ENZYMATIC SYNTHESIS AND BREAKDOWN OF A PYRIMIDINE, OROTIC ACID

II. DIHYDROOROTIC ACID, UREIDOSUCCINIC ACID, AND 5-CARBOXYMETHYLHYDANTOIN*

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(Received for publication, October 28, 1953)

Considerable evidence has accumulated from studies in vivo and with tissue slices indicating that erotic acid (4-carboxyuracil), or a closely related compound, is a precursor of the pyrimidines in nucleic acid (1–6). In order to study the pathway of erotic acid metabolism at the enzymatic level, a source material capable of metabolizing large amounts of erotic acid was sought. Such a system was found in an erotic acid-fermenting bacterium, Zymobacterium oroticum, isolated from mud by enrichment culture (7). Although fermentation studies with growing or resting cultures indicated a fairly extensive degradation of the pyrimidine to ammonia, CO₂, acetic acid, and a dicarboxylic acid (or acids), the use of enzyme fractions from broken cell preparations of the organism resulted in the accumulation of more complex derivatives.

With cell-free extracts and partially purified enzyme preparations, evidence has been obtained to support the accompanying scheme for erotic acid breakdown and synthesis.

Reaction 1, the reversible reduction of erotic acid, is catalyzed by dihydroorotic dehydrogenase, the purification and properties of which have been described (7). The reversible hydrolysis of L-dihydroorotic acid to yield the acyclic L-ureidosuccinic acid (Reaction 2) is mediated by an enzyme to be referred to as dihydroorotase. In Reaction 3, L-ureidosuccinic acid is recyclized to L-5-carboxymethylhydantoin; the enzyme responsible for this reaction will be referred to as 5-carboxymethylhydantoinase.

The purpose of this report is to present evidence for Reactions 2 and 3 and to describe some of their properties.

* This investigation was supported by a grant from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service.
† Aided by a fellowship from The National Foundation for Infantile Paralysis, Inc.
ENZYMATIC SYNTHESIS OF PYRIMIDINE

(1) $\text{HN-C}=\text{O}$
$\text{O}=\text{C} \, \text{CH}$
$\text{HN-C}=\text{O}$
$\text{O}=\text{C} \, \text{CH}$
$\text{O}=\text{C} \, \text{CH}_2$
$\text{O}=\text{C} \, \text{CH}_2$
$\text{O}=\text{C} \, \text{CH}_2$

Orotic acid
$\text{L-Dihydroorotic acid}$

(2) $\text{HN-C}=\text{O}$
$\text{O}=\text{C} \, \text{CH}_2$
$\text{HN-C}=\text{O}$
$\text{O}=\text{C} \, \text{CH}_2$
$\text{O}=\text{C} \, \text{CH}_2$

L-Dihydroorotic acid
$\text{L-Ureidosuccinic acid}$

(3) $\text{HN-C}=\text{O}$
$\text{O}=\text{C} \, \text{CH}_2$
$\text{HN-C}=\text{O}$
$\text{O}=\text{C} \, \text{CH}_2$
$\text{O}=\text{C} \, \text{CH}_2$

L-Ureidosuccinic acid
$\text{L-5-Carboxymethylhydantoin}$

Methods

Culture Methods and Preparation of Cell-Free Extract—Z. oroticum was cultured as described previously (7) in an orotate-yeast extract-tryptone medium under anaerobic conditions, and cell-free extracts were prepared as before (7). When growth was complete (16 to 18 hours), the cells were harvested with a Sharples supercentrifuge, briefly incubated in vacuo in an orotate solution, and broken by shaking with glass beads in a Mickle vibrator. The supernatant fluid obtained by centrifugation in a Servall centrifuge at about 10,000 × g was adjusted to 10 ml. per liter of culture (7).

Protamine Fraction—This fraction, which represents the first step in the purification of dihydroorotic dehydrogenase, was obtained as described previously (7). It contained only trace amounts of nucleic acid and was used in certain experiments with dihydroorotase. Glucose dehydrogenase was prepared from calf liver according to the procedure of Streeker and Korkes (8). Diphosphopyridine nucleotide (DPN+) was prepared as previously described (9).

