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AN ABSTRACT OF THE THESIS OF Penny Amy for the Master of Science in
Biology presented 2 August 1974.

Title: Enzyme Reactions using Ureidosuccinate as a Substrate during
Pyrimidine Biosynthesis and Degradation in Cl. oroticum

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

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Mary L. Taylor

Cells of Clostridium oroticum, an anaerobic bacterium, were grown on orotate as a carbon and energy source. Ureidosuccinase, an inducible enzyme in the pathway for pyrimidine degradation has been shown to convert ureidosuccinate to aspartate, CO₂ and NH₃ as reported by Lieberman and Kornberg (7). Aspartate and CO₂ were formed in approximately a 1:1 ratio from ureidosuccinase activity.

Ureidosuccinase was found to be a Mn⁺² requiring enzyme with a pH optimum of approximately pH 6.5. Enzyme activity is labile to O₂, temperature, pH, dilution and high ionic strength. The optimum conditions for storage of ureidosuccinase were found to be mixtures which contained Mn⁺², PO₄⁻³ and ureidosuccinate (the substrate of the enzyme) at -20°C in argon gassed serum vials.

Hydantoinase, an enzyme which converts ureidosuccinate to 5'-carboxymethylhydantoin in a ratio of two 5'-carboxymethylhydantoin to one ureidosuccinate, was present in cells grown on orotate. Hydantoinase was able to convert both the D and the L isomers of ureidosuccinate to carboxymethylhydantoin.

High levels of a Mg^{+2} dependent carbamyl phosphate kinase were found in extracts from cells grown on orotate. The activity was dependent upon addition of ADP or AMP to the reaction mixture. The pH optimum of the carbamyl phosphate kinase was approximately pH 6.5.

Cells grown on orotate contained high levels of an induced system for the degradation of pyrimidines whereas cells grown on glucose contained a constitutive level of enzymes for pyrimidine biosyntheses. Low levels of ureidosuccinase, carbamyl phosphate kinase and hydantoinase were found in glucose grown cells. Cells grown on glucose showed a high level of a Mn^{+2} dependent aspartate transcarbamylase (the analogous enzyme to ureidosuccinase which is operative during pyrimidine biosynthesis) which forms ureidosuccinate from aspartate and carbamyl phosphate.

A consideration of the energy and reducing power supply and demand was made for cells grown both on glucose and orotate as carbon and energy source.

ENZYME REACTIONS USING UREIDOSUCCINATE AS A SUBSTRATE
DURING PYRIMIDINE BIOSYNTHESIS AND DEGRADATION
IN CL. OROTICUM

by

PENNY AMY

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

in

BIOLOGY

Portland State University
1974

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

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INTRODUCTION

A pathway for pyrimidine biosynthesis is present in all organisms while a few organisms such as the anaerobic bacterium Clostridium oroticum also contain enzymes which are capable of pyrimidine degradation. Wachsman and Barker (14) described Cl. oroticum which had been isolated from San Francisco Bay mud. Lieberman and Kornberg (5) subsequently determined the pathway by which this organism metabolizes sodium orotate. Figure 1A shows a diagram of the biosynthesis of pyrimidines while Figure 1B shows a diagram of pyrimidine degradation.

Lieberman and Kornberg isolated and confirmed the presence of pyruvate, acetate and an unidentified dicarboxylic acid as fermentation products from cell free extracts of Cl. oroticum (6). After elucidating the pathway from orotate to aspartate, they hypothesized the further degradation of aspartate yielding products as designated in Figure 2. Aspartate, in the presence of alpha-ketoglutarate, was transaminated to oxaloacetate followed by decarboxylation to pyruvate. They predicted a dismutation of pyruvate to acetate and a dicarboxylic acid which would yield one ATP useful for cell growth.

Preliminary nutritional studies with Cl. oroticum indicated that the same amount of cell growth was produced when cells were provided orotate or glucose in equal molar amounts, and therefore the same amount of energy was made available from each substrate. Wachsman and Barker analyzed the fermentation products from glucose grown cells of Cl. oroticum (14). Although the carbon recovery was low, the fermentation pattern resembled yeast fermentations which yielded mainly CO₂, ethanol and acetate as products. Small quantities of lactate also were found.

Figure 1. Pathways for pyrimidine biosynthesis and degradation.

Figure 2. Lieberman and Kornberg's proposed scheme for pyrimidine degradation in *Cl. oroticum*.

From a yeast-like fermentation of glucose two ATP's resulted which were available to cells for growth processes. Likewise, two ATP's should be formed during the degradation of orotate to provide equal cell growth parameters. After reviewing these considerations the issue of prime interest to us was to determine which step in the degradative pathway contributed additional ATP for cell growth.

Due to the nature of enzymatic steps required for the formation of dihydroorotate (the addition of $2e^-$ across the double bond of orotate) and ureidosuccinate (addition of H_2O across a bond) no energy is likely to be produced in these two enzymatic reactions during pyrimidine degradation.

Shoesmith and Sherris (11) observed the motility of Psuedomonads when these strictly aerobic bacteria were maintained in an anaerobic environment. They found that under anaerobic conditions several species of Psuedomonas were motile in the presence of arginine and completely non-motile in the absence of arginine. Their experiments indicated that the arginine dihydrolase enzyme system was responsible for ATP production which provided energy for anaerobic motility. Arginine was degraded to citrulline which was degraded further in the presence of ADP to ornithine and ATP. Since ureidosuccinate contains a ureido group as does citrulline, ureidosuccinate may react similarly to yield ATP during ureidosuccinase activity.

The amount of growth by cells of Cl. oroticum provided with equal molar amounts of glucose or orotate as carbon and energy sources were equal whereas the amount of growth by cells provided with aspartate as a carbon and energy source was exactly one-half that observed for

orotate and glucose. The most reasonable explanation of this phenomenon was that energy for growth was released after the orotate step and before aspartate accumulated. Since dihydroorotate dehydrogenase and dihydroorotase are not likely to function as energy yielding processes of the degradative pathway, only ureidosuccinase activity remained as a potential energy producing reaction.

Energy in the form of ATP is required to produce carbamyl phosphate for synthesis of ureidosuccinate by the enzyme aspartate transcarbamylase. Likewise, ATP may be released by reversal of the same mechanism when ureidosuccinate is degraded.

Lieberman and Kornberg (7) disqualified ureidosuccinase as an energy producing enzyme involved in the pathway of orotate degradation because they could demonstrate no dependence of partially purified ureidosuccinase on ADP or phosphate ion. Since their partially purified enzyme showed extremely low activity, it is possible that they inhibited the enzyme activity by adding too much ADP; also, since the conditions under which these assays were studied are unclear, it is difficult to interpret their results. For these reasons the question of ATP production as well as other characteristics of ureidosuccinase were tested in this study.

