broth culture (10 ml of culture per liter of medium) and incubated at 30 C, with vigorous shaking. The cells were harvested in a Sharples supercentrifuge when approximately 80 per cent of the orotate (estimated spectrophotometrically) had been utilized (10–20 hours). The yield was 2–3 g of packed wet cells per liter of culture.

Cultures of corynebacterium II were prepared as described for corynebacterium I except that the medium included no yeast extract, contained orotic acid, 0.4 per cent, and, in order to prevent a lengthy lag period, larger inocula were used (50 ml of a 48 hour culture per liter of medium). Approximately 4 g of packed wet cells were obtained per liter of culture.[4]

Cell-free extracts of corynebacterium I were prepared by resuspending the cells in water (10 ml per liter of original culture, 2 C) and exposing 50 to 75 ml aliquots to sonic oscillation (Raytheon 10 kc sonic oscillator) for 25 minutes. Insoluble material was removed by centrifugation (*ca* 20,000 × G). The extracts were stable for several months at −13 C.

Extracts of corynebacterium II were prepared by grinding with alumina (Alcoa 301, 2 g per g of wet cells) (McIlwain, 1948) and extracting twice with potassium phosphate buffer (0.02 M, pH 7.0, 2.5 ml per g of wet cells). The extracts were combined, and insoluble material was removed by centrifugation (*ca* 20,000 × G). These extracts were also stable for several months at −13 C.

Glucose dehydrogenase was prepared from calf liver by the method of Strecker and Korkes (1952). DPN was prepared by the method of Kornberg and Pricer (1953) and TPN by the method of Kornberg and Horecker (1953). 2-C^{14}-Orotic acid was synthesized from C^{14}-potassium cyanate and DL-aspartic acid according to the method of Nyc and Mitchell (1947).[5] Nonisotopic orotic acid was a commercial preparation from Dougherty Chemicals. DL-5-Carboxymethylhydantoin was prepared from DL-ureidosuccinic acid by treatment with strong acid, as described by Nyc and Mitchell (1947). Isocitric dehydrogenase was obtained by extraction of pig heart acetone powder (Ochoa, 1945).

The enzymatic removal of orotate was estimated in the Beckman DU spectrophotometer by the decrease in optical density. A unit of enzyme was defined as the activity producing a decrease in optical density of 0.100 in 4 minutes at 290 mμ (corynebacterium I), or in 10 minutes at 280 mμ corynebacterium II). TPNH, which was required for orotate utilization by the enzyme preparations, was generated either by the addition of

[3] Abbreviations used: TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

[4] Often the packed cells were resuspended in a sodium orotate solution (0.2 per cent, 20 ml per g of wet cells) and incubated with shaking (50 minutes, 30 C) prior to the preparation of the cell-free extract. More active extracts appeared to be obtained when this step was included in the procedure.

[5] We are indebted to S. R. Kornberg for the synthesis of this compound.

TPN, glucose, and glucose dehydrogenase, or TPN, isocitrate, and isocitric dehydrogenase. Specific activity was defined as units of activity per mg of protein.

The products of orotate metabolism were separated by ion-exchange chromatography as previously described (Lieberman and Kornberg, 1954).

Protein was determined by the method of Lowry *et al.* (1951).

C^{14} containing compounds were plated in a thin layer on aluminum discs and measured in a gas flow counter.

RESULTS

Purification of enzymes. All steps were carried out at 0–2 C. The purification procedure with cell-free extracts of corynebacterium I was as follows: to 5.0 ml of cell-free extract (39.5 units, specific activity 0.61) 1.25 ml of a 1 per cent solution of protamine sulfate (Eli Lilly) were added with stirring. After 5 minutes the precipitate was removed by centrifugation (*ca* 16,000 × G). The supernatant solution (42.0 units, specific activity 0.95) was treated with 2.5 ml of calcium phosphate gel (16.5 mg solids per ml)[6] with occasional stirring for 5 minutes. The supernatant fluid was collected by centrifugation (*ca* 16,000 × G) and treated again with 5.0 ml of calcium phosphate gel. The mixture was centrifuged and the supernatant fluid represented the final fraction (25.0 units, specific activity 2.2). Because of the instability of this fraction, it was prepared fresh for use from the cell-free extract.

The purification of cell-free extracts of corynebacterium II was as follows: to 10.0 ml of cell-free extract (67.0 units, specific activity 2.4), 1.5 ml of a 1 per cent solution of protamine sulfate (Eli Lilly) were added with stirring. After 5 minutes, the precipitate was collected by centrifugation (*ca* 11,000 × G), and the supernatant solution discarded. The precipitated material was washed by stirring it with 1.0 ml of potassium acetate buffer (0.05 M, pH 6.0) for 10 minutes. The precipitate was suspended in 10.0 ml of sodium citrate buffer (0.05 M, pH 6.0), stirred for 10 minutes, and the supernatant solution (39.0 units, specific activity 5.0) collected by centrifugation. After dilution with an equal volume of cold distilled water, 4.2 g of ammonium sulfate were added, and the precipitate was discarded. Upon the addition of 1.4 g of ammonium sulfate, another precipitate formed which was collected and dissolved in potassium phosphate buffer (0.02 M, pH 7.0). This preparation (25 units, specific activity 11.3) showed no loss of activity after storage for 1 month at −13 C.