Dihydroorotic acid (2-C$^{14}$) was obtained as the crystalline free acid after the enzymatic reduction of orotic acid (2-C$^{14}$) (7).

DL-Ureidosuccinic acid (ureido-C$^{14}$) was synthesized from C$^{14}$-potassium

1 This compound has been referred to as 5-(acetic acid)-hydantoin in previous publications, but has been renamed 5-carboxymethylhydantoin at the suggestion of the editors.
cyanate and DL-aspartic acid by the method of Nyc and Mitchell (10). The unlabeled compound was obtained from Dougherty Chemicals. C¹⁴-Potassium cyanate was prepared from C¹⁴-urea (Tracerlab) and potassium carbonate (11). D- and L-ureidosuccinic acid (ureido-C¹⁴) were prepared from C¹⁴-potassium cyanate and the corresponding optical isomers of aspartic acid according to the procedure for the racemic compound. Since attempts at crystallizing optically active ureidosuccinic acid have been unsuccessful, ion exchange chromatography with Dowex 1 was used to purify each of the isomers. The details of the purification procedure are essentially the same as those used for the isolation of 5-carboxymethylhydantoin (see below). Unlabeled D- and L-ureidosuccinic acids were prepared in the same manner.

DL-5-Carboxymethylhydantoin (2-¹⁴C) was prepared from 2-C¹⁴-labeled DL-ureidosuccinic acid according to the procedure of Nyc and Mitchell (10). After crystallization the material was further purified by Dowex chromatography (see below). The final product had no contaminants detectable by chromatography on Dowex 1. The unlabeled compound was synthesized by the same method (10) and crystallized twice from water. The product melted at 214–217° (uncorrected) and gave the following analysis.

\[ \text{C₉H₆O₅N₂} \]
\[ \text{Calculated.} \quad \text{C} 37.97, \text{H} 3.79, \text{N} 17.72 \]
\[ \text{Found.} \quad \text{C} 38.05, \text{H} 3.03, \text{N} 17.45 \]

D- and L-5-Carboxymethylhydantoins (2-¹⁴C) were prepared from the corresponding optical isomers of ureidosuccinic acid (2-¹⁴C) (10) and purified by ion exchange chromatography (see below). Unlabeled material was similarly prepared and purified by three crystallizations from water.

Rat liver homogenate (6 gm. of rat liver) was prepared in a glass Potter-Elvehjem homogenizer in 25 ml. of K₃HPO₄ (0.05 m). A residue obtained by centrifugation (at about 4000 × g) for 5 minutes was discarded. All operations were carried out at 3°, and the homogenates were used immediately.

Chromatographic Separation, Identification, and Estimation of Dihydropyrimidinone—These three substances and orotic acid were separated from each other and quantitatively estimated by ion exchange chromatography of the C¹⁴-labeled compounds. The reaction mixture (0.5 to 3 ml.) was acidified to thymol blue by the addition of 4 N HCl, and the precipitated protein was removed by centrifugation. The supernatant solution was adjusted to pH 7.0 with 1 M KOH (brom thymol blue as an internal indicator) and adsorbed on a
column of Dowex 1, formate (200 to 400 mesh, 10 per cent cross-linkage, height 7 cm., diameter 1 cm.). The eluent was formate buffer (0.055 M sodium formate adjusted to pH 3.2 with formic acid), and the rate of flow was adjusted to 0.5 to 1.0 ml. per minute; fractions of 10 to 15 ml. were obtained with an automatic fraction collector.

Appearance of the C14-labeled compounds in the eluate was determined by measurement of radioactivity. The number of resin bed volumes of eluting solution required to elute each of the three compounds was found to be remarkably constant in a large number of trials. With solutions of each of these compounds, labeled with C14, or with known mixtures of them, the following pattern, expressed in resin bed volumes required for elution, was generally observed: 5-carboxymethylhydantoin, 6 to 12; dihydroorotic acid, 13 to 20; and ureidosuccinic acid, 25 to 50. The elution of orotic acid required the use of more concentrated formate buffer (0.1 M sodium formate adjusted to pH 3.2 with formic acid), and complete elution required between 40 and 50 resin bed volumes of this buffer.