MATERIALS AND METHODS

Cell Cultivation

Orotate Grown Cells

Clostridium oroticum cells were cultured at 30°C in the medium listed in Table 1. Cl. oroticum is a strictly anaerobic bacterium which is difficult to cultivate on a large scale. Therefore large inocula were always added to freshly steamed media in order to protect O₂ sensitive cells. One tenth ml of a 24 hour stock culture was inoculated into a 20 ml culture vial containing stock medium. After 24 hours incubation, this 20 ml culture was added to 100 ml of fresh stock medium; this preparation was cultivated another 24 hours at 30°C. The 120 ml culture was transferred to a 3 liter flask containing 2900 ml of fresh medium. When orotate was nearly depleted, the 3 liter culture was added carefully, observing sterile technique, into 10 liters of fresh medium. This culture was placed on a magnetic stirrer and was agitated slowly in order to distribute evenly the insoluble orotate. When gasing had ceased and the orotate was nearly depleted, 10-15 grams of orotate were added and incubation was continued. Prior to depletion of the additional orotate the culture was centrifuged using a CEPA continuous flow centrifuge, (Carl Padberg GMBH, 7630 Lahr/Schwarzwald) which provided a flow rate of 400 ml/minute at 40,000 rpm, and was equipped with a type K head. Wet weight yield was approximately 2.3 grams cells/liter. Cell yields have been shown to be proportionate to the amount of orotate utilized. The cell pellet was washed with approximately 150 ml of 0.05 M potassium phosphate buffer (pH 7.15). The washed cell

TABLE 1

OROTATE GROWTH MEDIUM FOR Clostridium oroticum

	<u>Amount/liter</u>
Tryptone.....	5.0 g
Yeast Extract.....	0.5 g
Sodium thioglycollate.....	0.5 g
K ₂ HPO ₄ KH ₂ PO ₄ (1 M pH = 7.54).....	50 ml
Sodium orotate.....	5.13 g
Riboflavin.....	0.015 g

For stock cultures add:

Agar.....	2 g
Resazurin.....	5 ml (0.2 mg/50 ml H ₂ O)

Final pH = 7.54

pellet was retained frozen at -20°C until used.

Glucose Grown Cells

Cl. oroticum was cultured on a semi-defined medium (Table II) under conditions described for the orotate grown cells. Cells were harvested in the same manner as orotate grown cells when a Klett-Summerson colorimeter reading of 75, using a #60 filter, was reached.

Stock Cultures

Stock cultures of Cl. oroticum were prepared by incubation of the organism at 30°C in freshly steamed 20 ml vials of stock medium for 48 hours. Stock cultures were maintained at room temperature and transferred every two weeks.

Cell Free Extracts

Cell-free extracts were prepared by suspending 7.5 g of cell paste (wet weight) to 15 ml volume in 0.05 M ACES (N-[2-Acetamido]-2-Aminoethane Sulfonic Acid) buffer pH 6.5 containing dithiothreitol (2.86×10^{-2} M [0.0665 g/15 ml]). This cell suspension was disrupted using a chilled French Pressure Cell (American Instrument Company, Inc.) held at 7000 psi. The extract was centrifuged at $34,000 \times g$ for 20 minutes at 4°C . The supernatant fluid was placed in argon gassed serum vials. Serum vials were gassed for an additional 2 minutes with argon, sealed with serum stoppers and stored at -20°C until used. This extract was considered the starting material for all enzyme work.

TABLE II

GLUCOSE GROWTH MEDIUM FOR Clostridium oroticum

	<u>g/liter</u>
Norite treated casitone.....	10.00
Glucose (autoclaved separately).....	20.00
Na citrate.....	0.25
MgSO ₄ · 7H ₂ O.....	0.20
NaCl.....	0.01
MnSO ₄ · 4H ₂ O.....	0.01
NH ₄ Cl.....	2.00
KH ₂ PO ₄	0.68
K ₂ HPO ₄	7.84
FeSO ₄ · H ₂ O.....	0.005
Na thioglycollate.....	0.50

	<u>mg/liter</u>
Pyridoxal-HCl.....	0.25
Thiamine.....	1.00
Calcium pantothenate.....	1.00
Riboflavin.....	1.00
Niacin.....	1.00
p-Aminobenzoic acid-HCl.....	0.50
Pyridoxine-HCl.....	2.00
Folic acid.....	0.25
Biotin.....	0.005
Vitamin B ₁₂	0.002

Final pH = 7.4

Purification

Streptomycin-Manganese Treatment

A test tube containing crude extract was placed in an ice bath under a light flow of 100% argon. To this extract, a 10% streptomycin sulfate solution (0.25 volume) was added slowly. Stirring was continued for an additional 20 minutes. The resulting extract was placed in glass centrifuge tubes, gassed with argon, covered with parafilm and centrifuged for 20 minutes (34,000 X g at 4°C). This supernatant fluid was labelled streptomycin-manganese extract and stored in gassed serum vials in the same manner as the crude extract.

Sephadex G-25 Chromatography

A 5 ml sample of streptomycin-manganese extract was applied to a 1.5 X 30 cm column of Sephadex G-25 equilibrated with 0.05 M ACES buffer of pH 6.5 containing 1×10^{-3} M dithiothreitol. Elution of the column was performed at 4°C using a flow rate of 1 ml/minute. Approximately 0.5 ml sample volumes were collected in an automatic fraction collector (Gilson Microfractionator). Conventional CO_2 assays were used to determine ureidosuccinase activity (13). In accordance with activity, appropriate samples were combined.

Sephadex G-50 Chromatography

A 3 ml sample of streptomycin-manganese extract was applied to a 2.5 X 40 cm column of Sephadex G-50 equilibrated with 0.1 M potassium phosphate pH 7.05 containing 1×10^{-3} M dithiothreitol. Approximately 0.5 ml samples were collected with a flow rate consistent with a 19 cm pressure head. Samples were combined by activity using a conventional

CO₂ assay. Samples were stored in gassed serum vials as before.