Requirement for TPNH. Although the cell-free extracts showed no requirement for TPN or a reducing system, a source of TPNH was necessary for a maximum rate of orotate utilization by the partially purified enzyme preparations obtained from both organisms (table 1). The relatively rapid rate of orotate removal observed with glucose dehydrogenase and the enzyme preparation from corynebacterium I in the absence of added glucose may be due to a contamination of the enzyme preparation with glucose or another substrate for the dehydrogenase. Es-

TABLE 1

TPNH requirement for the utilization of orotic acid

Expt. No.	TPN	Glucose Dehydrogenase (GD) or Isocitric Dehydrogenase (ID)	Glucose (G) or Isocitrate (I)	Decrease in Optical Density	
				Corynebacterium I	Corynebacterium II
1	TPN	GD	G	0.172	0.261
	TPN	—	—	0.034	0.021
	TPN	—	G	0.031	0.027
	TPN	GD	—	0.116	0.062
	—	GD	G	0.029	0.031
	—	—	—	0.021	0.008
2	TPN	ID	I	0.195	
	TPN	ID	—	0.025	
	TPN	—	I	0.036	

Each cuvette contained in 3.0 ml, 0.1 ml of potassium maleate buffer (0.5 M, pH 6.5), 0.02 ml of $MgCl_2$ (0.3 M), 0.04 ml of sodium orotate (0.01 M), and 1.7 units (corynebacterium I, specific activity 2.2) or 2.6 units (corynebacterium II, specific activity 11.3) of enzyme preparation. Added as indicated were 0.01 ml of TPN (0.01 M), 0.1 ml of glucose (1), 0.05 ml of DL-isocitrate (0.18 M), 2 units of isocitric dehydrogenase, and 250 units of glucose dehydrogenase. Dihydroorotic dehydrogenase activities of the partially purified enzyme preparations were measured according to the standard assay procedures (see Materials and Methods).

[6] The calcium phosphate gel suspensions were centrifuged and the supernatant fluid removed prior to the addition of the enzyme preparations.

sentially no orotate removal was observed with this preparation and isocitric dehydrogenase in the absence of added isocitrate. DPNH either failed to replace the TPNH requirement with the enzyme preparations from both organism or was less than 5 per cent as active as TPNH.

Isolation and identification of reaction products. The requirement for reduced TPN suggested that the first step in the metabolism of orotate is its reduction to dihydroorotate. To test this possibility a reaction mixture was prepared containing in 3.0 ml, 100 μM of potassium phosphate buffer (pH 6.5), 6 μM of $MgCl_2$, 0.2 μM of TPN, 100 μM of glucose, 250 units of glucose dehydrogenase, 2.6 μM of 2-C^{14}-orotate (7.1×10^5 cpm), and 15 units of the enzyme preparation from corynebacterium I (specific activity 1.0). Incubation was carried out for 150 minutes at 34 C. The reaction was stopped by heating in a boiling water bath for 2 minutes. Insoluble material was removed by centrifugation, and the supernatant solution, adjusted to pH 7.0, was subjected to chromatography on a column of Dowex-1-formate, 10 per cent cross-linked (1 cm diam, 7 cm high). Elution was carried out as previously described (Lieberman and Kornberg, 1954) with 0.055 M sodium formate (adjusted to pH 3.2 with formic acid). A radioactive compound (6.8×10^4 cpm), presumably dihydroorotic acid, was eluted as a discrete band between 11.5 and 16 resin-bed volumes of eluent. A second radioactive band (3×10^5 cpm) was eluted in an area (25–35 resin-bed volumes of eluent) suggesting its identity with ureidosuccinic acid.

Each radioactive product was passed through "Dowex 50", hydrogen ion form (resin-bed volume of 9.4 ml), to remove the sodium ions. Water and formic acid were removed under reduced pressure (water bath temperature of 40–45 C). The residues were dried over KOH in a vacuum desiccator.

The first compound eluted from the column was identified as dihydroorotic acid by its enzymatic oxidation to orotic acid with dihydroorotic dehydrogenase (Lieberman and Kornberg, 1953*b*) (table 2). Confirmation of the identity of the second compound as ureidosuccinic acid was obtained by its conversion to 5-carboxymethylhydantoin (Nyc and Mitchell, 1947), identified by its properties on ion exchange chromatography (table 3).

TABLE 2

Identification of a reaction product as dihydroorotic acid by its enzymatic oxidation to orotic acid

Source of Enzyme Producing the Reaction Product	Test Material Added†	Orotic Acid Formed	Ratios of Optical Densities of Product*	
			$\frac{\lambda 280}{\lambda 260}$	$\frac{\lambda 280}{\lambda 300}$
	μM	μM		
Corynebacterium I	0.13	0.12	1.61	2.71
Corynebacterium II	0.13	0.11	1.61	2.82

* The ratios obtained with authentic orotic acid under the same conditions were 280/260 = 1.59; 280/300 = 2.69.