Ureidosuccinic acid was estimated colorimetrically according to the unpublished procedure for carbamoyl compounds of Dr. S. B. Koritz and Dr. P. P. Cohen, kindly furnished by Dr. S. Grisolia. Hydantoin (an Eastman product) was estimated after deproteinization in a boiling water bath for 2 minutes by the colorimetric method of Borsnes and Taussky (12) for creatine. Color intensity was measured in the Beckman model DU spectrophotometer at 525 mp after 14 hours incubation at room temperature.

Samples containing C14 were plated on aluminum disks and measured in a gas flow counter.

**Results**

**Products of Orotate Breakdown with Cell-Free Extract**—When cell-free extract was incubated with C14-labeled orotate until ultraviolet measurements indicated removal of the pyrimidine absorption, Dowex chromatography of the reaction mixture revealed three distinct radioactive compounds (Fig. 1). One of these, dihydroorotic acid, had been previously isolated in crystalline form and identified (7) and was now recognized by its chromatographic behavior on Dowex 1 and on paper (with a butanol-acetic acid-water solvent). The presence of two sharply defined radioactive zones in the precise areas known to be occupied by ureidosuccinic acid and 5-carboxymethylhydantoin suggested that these two compounds had been formed in the reaction, and further evidence for this will be presented below.

**Optical Activity of Enzymatically Formed Dihydroorotic Acid**—Polarimetric measurements, not included in the previous report (7), have indicated a specific rotation of +66.0° for the free acid in water.4 On the

4 We are grateful to Dr. D. Lipkin for assisting us in the use of his polarimeter.
basis of the enzymatic specificity of the L isomer of ureidosuccinic acid and the correspondence of the optical rotation of the enzymatic 5-carboxymethylhydantoin with that of the synthetic L isomer (see below), the enzymatically produced dihydroorotate has been tentatively assigned the L configuration.5

**Fig. 1.** Formation of 5-carboxymethylhydantoin, dihydroorotic acid, and ureidosuccinic acid as products of orotic acid breakdown. The composition of the reaction mixture, incubation conditions (Experiment 4, Table II), and chromatographic procedure ("Methods") are described in the text.

**Interconversion of Dihydroorotate and L-Ureidosuccinate**

**Identification of Ureidosuccinic Acid**—To confirm the identity of the radioactive product in the ureidosuccinic acid area, an aliquot of the reaction mixture containing approximately 1 μM of the C14-labeled product

5 Cooper and Wilson prepared a compound by the catalytic hydrogenation of orotic acid having the elementary composition of dihydroorotic acid and the same melting point as our enzymatically prepared compound. When this synthetic material was oxidized with dihydroorotic dehydrogenase, 46 per cent of the theoretical yield of orotic acid was obtained. This result suggests that the chemically synthesized dihydroorotic acid is a racemic mixture, of which only one isomer can be enzymatically utilized. We are indebted to Mr. C. Cooper and Dr. D. W. Wilson for furnishing us with a sample of their compound.
was mixed with 100 μM of unlabeled DL-ureidosuccinic acid and chromatographed on Dowex 1 (formate) resin. The ureidosuccinic acid content of the eluted fractions was assayed colorimetrically and the C\textsuperscript{14} content was determined. As shown in Table I, the elution patterns of the authentic ureidosuccinic acid and the C\textsuperscript{14}-labeled product were identical.