Enzyme Assays

Ureidosuccinase

The degradation of dihydroorotate, 5'-carboxymethylhydantoin, and ureidosuccinate was followed by measurement of CO₂ evolution under anaerobic conditions at 20°C using Warburg techniques (13). The enzymatic conversion of ureidosuccinate yielded CO₂, NH₃ and aspartate. The standard assay consisted of 180 µmoles ACES buffer (pH 6.5), 5 µmoles MgCl₂, 1.5 µmoles MnCl₂, 10 µmoles potassium phosphate, 30 µmoles D,L-ureidosuccinate or 15 µmoles of L-5'-carboxymethylhydantoin, enzyme and water to a 2.9 ml volume. Total volume in the reaction vessel was 3.0 ml since 0.1 ml of 90% trichloroacetic acid was present in the sidearm for terminating the reaction. The reaction was initiated by addition of enzyme after 10 minutes gassing of the reaction mixture with 99% N₂-1% H₂ followed by 10 minutes equilibration after sealing the flasks. Readings were taken at timed intervals thus determining the initial rate of CO₂ evolution. The readings were converted to µmoles CO₂ by standard manometric techniques (13). The reaction was terminated by adding trichloroacetic acid to the reaction mixture, thus releasing any dissolved CO₂ from solution.

Carbamyl Phosphate Kinase

Carbamyl phosphate kinase activity was assayed by the evolution of CO₂ at 20°C in a N₂-H₂ atmosphere in a Warburg respirometer. The reaction mixture contained 180 µmoles ACES buffer (pH 6.5), 5 µmoles MgCl₂, 5 µmoles carbamyl phosphate, 1 µmole ADP and water to 2.95 ml

total volume. Trichloroacetic acid (0.05 ml) was present in the second sidearm thereby giving a total volume in the Warburg vessel of 3.0 ml. The unsealed flasks were gassed for 10 minutes, sealed, and allowed to equilibrate for an additional 10 minutes. Enzyme assays were initiated by the addition of enzyme from the sidearm. Following the cessation of CO₂ evolution, the reaction was terminated by the addition of trichloroacetic acid.

Aspartate Transcarbamylase

Aspartate transcarbamylase activity was assayed in both orotate and glucose grown cell extracts according to the method of Jones (4). Reaction mixtures contained 100 μ moles Tris-HCl (tris[hydroxymethyl]aminomethane) (pH 8.5) or 100 μ moles ACES buffer (pH 6.5), 10 μ moles carbamyl phosphate, 10 μ moles L-aspartate, enzyme and water to a final volume of 0.70 ml. Triplicate mixtures were incubated in a 30°C water bath. At 10, 20, and 30 minute intervals, tubes were removed and reactions were terminated by the addition of 0.05 ml 90% trichloroacetic acid. Mixtures were centrifuged and supernatant fluids were tested for the appearance of ureidosuccinate and 5'-carboxymethylhydantoin.

5'-Carboxymethylhydantoinase

The conversion of D,L-ureidosuccinate to D,L-5'-carboxymethylhydantoin by an enzyme present in cell free extracts was described by Lieberman and Kornberg (7). A typical assay mixture contained 120 μ moles buffer (ACES pH 6.5 or Tris-HCl pH 8.5), 5 μ moles MgCl₂, 10 μ moles D,L-ureidosuccinate or 5 μ moles L-5'-carboxymethylhydantoin, enzyme and water to give a total volume of 0.70 ml. The mixture was incubated at

30°C for 20 minutes followed by the addition of 0.05 ml of 90% tri-chloroacetic acid to terminate the reaction. Samples were centrifuged and supernatant fluids were used for the measurement of D,L-ureidosuccinate and 5'-carboxymethylhydantoin.

Chemical Synthesis

L-ureidosuccinate

L-ureidosuccinate was synthesized by Dr. Mary Taylor following the method described by Nyc and Mitchell (9). Separation of products was accomplished as outlined by Lieberman and Kornberg (6). An infrared spectrum (Nujol Mull) of the synthetic L-ureidosuccinate was run on an IR spectrophotometer (Perkin-Elmer Model 137 NaCl) and the spectrum obtained compared with a spectrum of commercial D,L-ureidosuccinate prepared and run under the same conditions. Due to the extreme hydroscopic nature of L-ureidosuccinate, peaks in the spectrum were depressed. However, the spectrum matched well enough so that it was possible to identify the synthetic compound as ureidosuccinate.

L-5'-carboxymethylhydantoin

L-5'-carboxymethylhydantoin was synthesized from L-ureidosuccinate using the method reported by Lieberman and Kornberg (6). An infrared spectrum of this compound matched exactly the spectrum published by Lieberman and Kornberg (6).

Chemical Determinations

Protein Determination

Protein concentrations were measured according to two methods. Various fractions were assayed for protein content using the Folin-phenol

reagent procedure of Lowry et al (8). Unknown samples and bovine serum albumin standards were read using a #66 filter in a Klett-Summerson colorimeter.

Elution profiles were assayed for protein by determining the ratio of absorbance at 280/260 nm in a Cary 14 spectrophotometer according to the procedure of Warburg and Christian (15).

Ureidosuccinate

Quantities of D,L-ureidosuccinate and L-ureidosuccinate were determined by an extremely sensitive colorimetric method described by Prescott and Jones (10). This assay was specific for ureido compounds. Carboxymethylhydantoin, dihydroorotate, aspartate, carbamyl phosphate, ACES buffer, and ions included in the standard assays gave no color in this test.

5'-carboxymethylhydantoin

5'-carboxymethylhydantoin was measured colorimetrically by the method of Bonsnes and Taussky (1). The procedure was specific for compounds containing a hydantoin ring; no interfering compounds were found.

Aspartate

The presence of aspartate was detected by means of two methods. One method as outlined in Figure 3, was a spectrophotometric assay accomplished by coupling two enzymatic reactions to yield NAD, ultimately. Each 3.0 ml reaction mixture contained 122.5 μ moles ACES buffer (pH 7.5), 0.3 μ mole $MnCl_2$, 2.5 μ moles $MgCl_2$, 10 μ moles alpha-ketoglutarate, 10 μ moles NADH, 0.05 ml each glutamic-oxaloacetic transaminase

Figure 3. A spectrophotometric assay for aspartate formed in the supernatant fluids from ureidosuccinase activity.

and malic dehydrogenase and varying amounts of aspartate. The blank contained all reaction components except NADH. The reaction was initiated by addition of aspartate. Decreased absorption at 340 nm was followed with time. Values were taken from the linear portion of the graph. Controls where either enzyme or substrate were omitted showed no activity.

The second method was a thin layer chromatographic separation for aspartate, dihydroorotate, and ureidosuccinate using DEAE-cellulose thin layer plates (3). DEAE-cellulose slurry was spread on clean alcohol rinsed glass plates (8 X 8 cm). Plates were allowed to equilibrate and were stored in a chromatography chamber containing 0.6 N formic acid. Plates were removed as needed, partially dried, and spotted with 1 to 10 μ moles of standard solutions and 10 μ liters of unknown samples. After drying, the plates were placed in a chamber containing 0.6 N formic acid until the solvent front approached the top of the plate. Then plates were removed, dried and sprayed with Ninhydrin Reagent (Sigma), and allowed to develop for 24 hours.