† Estimated by radioactivity measurements.

The enzymatic products tentatively identified as dihydroorotic acid were isolated by ion exchange chromatography (see Results).

The dihydroorotic dehydrogenase reaction mixtures contained in 3.0 ml, 0.1 ml of potassium phosphate buffer (1 M, pH 6.1), 0.05 ml of $MgCl_2$ (0.3 M), 0.026 ml of DPN (0.001 M), and 20 units of dihydroorotic dehydrogenase (*Z. oroticum*, specific activity 26.5).

TABLE 3

Identification of a reaction product as ureidosuccinic acid by its conversion to 5-carboxymethylhydantoin

Fraction No.	cpm	μM CMH*	cpm/μM CMH
8	1,240	0.80	
9	5,020	3.20	1,570
10	8,540	5.08	1,680
11	12,860	7.88	1,630
12	16,320	10.60	1,535
13	15,100	11.60	1,300
14	13,580	9.00	1,510
15	6,540	4.88	1,340
16	1,920	0.80	

* 5-Carboxymethylhydantoin.

An aliquot of the enzymatic product (9.8×10^4 cpm) suspected of being ureidosuccinic acid was heated with strong acid as described by Nyc and Mitchell (1947). After neutralization, it was mixed with 53.5 μM of synthetic, unlabeled 5-carboxymethylhydantoin, and the mixture was chromatographed on a column of Dowex 1, formate (diameter 1 cm; height 2 cm). The eluting fluid was 0.035 N HCl. Four ml fractions were collected, and 5-carboxymethylhydantoin, which was eluted between 15 and 35 resin-bed volumes of eluent, was estimated spectrophotometrically (E = 4,800 at λ235 in N NaOH) and by radioactivity measurements.

Experiments carried out with the partially purified enzyme preparations from corynebacterium II yielded the same products, which were isolated and identified as described for corynebacterium I (see table 2).

Oxidation of dihydroorotate. The reversibility of the conversion of orotate to dihydroorotate was demonstrated with the partially purified enzyme preparation from corynebacterium I. One and six-tenths units of enzyme were incubated with 0.22 μM of synthetic DL-dihydroorotate (kindly supplied by Dr. C. S. Miller), 0.1 μM of TPN, 6 μM of $MgCl_2$, and 100 μM of potassium maleate buffer (pH 6.5), in a volume of 3.0 ml. The control mixture contained no dihydroorotate. An increase in density corresponding to 0.075 μM of orotate was observed. Assuming that only the L-isomer of dihydroorotate is active, this represents a 65 per cent conversion to orotate.

DISCUSSION

The metabolism of the pyrimidine orotic acid in the strictly aerobic bacteria studied here appears to involve the same pathway as was previously demonstrated with the obligate anaerobe *Z. oroticum*. This is particularly noteworthy since the first step in the metabolic pathway, the conversion of orotic acid to dihydroorotic acid, is a reductive rather than an oxidative reaction.

The accumulation of ureidosuccinic acid by enzyme preparations from the aerobic organisms suggests that they also catalyze the hydrolysis of dihydroorotic acid, and that this hydrolysis represents the second step in the breakdown of orotic acid. With regard to this reaction, the ratios of ureidosuccinic and dihydroorotic acids accumulated by the corynebacterium I and corynebacterium II systems were 4.2 and 3.2, respectively, values which exceed the equilibrium value of 2.0 reported in studies with preparations from *Z. oroticum* (Lieberman and Kornberg, 1954). The basis for this lack of agreement is not apparent.

Another point of difference between the aerobic and anaerobic systems was the apparent absence in the former of an enzyme catalyzing the conversion of ureidosuccinic acid to 5-carboxymethylhydantoin (equation 1), a reaction now recognized to represent a spur from the main pathway of orotic acid metabolism (Lieberman and Kornberg, 1954).

The partially purified enzyme preparations from the two corynebacteria studied were unable to degrade orotic acid beyond ureidosuccinic acid, as indicated by the complete recovery of carbon 2 of orotic acid in dihydroorotic and ureidosuccinic acids. On the other hand, with the unfractionated extract from one of these organisms (corynebacterium I), large amounts of $C^{14}O_2$ were evolved from 2-C^{14}-orotic acid. It seems likely that the immediate source of the $C^{14}O_2$ was ureidosuccinic acid, the further metabolism of which by the *Z. oroticum* system has been shown to yield aspartic acid, CO_2, and NH_3 (equation (1)) (Lieberman and Kornberg, *in preparation*).

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SUMMARY

Two corynebacterium strains capable of metabolizing orotic acid were isolated from soil by enrichment culture.

With partially purified enzyme preparations obtained from these aerobic bacteria, evidence has been provided for a pathway of orotate metabolism essentially the same as that previously described for an obligate anaerobe from soil, *Zymobacterium oroticum*, and represented by the scheme:

$$\text{Orotate} \underset{\text{TPN}}{\overset{\text{TPNH}^+}{\rightleftharpoons}} \text{dihydroorotate} \overset{H_2O}{\rightleftharpoons} \text{ureidosuccinate} \rightleftharpoons \text{aspartate} + NH_3 + CO_2$$

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