Further identification of the enzymatic product with ureidosuccinic acid was provided by the results of paper chromatography. A sample of the

### Table I

**Chromatographic Identification of Ureidosuccinic Acid in Reaction Products**

The C\textsuperscript{14}-labeled compound presumed to be ureidosuccinic acid was prepared in a reaction mixture containing 200 μM of glucose, 15 μM of MgCl\textsubscript{2}, 50 μM of potassium phosphate buffer (pH 6.1), 30 μM of cysteine (pH 7.0), 0.06 μM of DPN, 500 units of glucose dehydrogenase, 10 μM of 2-C\textsuperscript{14}-potassium orotate (30,000 c.p.m. per μM), and 0.5 ml. of cell-free extract in a volume of 3.0 ml. When the disappearance of orotate, followed spectrophotometrically at 280 nm, was complete (97 minutes), 0.05 ml. of 4 N HCl was added; the insoluble material which formed was discarded after centrifugation. 1.0 ml. of the supernatant solution (containing approximately 1 μM of product presumed to be ureidosuccinic acid) was mixed with 100 μM of unlabeled DL-ureidosuccinic acid. The mixture was neutralized with 1 M KOH and subjected to ion exchange chromatography on Dowex 1 formate (see "Methods"). Fractions of 12.8 ml. were collected, assayed colorimetrically for ureidosuccinic acid, and by radioactivity measurement for C\textsuperscript{14} content. 86.7 μM of ureidosuccinic acid were recovered in Fractions 13 to 17; the formate present in the eluent did not affect the colorimetric test.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>C.p.m. per ml.</th>
<th>μM ureidosuccinic acid per ml.</th>
<th>C.p.m. per μM ureidosuccinic acid</th>
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<tbody>
<tr>
<td>12</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>75</td>
<td>0.55</td>
<td>136</td>
</tr>
<tr>
<td>14</td>
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<tr>
<td>15</td>
<td>333</td>
<td>2.36</td>
<td>142</td>
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<tr>
<td>16</td>
<td>155</td>
<td>1.15</td>
<td>135</td>
</tr>
<tr>
<td>17</td>
<td>50</td>
<td>0.35</td>
<td>143</td>
</tr>
<tr>
<td>18</td>
<td>12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C\textsuperscript{14}-labeled enzymatic product was mixed with a sample of C\textsuperscript{14}-DL-ureidosuccinic acid of approximately equal specific activity and then subjected to ascending two-dimensional chromatography in three solvent pairs. In each case only one radioactive spot was found.\textsuperscript{6}

**Enzymatic Synthesis of Orotate from L-Ureidosuccinate**—The reversibility of orotate reduction (Reaction 1) was previously demonstrated (7) spectro-

\textsuperscript{6} Propanol-water, 10:3 (R\textsubscript{F} 0.63), and butanol-acetic acid-water, 80:16:40 (13) (R\textsubscript{F} 0.52); propanol-water and butanol saturated with 10 per cent aqueous urea solution (14) (R\textsubscript{F} 0.0); butanol-acetic acid-water and aqueous 5 per cent solution of Na\textsubscript{2}HPO\textsubscript{4} saturated and layered with isoamyl alcohol (14) (R\textsubscript{F} 0.90).
photometrically by incubating dihydroorotate with dihydroorotic dehydrogenase and a catalytic amount of DPN. Oxidation of reduced DPN was presumably achieved by the action of DPNH oxidase which was

![Graph Figure 2](image2)

**FIG. 2.** Synthesis of orotate from ureidosuccinate. The incubation mixture in the experimental cuvette contained 15 μM of MgCl₂, 100 μM of potassium phosphate buffer (pH 6.1), 0.05 μM of DPN, 30 μM of cysteine (pH 7.0), 20 μM of DL-potassium ureidosuccinate, and 2.0 ml. of protamine fraction (containing 26.6 units of dihydroorotic dehydrogenase) in a volume of 3.0 ml. The incubation mixture in the blank cell contained no ureidosuccinate. 250 units of glucose dehydrogenase and 200 μM of glucose were added at the indicated times to both cells.