Phosphate

The small volume test as described by Fiske and Subbarow (2) was used for the determination of phosphate. Since reading values continued to rise with time, reactions were started at one minute intervals so that all tubes could be read immediately following 15 minute incubation.

Carbon Dioxide

Carbon dioxide evolution was assayed by conventional techniques (13) in a Warburg respirometer (Gilson Medical Instruments).

Materials

L-ureidosuccinate and L-5'-carboxymethylhydantoin were synthesized and furnished to me by Dr. Mary L. Taylor. ACES, TRIS, PIPES, ADA and MES buffers, aspartate, L-dihydroorotate, sodium orotate, D,L-ureidosuccinate, carbamyl phosphate, dithiothreitol, NADH, bovine serum albumin, protamine sulfate, streptomycin sulfate, AMP, ADP, ATP, malic dehydrogenase, glutamic-oxaloacetic transaminase, DEAE, and Ninhydrin spray reagent were all purchased from Sigma Chemical Company. Cellulose MN 300 was purchased from E. Merck. All other chemicals and media were analytical or reagent grade.

RESULTS

Preparation of Ureidosuccinase

The purification of ureidosuccinase from orotate grown Cl. oroticum cells was hindered at every step by the instability of the enzyme. Enzyme extracts were kept at 4°C or colder during purification and storage. Extracts were stored, purified and assayed under argon or N₂-H₂ gas phases.

Several attempts were made to purify ureidosuccinase according to the method of Lieberman and Kornberg (7). They measured enzymatic activity by the evolution of ¹⁴CO₂ in such small quantities that it became difficult to compare their results with the results obtained in our experiments.

Apparently acid conditions greatly reduced enzyme activity since merely lowering the pH of the extract to pH 5.5 destroyed approximately 50% of the ureidosuccinase activity and attempts to precipitate the enzyme with acid (NH₄)₂SO₄ totally destroyed the enzyme activity. It is not known why the extract prepared from french pressed cells used here did not behave as did those broken by sonic oscillation described by Lieberman and Kornberg (7).

Precipitation with alkaline (NH₄)₂SO₄ (pH 7.87) was more successful than precipitation with acid (NH₄)₂SO₄. Ureidosuccinase activity was found in the resuspended pellet; however this activity decreased to zero overnight. No stabilization of ureidosuccinase activity was obtained by dialysis against 0.05 M ACES buffer, pH 6.5, containing 1 X 10⁻³ M dithiothreitol. Addition of 15 μmoles each of NH₄⁺ ion as NH₄Cl or SO₄⁻² ion as Na₂SO₄ to standard assays showed no inhibition of

ureidosuccinase activity. It is possible that the high ionic strength and acid conditions denatured the enzyme.

Streptomycin sulfate and manganese chloride were used to precipitate nucleic acids from crude extracts (Table III). Crude and streptomycin-manganese treated preparations were stable for 2-3 months when stored under argon at -20°C .

Precipitation of nucleic acids with MnCl_2 was not successful in terms of purification or specific activity, and did not significantly remove nucleic acids from the extract as shown by the ratio of absorbance at 280/260 nm. Manganese chloride supernatant fluid eluted from a Sephadex G-25 column retained nearly all its original specific activity and removed the residual nucleic acids from the sample as shown by the ratio of absorbance at 280/260 nm. Streptomycin sulfate treatment of crude extracts removed a small amount of nucleic acid from the sample and increased the specific activity of the sample.

Although not shown in Table III, the use of a combination of NaCl_2 and streptomycin sulfate as described in Materials and Methods was more efficient for the removal of nucleic acids. Treated extract was more than forty times higher than the value reported by Lieberman and Kornberg (7) after full purification. Using their definition of specific activity as $1 \mu\text{mole CO}_2/\text{hour}/\text{mg protein}$, the levels of $^{14}\text{CO}_2$ they measured were so low that spontaneous decarboxylation of ureidosuccinate may have been taking place.

Problems in attempting to purify the kinase mimicked those encountered with attempts to purify ureidosuccinase. Precipitation of a streptomycin-manganese treated extract, at 55% saturated alkaline

TABLE III

PREPARATION OF UREIDOSUCCINASE FROM
Clostridium oroticum

EXTRACT	280/260 nm	SPECIFIC ACTIVITIES (Units/mg Protein)
Crude extract	0.633	20.0
Streptomycin-sulfate supernatant	0.844	26.7
Manganese chloride supernatant	0.690	13.3
G-25 Eluate (combined active fractions using a manganese chloride supernate as the starting material)	1.500	11.6

(NH₄)₂SO₄ concentration, split the kinase activity equally between the precipitate and supernatant fluid; total activity recovered was only one-half the original value.

Enzyme Stability

Ureidosuccinase activity showed a severe instability to dilution. For example, when one half the sample volume of extract was used in the standard assay, a threefold decrease in the rate of CO₂ production was found. Presumably the disproportionate decrease in enzyme activity when smaller quantities of enzyme were used (dilution effect) was due to dilution of the enzyme protein, since the addition of bovine serum album, protamine sulfate, or boiled extracts from Cl. oroticum to the assay mixture so that the enzyme-protein concentration was not diluted did not show a decreased rate of ureidosuccinase activity.

In order to test the possibility that freezing and thawing destroyed the enzyme activity, two identical gassed sealed vials containing the extracts were stored at -20°C. One sealed vial was thawed and re-frozen every two weeks over a two month interval while the other vial remained at -20°C for the entire period. In the same experiment both samples were tested at the end of the two months under standard conditions. A 20 to 30% decrease in initial rate and total CO₂ production was found in frozen-thawed samples as compared with samples which had been stored two months. Even though freezing and thawing of samples did not destroy very much enzyme activity, sample volumes were kept small in order to avoid repeated exposure to air by freezing and thawing during storage. Storage of extracts at -20°C was found to be superior to storage at 4°C.

Samples placed in gassed serum vials show increased stability over those stored in parafilm covered test tubes. It is known now that some O_2 penetrates even the serum vial plugs and perhaps with time this penetration of O_2 causes some decrease in ureidosuccinase activity.

Oxygen lability of ureidosuccinase activity was demonstrated by the inability to measure any enzyme activity without the addition of dithiothreitol (1 to 10 mM final concentration) and the replacement of an air atmosphere by argon or N_2-H_2 at every step. No ureidosuccinase activity was obtained when an air atmosphere was used in the standard assay system.