![Graph Figure 3](image3)

**FIG. 3.** A comparison of the absorption spectra of orotate and the product derived from ureidosuccinate. The curve represents the absorption spectrum of sodium orotate in phosphate buffer (0.033 M, pH 6.4). ●, spectrum of reaction mixture (Fig. 2) at 41 to 45 minutes; ○, spectrum of reaction mixture (Fig. 2) at 66 to 70 minutes. In each case the values are corrected for the optical density of a blank cell containing all the reactants except ureidosuccinate.
present in the enzyme preparation. When ureidosuccinate was incubated with the protamine enzyme fraction under essentially similar conditions, it was converted to a substance having the ultraviolet absorption spectrum of orotate, and complete removal of this material could be effected when glucose and glucose dehydrogenase were added as a source of reduced DPN (Figs. 2 and 3).

Only the L isomer of ureidosuccinate was capable of conversion to orotate; the D isomer was completely inactive. Thus, under the conditions shown in Fig. 2, with 1.0 ml. of protamine fraction (containing 4.6 units of dihydroorotic dehydrogenase) the increase in optical density at 280 m\(\mu\) in 30 minutes was 0.000, 1.034, and 0.913 with the D (0.003 M), the L (0.003 M), and a mixture of both isomers (each 0.003 M), respectively. In two quantitative experiments with 0.176 \(\mu\)M of the L isomer, 0.127 and 0.134 \(\mu\)M of orotate were formed, indicating conversions of 72 and 75 per cent, respectively. With the racemic compound (0.200 \(\mu\)M) a 40 per cent conversion to orotate (0.080 \(\mu\)M) was observed, or 80 per cent conversion, assuming activity of only the L isomer.

**Influence of Ureidosuccinate Concentration on Rate of Orotate Synthesis**—When the rate of orotate synthesis was studied as a function of ureidosuccinate concentration and the data were plotted according to Lineweaver and Burk (15), a straight line was obtained (Fig. 4). \(K_s\) was calculated to be \(2.8 \times 10^{-4}\) M.
Determination of Equilibrium Constant for Dihydroorotase Reaction—The equilibrium of the reversible hydrolysis of dihydroorotate to ureidosuccinate was studied, starting with each compound as substrate. In order to

TABLE II

Equilibria of Dihydroorotase and 5-Carboxymethylhydantoinase Reactions

The reaction mixtures contained 200 \( \mu M \) of glucose, 15 \( \mu M \) of MgCl\(_2\), 50 \( \mu M \) of potassium phosphate buffer (pH 6.1), 30 \( \mu M \) of cysteine (pH 7.0), 0.05 \( \mu M \) of DPN, 500 units of glucose dehydrogenase, and 0.4 or 0.5 ml. of cell-free extract in a volume of 3.0 ml. In Experiments 1, 2, and 6, a cell-free preparation very weak in 5-carboxymethylhydantoinase activity was used. The amount and specific activity (in counts per minute per micromole) of the substrates were as follows: Experiment 1, 10 \( \mu M \) (970); Experiment 2, 0.175 \( \mu M \) (3 \( \times \) 10^4); Experiment 3, 10 \( \mu M \) (4.2 \( \times \) 10^4); Experiments 4 and 5, 10 \( \mu M \) (4 \( \times \) 10^4); Experiment 6, 2 \( \mu M \) (4 \( \times \) 10^4). Incubation was carried out in cuvettes and the reaction measured in a model DU Beckman spectrophotometer at about 30° when orotate was the substrate; in the other experiments incubation was in a 34° water bath. In Experiments 4, 5, and 6, the reduction of orotate was complete in 110, 105, and 22 minutes, and the reactions were stopped at 120, 115, and 97 minutes, respectively. At the end of the incubation period, the reaction mixtures were acidified and the neutralized supernatant solutions chromatographed on Dowex 1, formate, as described under "Methods." Recoveries of radioactivity applied to the columns, uncorrected for self-absorption by sodium formate, expressed as per cent, were 89, 82, 91, 74, 71, and 90 in Experiments 1 to 6, respectively.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Substrate</th>
<th>Products, per cent of total radioactivity</th>
<th>( K_{eq} )*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dihydro-</td>
<td>Ureido-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>orotic</td>
<td>succinic</td>
</tr>
<tr>
<td>1</td>
<td>L-Dihydro-orotic (0 min.)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>34.5</td>
<td>58.8</td>
</tr>
<tr>
<td>2</td>
<td>L-Ureidosuccinic (0 min.)</td>
<td>0</td>
<td>93.8</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>27.9</td>
<td>57.9</td>
</tr>
<tr>
<td>3</td>
<td>DL-Carboxymethylhydantoin (0 min.)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>DL-Carboxymethylhydantoin (80 min.)</td>
<td>14.5</td>
<td>32.6</td>
</tr>
<tr>
<td>4</td>
<td>Orotic</td>
<td>14.9</td>
<td>30.2</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>18.5</td>
<td>25.8</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>23.8</td>
<td>52.0</td>
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<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* These calculations neglect the participation of water in the equilibria.
† The values in parentheses do not represent equilibrium values, owing to the low 5-carboxymethylhydantoinase activity in the enzyme preparation used in these experiments.
‡ The results have been calculated on the basis of activity of only one optical isomer.
minimize the removal of ureidosuccinate by 5-carboxymethylhydantoinase (Reaction 3), an enzyme preparation was used that had been found to contain relatively little of this activity. At equilibrium, the ratio of ureidosuccinate to dihydroorotate was found to be approximately 1.9 (Table II).