Originally, the temperature used for the standard assay was $30^\circ C$ which was the temperature used for cell growth. It was discovered later that a continued linear rate reaction could be lengthened by lowering the temperature to $17-20^\circ C$ (Figure 4). The initial rate of CO_2 production was not increased by the lowered temperature. In all assays in which an initial linear rate was measured, an abrupt drop in rate occurred after approximately 10 minutes (Figure 4). The length of time over which the second rate was linear was related in part to the assay temperature. Perhaps temperature lower than $17^\circ C$ for assaying ureidosuccinase activity would have eliminated the ten minute change in rate; however, there may be other explanations for the phenomenon such as end product inhibition.

Because of the severe instability of the enzyme to O_2 , temperature, dilution, low pH, and various ions, it was necessary to determine the optimal conditions for enzyme activity. Solubility of CO_2 increases as the pH of the solution rises and therefore with higher initial pH values

Figure 5. The pH dependence of ureidosuccinase activity.

Figure 6. Comparison of ureidosuccinase activity in non-phosphate buffers.

Sephadex G-25 column, equilibrated with 0.05 M ACES buffer pH 6.5 and 10^{-3} M dithiothreitol, was prepared as follows: the sample was divided into 4 sets of 4 fractions with each containing 0.25 ml of extract. One set contained ACES buffer, 12.5 μ moles of Tris-HCl buffer pH 6.95 was added to the second set, 12.5 μ moles of potassium phosphate pH 7.0 was added to the third set and 12.5 μ moles of MES pH 6.5 was added to the fourth set. To one fraction from each set was added 0.025 μ moles of the substrate, ureidosuccinate. To a second tube of each set was added 0.025 μ moles ureidosuccinate + 0.025 μ moles MnCl_2 . To a third tube of each set was added 0.025 μ moles of aspartate, the end product of ureidosuccinase activity. To the fourth tube of each set was added 0.025 μ moles of aspartate and 0.025 μ moles MnCl_2 . The final volume was made 0.50 ml with water. The results are shown in Table IV. In all cases, the addition of the end product aspartate definitely helped in stabilizing ureidosuccinase activity. The substrate ureidosuccinate stabilized activity in the presence of ACES, phosphate or Tris-HCl but not MES. The presence of phosphate in the storage vials gave stability under all conditions and in the presence of ureidosuccinate plus MnCl_2 gave a four fold increase in activity over the control. (This also represents a two fold stimulation over the initial activity of the extract.)

Manganese has been reported to be a cofactor required for ureidosuccinase activity (7). Phosphate is a product of aspartate transcarbamylase (aspartate + carbamyl phosphate $\rightarrow$ ureidosuccinate + inorganic phosphate) activity. During degradation of ureidosuccinate, perhaps the enzyme takes up inorganic phosphate and thus is stabilized by its presence. Although attempts to demonstrate the uptake of phosphate during

TABLE IV

THE EFFECT OF CHEMICALS ON THE STABILITY
OF UREIDOSUCCINASE DURING STORAGE*

ADDITIONS	ACES	BUFFER ADDITIONS		
		PHOSPHATE	MES	TRIS
None	50	---	---	---
Aspartate	84	81.5	71.0	68.5
Aspartate + Mn^{+2}	36	74.0	47.0	58.5
Ureidosuccinate	75	80.5	35.0	56.5
Ureidosuccinate + Mn^{+2}	52	207.0	84.5	47.0

* Percent ureidosuccinase activity after five days storage at $-20^{\circ}C$ using the standard ureidosuccinase assay.

ureidosuccinase activity failed, active or heat treated, phosphate free G-25 samples showed nonspecific adsorption of phosphate and sometimes showed stimulation of CO₂ production when low amounts of phosphate were included in the reaction mixture. It is interesting to note that the exact conditions of the standard assay were those which stabilized the enzyme most. In retrospect it appears that potassium phosphate buffer was the buffer of choice; however, every attempt was made to exclude phosphate during purification so that any phosphate uptake or stimulation occurring could be measured.

Enzyme Activity

Ureidosuccinase and Hydantoinase

Ureidosuccinase was found to be specific for L-ureidosuccinate. When saturating concentrations of D,L-ureidosuccinate were compared with L-ureidosuccinate in the standard assay, the rates of CO₂ production were equal, thereby indicating that with saturating quantities of L-ureidosuccinate, the presence of D-ureidosuccinate was not inhibitory. A closely related compound, citrulline, was not a substrate for ureidosuccinase. Although citrulline contains a ureido group it is not adjacent to the end carboxyl group as in ureidosuccinate. Perhaps it is this specification which is needed for ureidosuccinase activity.

The formation of 5'-carboxymethylhydantoin from ureidosuccinate is a side reaction off the main degradative pathway. The enzyme involved is called 5'-carboxymethylhydantoinase and interconverts ureidosuccinate and 5'-carboxymethylhydantoin.

When 5'-carboxymethylhydantoin was the only substrate available a lag of up to 10 minutes preceded any CO₂ production. After the lag period

the rate of CO_2 evolution was equal to the second rate (that rate obtained after 10 minutes) from the same extract supplied with ureidosuccinate as the substrate. Because of this phenomenon, initial rates of CO_2 or total $\mu\text{moles CO}_2$ were compared as a measure of enzyme activity. This phenomenon implicated the hydantoinase conversion of 5'-carboxymethylhydantoin to ureidosuccinate as the controlling factor for the second rate observed after approximately 10 minutes reaction time, when ureidosuccinate was the substrate. Ureidosuccinate initially saturated the ureidosuccinase enzyme allowing it to react at a true rate for approximately 10 minutes until the ureidosuccinate pool was limiting due to competition between the hydantoinase, which favored 5'-carboxymethylhydantoin production by 2:1, and ureidosuccinase which rapidly removed ureidosuccinate from the substrate pool. Extracts stored at -20°C gradually lost hydantoinase activity and standard ureidosuccinase assays performed using these old extracts did not show the abrupt decrease in rate of CO_2 production after 10 minutes reaction time (Figure 7).

As shown in Table V, when the enzyme concentration was doubled, the initial rate of CO_2 production was increased by a factor of three. Another result of varying the enzyme concentration was a large variation in total $\mu\text{moles CO}_2$ produced. The theoretical number of $\mu\text{moles CO}_2$ expected from 30 $\mu\text{moles D,L-ureidosuccinate}$ would be 15 μmoles . Using 0.05 ml extract, approximately the theoretical number of $\mu\text{moles CO}_2$ were produced; using 0.1 ml extract, a tripled initial rate of CO_2 was observed, one third more CO_2 than theoretical was produced, and the second rate of the reaction continued for 145 minutes. In contrast to the results

Figure 7. The stimulation of ureidosuccinase activity by ADP.

TABLE V

THE EFFECT OF VARYING UREIDOSUCCINASE CONCENTRATION ON
ENZYMATIC ACTIVITY USING THE STANDARD
UREIDOSUCCINASE ASSAY

ml Extract	$\mu\text{moles CO}_2/\text{min}$	Total $\mu\text{moles CO}_2$	Reaction Time (min)
0.05	0.1	16.4	70
0.10	0.3	19.0	145
0.20	0.9	13.4	20

obtained with lower enzyme concentrations, using 0.2 ml extract, the initial rate was the only measurable rate found, with the reaction ending in just 20 minutes when less than the theoretical number of μ moles of CO_2 had been produced. These results seemed to suggest the possibility of end product inhibition during the enzyme reaction as well as instability of the enzyme to dilution as was mentioned earlier. In order to check for end product inhibition, fifteen μ moles of NH_4^+ ion were added to the standard reaction mixture. No change in initial rate of CO_2 production was observed; however, the addition of 15 μ moles of L-aspartate to the standard assay mixture lowered the total μ moles CO_2 produced by approximately 20% while the initial enzyme rate was lowered only slightly. Perhaps a rapid build-up of L-aspartate triggered a premature end to the enzyme reaction.

The original hypothesis of this study was to question whether or not energy in the form of ATP is evolved in the ureidosuccinase reaction. Since ATP is required in pyrimidine biosynthesis to form carbamyl phosphate, it was thought possible the primary products of ureidosuccinase activity might be carbamyl phosphate and aspartate. The further conversion of carbamyl phosphate to NH_3 , CO_2 , and ATP might result from the activity of a carbamyl phosphate kinase. This hypothesis was rejected by Lieberman and Kornberg (7) because they could show no stimulation of ureidosuccinase activity with inorganic phosphate. In this study, experiments designed to check for phosphate stimulation of ureidosuccinase were inconsistent; however, the enzyme was stabilized and stimulated when stored in the presence of phosphate as was shown in Table IV.

In order for ATP to be released during the breakdown of carbamyl

phosphate, an acceptor for the high energy phosphate of carbamyl phosphate must be present. This acceptor is usually ADP. As shown in Figure 7, ureidosuccinase activity was stimulated 25% when 1 μ mole ADP was added to the reaction mixture. A maximum initial rate of CO_2 production was obtained with the addition of 1 μ mole of ADP to the standard assay mixture. The activity of ureidosuccinase was inhibited with increased ADP concentration. Adenosine monophosphate (AMP) also stimulated ureidosuccinase activity in this reaction. The addition of 5 μ moles of carbamyl phosphate does not increase the initial rate of CO_2 produced by ureidosuccinase; however, it does lengthen the total reaction time as might be expected if it is enzymatically converted to CO_2 and NH_3 .

Since non-stoichiometric stimulation of ureidosuccinase is shown with the addition of AMP and ADP, some type of recycling process for high energy phosphate carriers must be involved. No adenosine triphosphatase activity was shown as measured by the release of inorganic phosphate from ATP with enzyme extracts. Some recycling system must be involved in this reaction since only 1 μ mole of AMP or ADP is required to produce five or more μ moles CO_2 from carbamyl phosphate. The K_m of carbamyl phosphate kinase for most organisms lies greatly in the direction of ATP production (4), and thus provides an efficient energy generating system for cell growth. It may be that ATP is degraded to AMP and pyrophosphate as diagrammed in Figure 8, or by some other high energy phosphate recycling scheme.

Carbamyl Phosphate Kinase

The carbamyl phosphate kinase enzyme showed some similarity to

Figure 8. A possible high energy recycling scheme during energy production in Cl. oroticum.

ureidosuccinase in its instability to dilution during assay. For example, when carbamyl phosphate, Mg^{+2} and enzyme were incubated for 50 minutes under standard assay conditions prior to the addition of ADP, only one-half the total μ moles CO_2 were produced as compared to a control flask where ADP was added at zero time. Extracts stored at $-20^\circ C$ lost enzymatic activity with time, especially in the absence of argon in spite of the fact that they contained 0.1 M potassium phosphate (pH 7.05) which had been shown to stabilize ureidosuccinase.

In an air atmosphere, the carbamyl phosphate kinase activity was reduced to one-half that found when assayed under a 99% N_2 -1% H_2 atmosphere. This was in contrast to the lack of any CO_2 evolution from ureidosuccinate when assayed under an air atmosphere. It is not known whether ureidosuccinase is one enzyme with an active site for the formation of carbamyl phosphate and a second site for carbamyl phosphate kinase activity or whether two different enzyme proteins are involved. In either instance, it would appear that air inhibited the initial reaction of ureidosuccinate with the enzyme totally, but allowed some kinase activity to take place.

Extracts of Cl. oroticum contained high levels of carbamyl phosphate kinase activity as compared to ureidosuccinase so that very little protein was necessary for enzyme assays. No enzymatic activity was observed when carbamyl phosphate, ADP, Mg^{+2} or enzyme was omitted from the standard kinase assay. Substitution of ATP for ADP or inclusion of 1 μ mole ATP to the assay mixture totally inhibited the reaction. It appeared that end product inhibition could have a great effect on kinase activity. Carbamyl phosphate kinase activity showed the same

inhibition with high ADP concentration as was found for the ureidosuccinase reaction. As shown in Table VI, one μ mole ADP gave a greater rate of CO_2 evolution than did 2 μ moles. Decrease in enzyme concentration by one-half did not produce exactly one-half the initial rate of CO_2 production; but it did give one-half the total μ moles CO_2 .

As shown in Table VII, a study of pH versus kinase activity showed a broad optimum between pH values 5.8 and 6.5 for the assay. It appears that the optimum pH for both the carbamyl phosphate kinase and ureidosuccinase coincide.

Orotate vs Glucose Grown Cell-Free Extracts

Studies done on dihydroorotate dehydrogenase and dihydroorotase, the two enzymes immediately preceding ureidosuccinase in pyrimidine metabolism, showed that separate enzymes were responsible for enzymatic conversion during degradation and biosynthesis (12). Pyrimidine degradative and biosynthetic pathways were outlined in Figure 1. Lieberman and Kornberg (7) described ureidosuccinase as that enzyme which degraded ureidosuccinate to CO_2 , NH_3 , and aspartate. If ureidosuccinase were involved in the release of carbamyl phosphate and aspartate, as shown in Figure 8, then its activity would be the reverse of aspartate transcarbamylase, the enzyme responsible for synthesizing ureidosuccinate from aspartate and carbamyl phosphate. It would then be possible for ureidosuccinase and aspartate transcarbamylase to be the same enzyme undergoing a reversible reaction. Thus, aspartate transcarbamylase activity in extracts prepared from cells grown under conditions of pyrimidine synthesis and degradation was tested.