Interconversion of Ureidosuccinate and 5-Carboxymethylhydantoin

Isolation and Identification of 5-Carboxymethylhydantoin—To obtain a sufficient amount of the product of orotate breakdown presumed to be 5-carboxymethylhydantoin for positive identification, a large scale reaction was carried out with 1.43 mM of 2-C\textsuperscript{14} orotate (2.0 × 10\textsuperscript{6} c.p.m.) and cell-free extract from cells obtained from 12 liters of culture medium. The reaction mixture (200 ml.) also contained glucose (0.18 M), MgCl\textsubscript{2} (0.009 M), potassium phosphate buffer (0.04 M, pH 6.4), cysteine (0.009 M, pH 7.0), DPN (8 × 10\textsuperscript{−8} M), and glucose dehydrogenase (25,000 units). At intervals during incubation at 34\degree, aliquots of the reaction mixture were removed and, after proper dilution, examined spectrophotometrically for orotate disappearance. When orotate removal was complete (4 hours), 4 N HCl was added until the solution became acid to thymol blue and precipitated protein was discarded after centrifugation. The supernatant solution was neutralized with 1 M KOH and subjected to ion exchange chromatography on a column of Dowex 1, formate (height 7.5 cm., diameter 6.5 cm.). The eluting fluid was formate buffer (0.055 M sodium formate adjusted to pH 3.2 with formic acid). The unknown material was eluted from the column between 6 and 9 resin bed volumes of eluent, with a peak at 7.8. It represented approximately 60 per cent of the radioactivity recovered from the column. Two other radioactive compounds were found, one with a peak at 15.1 (dihydroorotic acid) and the other at 30.1 (ureidosuccinic acid) resin bed volumes of eluent.

Fractions of the first radioactive material eluted from the column were combined (740 ml.) and passed through a column of Dowex 50, hydrogen ion form (200 to 400 mesh, height 6.8 cm., diameter 5.0 cm.), to remove the sodium ions. Water and formic acid were removed under reduced pressure at a water bath temperature of 40–50\degree. The residue was dried over KOH in vacuo, dissolved in hot water, and crystallized in the cold. Recrystallization from water yielded 43 mg. of a white product, representing a yield of 19.3 per cent based on the orotate as starting material.

5-Carboxymethylhydantoinase appears to be the least stable of the three enzymes under consideration. Thus, cell-free preparations stored at −16\degree for several weeks have been found to possess little 5-carboxymethylhydantoinase activity, whereas dihydroorotic dehydrogenase and dihydroorotase were not markedly reduced.
The enzymatic product was identified as 5-carboxymethylhydantoin on the basis of the following properties: (1) identity of its infra-red absorption spectrum with that of the synthetic compound (Fig. 5); (2) identical chromatographic behavior on Dowex 1, formate, resin (Table III); (3) elementary analysis for C and H in agreement with the theoretical values, although the N value was 5 per cent low; (4) melting points of DL-5-carboxymethylhydantoin, of the enzymatic compound, and of a mixture of the two, 214–217°, 215–218°, and 212–214° (all uncorrected), respectively.