Using Tris-HCl buffer pH 8.5, and the assay procedure as described

TABLE VI

THE EFFECT OF ENZYME AND ADP CONCENTRATION ON
CARBAMYL PHOSPHATE KINASE ACTIVITY*

ADP (μ moles)	Total CO ₂ (μ moles)	Initial Rate (μ moles/min)
1	3	0.3
1 ($\frac{1}{2}$ enzyme concentration)	1.5	0.18
2	2.3	0.24

* 3 ml standard kinase assay

TABLE VII

THE pH DEPENDENCE OF CARBAMYL
PHOSPHATE KINASE

pH Value	Buffer	Total $\mu\text{mole CO}_2$	% Maximum
4.2	Acetate	0.85	17
5.8	MES	4.70	94
6.5*	ACES	5.00	100
7.5	ACES	3.50	70
8.5	Tris-HCl	3.80	76

* The number of $\mu\text{moles of CO}_2$ released in ACES pH 6.5 was used as a 100% value. All other values were determined by comparison to that value. All flasks contained the standard reaction mixture using different buffers, 180 μmoles each.

in Materials and Methods, only a small quantity of ureidosuccinate was formed by extracts prepared from orotate grown cells, while a large quantity of ureidosuccinate was formed by extracts from glucose grown cells as shown in Figure 9. Neither ureidosuccinate nor 5'-carboxymethylhydantoin was formed when ACES buffer pH 6.5 was used in place of Tris-HCl buffer pH 8.5. Aspartate transcarbamylase from Cl. oroticum thus displayed a pH dependency similar to the same enzyme obtained from Streptococcus faecalis (4).

Table VIII summarizes the levels of aspartate transcarbamylase, ureidosuccinase carbamyl phosphate kinase, and hydantoinase in extracts from cells grown under conditions of pyrimidine synthesis and degradation.

During an aspartate transcarbamylase assay with a cell-free extract of glucose grown cells only 0.05 to 0.06 μ moles of 5'-carboxymethylhydantoin were found compared to 0.159 μ moles of ureidosuccinate. Although a small quantity of ureidosuccinate was converted to 5'-carboxymethylhydantoin by streptomycin-manganese treated extracts from glucose grown cells, the 2:1 ration of 5'-carboxymethylhydantoin to ureidosuccinate found with orotate grown cell extracts was not found. The formation of 5'-carboxymethylhydantoin from ureidosuccinate was apparently limited by the decreased level of hydantoinase in extracts of glucose grown cells because from 10 μ moles D,L-ureidosuccinate only 0.04 μ moles 5'-carboxymethylhydantoin was formed in 20 minutes.

Addition of Mn^{+2} was required for aspartate transcarbamylase activity even though streptomycin-manganese treated extracts were used. Extracts were prepared and tested the same day due to loss in activity

Figure 9. Ureidosuccinate formation by aspartate transcarbamylase from glucose and orotate grown cell-free extracts.

0.5 mg protein was used for the glucose cell extract assay whereas 1.0 mg protein was used for the orotate grown cell free extract assay for aspartate transcarbamylase activity.

TABLE VIII

ENZYMATIC COMPARISON OF EXTRACTS FROM GLUCOSE AND OROTATE GROWN CELLS

(A)	ENZYME	Glucose Grown Cells		Orotate Grown Cells	
		Total μ moles CO ₂	Specific Activity	Total μ moles CO ₂	Specific Activity
<u>UREIDOSUCCINASE</u>					
	Crude extract	0.43	24.6	---	---
	Streptomycin-manganese supernatant	0.084	10.1	6.0	300
<u>KINASE</u>					
	Crude extract	2.04	116.0	---	---
	Streptomycin-manganese supernatant	1.86 (1.9)	223.2	5.0	300
(B)	ENZYME	μ moles Ureidosuccinate		μ moles Ureidosuccinate	
	Aspartate transcarbamylase (30 min reaction)	0.159	2.38	0.0115	0.17
(C)	ENZYME*	5'-carboxymethylhydantoin		5'-carboxymethylhydantoin	
	<u>HYDANTOINASE</u> (20 min re-action)	0.04	0.9	4.0	150

* Glucose grown hydantoinase measured from standard hydantoinase assay. Orotate grown hydantoinase measured from standard ureidosuccinase assay.

during overnight storage at -20°C .

Two striking differences between aspartate transcarbamylase and ureidosuccinase are apparent. First, in streptomycin-manganese treated extracts, ureidosuccinase is stable at -20°C for 2 to 3 months whereas aspartate transcarbamylase is unstable to storage overnight under the same conditions. Second, the ratio of products to reactants of the two enzymes is significantly different, which indicates two different enzymes perform the reactions during pyrimidine biosynthesis and degradation. As shown in Table VIII the specific activity of ureidosuccinase in orotate grown cell extracts was 30 times greater than ureidosuccinase activity in glucose grown cell extracts. In contrast, the ratios of specific activity for aspartate transcarbamylase are 14:1 for ureidosuccinate formation for glucose grown cells as compared to orotate grown cells. Although ureidosuccinase and aspartate transcarbamylase catalyze the same reactions they probably represent two different enzymes; one for ureidosuccinate synthesis and one for ureidosuccinate degradation. This, of course, cannot be confirmed until both enzymes are purified and shown to be different in action after separation.

A carbamyl phosphate kinase is needed both to synthesize carbamyl phosphate for biosynthesis of pyrimidines and to form ATP from it under degradative conditions, and therefore should show reversibility. Although synthesis of carbamyl phosphate was not tested, as shown in Table VIII glucose grown cell extracts show a repressed level of kinase activity for the degradation of carbamyl phosphate whereas extracts from orotate grown cells show a high level of activity for degradation of carbamyl phosphate to ATP, CO_2 , and NH_3 . The activity of ureidosuccinase

is severely repressed in glucose grown cell extracts as compared with the activity in orotate grown cell extracts. Conditions within the growing cells seem to direct either mainly "biosynthesis" or "degradation" depending upon cell need.

Chemical Balance

Dihydroorotate or 5'-carboxymethylhydantoin as converted enzymatically to ureidosuccinate which was decomposed enzymatically to CO_2 , NH_3 , aspartate and possibly released energy in the form of ATP. A balance showing 5'-carboxymethylhydantoin and CO_2 production from 30 μmoles D,L-ureidosuccinate is summarized in Table IX. As can be seen, the ratio of 5'-carboxymethylhydantoin to ureidosuccinate is 2:1 even after nearly all the L-ureidosuccinate is removed by degradation to CO_2 and NH_3 . D-ureidosuccinate apparently is converted to D-5'-carboxymethylhydantoin since less than the 15 μmoles of D-ureidosuccinate remain. Thus, ureidosuccinase appears to be specific for L-ureidosuccinate whereas hydantoinase can convert either the D or L isomer to 5'-carboxymethylhydantoin.