The enzymatically produced compound was found to be optically active, having a specific rotation for the free acid in water of $-98.9^\circ$. The specific rotations of synthetic L- and D-5-carboxymethylhydantoin under identical conditions were $-90.1^\circ$ and $+94.6^\circ$, respectively. These discrepancies are not considered significant in view of the errors encountered in these micro scale determinations.

Reversibility of 5-Carboxymethylhydantoinase Reaction and Determination of Its Equilibrium Constant—With 5-carboxymethylhydantoin as starting material, it was observed that both ureidosuccinate and dihydroorotate were formed (Table II). From the concentrations of ureidosuccinate and 5-carboxymethylhydantoin after a steady state had been reached, an equilibrium ratio of 5-carboxymethylhydantoin to ureidosuccinate of 1.9 was observed. This value is approximately the same as that calculated in experiments starting with orotate as substrate. It should be noted that

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8 The infra-red absorption analyses were provided through the kindness of Dr. T. C. Stadtman of the National Institutes of Health, Bethesda, Maryland.

9 Polarimetric measurements were made in a universal high precision polarimeter, No. 126, D. C. Rudolph and Sons, with solutions containing 7 to 12 mg. of the compound per ml. in a micro cell with a 1 dm. path.
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with heated or acid-treated enzyme preparations no interconversion of 5-carboxymethylhydantoin and ureidosuccinate was detectable.

Inactivity of Rat Liver Hydantoinase toward 5-Carboxymethylhydantoin—Bernheim and Bernheim (16) have described an enzyme in rat liver homogenate which hydrolyzes hydantoin to hydantoic acid (ureidoacetic acid). It was of interest to determine whether similar preparations were also able to hydrolyze 5-carboxymethylhydantoin. To test this point, 3.6 ml. of rat liver homogenate were incubated with 0.4 ml. of $1.67 \times 10^{-4} \text{M}$ DL-5-carboxymethylhydantoin ($4.2 \times 10^5 \text{ c.p.m. per \mu M}$) for 4 hours at 37°. Another portion of the homogenate (4.0 ml.) was incubated with 0.01 M hydantoin under similar conditions. Whereas 66 per cent of hydantoin (33 \mu M) disappeared, presumably by hydrolysis to hydantoic acid, ion exchange analysis of the reaction mixture containing 5-carboxymethylhydantoin revealed no ureidosuccinic acid, and 97 per cent of the initial radioactivity was recovered as 5-carboxymethylhydantoin.

**DISCUSSION**

The metabolism of orotic acid by this soil bacterium has been found to involve initially a DPN-linked reduction to form L-dihydroorotic acid which is then hydrolyzed to yield L-ureidosuccinic acid. As previously discussed (7), these reactions are in all likelihood also to be found in rat

**Table III**

Chromatographic Identification of Reaction Product As 5-Carboxymethylhydantoin

The C¹⁴-labeled compound presumed to be 5-carboxymethylhydantoin was prepared essentially as described in Table I. An aliquot (0.5 ml.) of the supernatant fluid (obtained on removal of the protein precipitate from the acidified reaction mixture), containing approximately 1 \mu M of the product, was mixed with 100 \mu M of unlabeled DL-5-carboxymethylhydantoin. The mixture was neutralized and chromatographed on Dowex 1, formate, as described under "Methods." Fractions of 14.5 ml. were collected and freed of sodium ions by shaking for 10 minutes with 3 ml. of well washed Dowex 50 (hydrogen ion form) resin. The resin was removed by filtration; formic acid was removed by evaporation to dryness on a steam bath. The residues were each dissolved in 10 ml. of water, and aliquots were titrated with 0.010 N NaOH with a micro burette. As controls, 15 ml. of the sodium formate solution used for elution and 15 ml. of water containing 100 \mu M of 5-carboxymethylhydantoin were similarly treated; 0.0 and 94.6 \mu M of titratable acid were recovered, respectively.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>C.p.m. per ml</th>
<th>Carboxymethylhydantoin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>\mu M per ml.</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>0.00</td>
</tr>
<tr>
<td>3</td>
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</table>

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liver and *Lactobacillus bulgaricus* 09, in which ureidosuccinic acid, like orotic acid, has been found to serve as an effective precursor of pyrimidine nucleotide. The question whether 5-carboxymethylhydantoin, which is rapidly formed from ureidosuccinic acid by these cell-free preparations, is on the main pathway of orotic acid metabolism or on a side path has not been answered. It should be remembered that neither ureidosuccinic acid nor 5-carboxymethylhydantoin was further degraded by these preparations, and it is quite possible that, in the absence of enzymes to metabolize ureidosuccinic acid along the main pathway, the presence of 5-carboxymethylhydantoinase permitted the accumulation of an equilibrium concentration of 5-carboxymethylhydantoin. Indeed, work in progress with another type of cell-free preparation from this organism, which liberates ammonia and CO$_2$ to form L-aspartic acid from both ureidosuccinic acid and 5-carboxymethylhydantoin, indicates that the latter is metabolized only by way of ureidosuccinic as the intermediate. On this basis then the scheme for orotate metabolism should be provisionally regarded as follows:

\[
\text{Orotate} \rightleftharpoons \text{L-dihydroorotate} \rightleftharpoons \text{L-ureidosuccinate} \rightleftharpoons \text{L-aspartate} \\
\text{L-5-carboxymethylhydantoin}
\]

The natural occurrence of hydantoins appears to be very limited, this being the first known instance of 5-carboxymethylhydantoin, and the only other example being that of allantoin (5-ureidohydantoin), a metabolic product of purines in most mammals. The further breakdown of allantoin is known to proceed by way of allantoic acid (diureidoacetic acid) to urea and glyoxylic acid. Whether other examples of the natural formation of 5-carboxymethylhydantoin will be found and what the general metabolic significance of 5-carboxymethylhydantoin is, remain to be determined. In this connection, it is of interest that unsubstituted hydantoin is hydrolyzed to hydantoic acid (ureidoacetic acid) by preparations from a number of animal and plant tissues (16, 17); from our studies 5-carboxymethylhydantoin does not appear to be a substrate for this enzyme.

**SUMMARY**

1. The metabolism of orotic acid has been studied with enzyme preparations from an anaerobic soil bacterium obtained by enrichment culture for orotic acid.
2. The following reactions have been established.

\[
\text{Orotic acid} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{L-dihydroorotic acid} + \text{DPN}^+ \\
\text{L-Dihydroorotic acid} + \text{H}_2\text{O} \rightleftharpoons \text{L-ureidosuccinic acid} \\
\text{L-Ureidosuccinic acid} \rightleftharpoons \text{L-5-carboxymethylhydantoin} + \text{H}_2\text{O}
\]
3. Equilibria between dihydroorotic acid and ureidosuccinic acid, and between the latter and 5-carboxymethylhydantoin, were readily established, starting with any of these three compounds or with orotic acid. The concentrations at equilibrium of dihydroorotic acid, ureidosuccinic acid, and 5-carboxymethylhydantoin are in the approximate ratio of 1:2:4.

4. The conversion of ureidosuccinic acid, an acyclic compound, to the pyrimidine, orotic acid, is specific for the L isomer and proceeds readily when linked to a DPNH-oxidizing system.

BIBLIOGRAPHY

ENZYMATIC SYNTHESIS AND BREAKDOWN OF A PYRIMIDINE, OROTIC ACID: II. DIHYDROOROTIC ACID, UREIDOSUCCINIC ACID, AND 5-CARBOXYMETHYLHYDANTOIN
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