Support of the appearance of L-aspartate from ureidosuccinase activity was found by assaying supernatant fluids from standard ureidosuccinase assays for L-aspartate by coupling several enzymatic reactions to create a spectrophotometric assay as outlined in Figure 3. L-aspartate from the supernatant fluids was compared to a series of standard aspartate concentrations. The degradation of ureidosuccinate resulted in approximately a 1:1 ratio of aspartate to CO_2 as shown in Table X. Also, aspartate was detected chromatographically from the same ureidosuccinase

TABLE IX

CHEMICAL BALANCE FROM 30 MICROMOLES D,L-UREIDOSUCCINATE
IN A STANDARD UREIDOSUCCINASE ASSAY

G-25 Elute	Total μ moles CO ₂	5'-Carboxymethylhydantoin μ moles	Ureidosuccinate μ moles
(A)	12.0	12.70	6.10
(B)	10.2	12.90	6.30
(C)	11.5	12.00	5.90
(C') ($\frac{1}{2}$ amount of extract)	3.9	16.35	9.65

TABLE X
L-ASPARTATE AND CO₂ FORMED DURING
UREIDOSUCCINASE ACTIVITY

D,L-Ureidosuccinate	Extract ml*	Total CO ₂ μmoles	Total Aspartate μmoles	Theoretical Total Aspartate μmoles
None	0.05	1.30	0.0	0.00
30 μmoles	None	0.996	0.0	0.00
30 μmoles	0.05	16.40	23.2	16.40
30 μmoles	0.10	19.00	19.8	19.00
30 μmoles	0.10	17.25	19.8	17.25

* Streptomycin treated extract. The aspartate assay described in Materials and Methods was used. Supernatants from ureidosuccinase assays were used as a source of aspartate. The values obtained are compared to the amount of CO₂ evolved during ureidosuccinase activity.

standard assay supernatant preparations. The R_f values for the standard and unknown were the same, which identified it as L-aspartate.

DISCUSSION

Previous work has shown that the two reactions preceeding ureidosuccinase in the pyrimidine pathway involve separate enzymes depending upon whether cell conditions require biosynthesis or degradation of pyrimidines (12). The biosynthetic dihydroorotate dehydrogenase and dihydroorotase are constitutive. The analogous degradative enzymes are induced when orotate is present as a carbon and energy source. From the work described here it would appear that ureidosuccinase is a different enzyme than aspartate transcarbamylase or at least a different active site on the enzyme. Aspartate transcarbamylase activity was found in extracts of glucose grown cells whereas very little aspartate transcarbamylase activity was found in extracts of orotate grown cells. On the other hand, high levels of a presumably induced enzyme system for pyrimidine metabolism are found in extracts of orotate grown cells and low levels of the same degradative enzymes in extracts of glucose grown cells. Ureidosuccinase and carbamyl phosphate kinase activities were high in orotate grown cell-free extracts, while low in extracts of glucose grown cells. Thus, ureidosuccinase appears to represent another inducible enzyme in the pathway for pyrimidine degradation.

The enzyme hydantoinase may represent a metabolic control point in the pyrimidine pathway. Hydantoinase was described by Lieberman and Kornberg as merely a side reaction (7), however it could play an important role in regulating the availability of ureidosuccinate during pyrimidine degradation and thus would be essentially inactive during pyrimidine biosynthesis. The lack of hydantoinase activity in glucose grown cell extracts as compared to orotate grown cell extracts probably represents an important difference in metabolic regulation of dihydroorotase activity

by controlling ureidosuccinate availability. Dihydroorotase interconverts dihydroorotate and ureidosuccinate. Thus ureidosuccinate could be removed to 5'-carboxymethylhydantoin during pyrimidine degradation to allow for maximum production of ureidosuccinate and allow orotate to serve as an electron "sink" during fermentation. Whereas the ureidosuccinate formed during biosynthesis is probably immediately converted to dihydroorotate by dehydroorotase.

Following breakdown of ureidosuccinate to aspartate during pyrimidine degradation, the pathway may rely on glutamic-oxaloacetic transaminase to convert aspartate to oxaloacetate (Figure 3). The presence of this enzyme was demonstrated with fresh streptomycin extracts under standard ureidosuccinase assay conditions where 20 μ moles each of L-aspartate and alpha-ketoglutarate were substituted for 30 μ moles D,L-ureidosuccinate. No CO₂ was produced when enzyme was incubated with aspartate or alpha-ketoglutarate alone. However, 10 μ moles of CO₂ were produced when both were present in the assay mixture. Although equal quantities of aspartate and alpha-ketoglutarate were most active in producing CO₂, more than 2 μ moles CO₂ were produced when only 2 μ moles of alpha-ketoglutarate were included with 20 μ moles aspartate. More than the theoretical 5 μ moles of CO₂ were produced when alpha-ketoglutarate was added to 10 μ moles D,L-ureidosuccinate in a standard assay. Greater than the theoretical number of μ moles of CO₂ could often be produced when fresh extracts were used under conditions which allowed for a long slow reaction rate. The continued availability of alpha-ketoglutarate in streptomycin extracts was probably responsible for the additional CO₂ production. Carbon dioxide was released in these reactions from the

enzymatic or spontaneous decarboxylation of oxaloacetate to pyruvate. This scheme for CO_2 production from aspartate and alpha-ketoglutarate was also proposed by Lieberman and Kornberg (Figure 2). As outlined in Figure 10, alpha-ketoglutarate was probably recycled by means of glutamic dehydrogenase releasing NADH and NH_3 . If this enzyme mechanism were operative in orotate grown cells the release of NADH at this point would neutralize the deficit in NADH molecules occurring when one NADH molecule was used to reduce orotate to dihydroorotate.

Transaminase activity was only found in fresh extracts, therefore the enzymes responsible for the further breakdown of aspartate are quite labile under the assay and storage conditions used here. Several attempts were made to demonstrate the presence of keto-acids in reaction supernatants from standard ureidosuccinase assays, but no keto-acids were found. Perhaps rapid conversion of pyruvate to acetate and CO_2 took place, removing keto-acids from the reaction mixture.

Acetate production from pyruvate would provide both an NADH and one ATP. The reducing power of NADH molecules would be in excess and reduced molecules such as lactate, malate or ethanol could be formed to relieve this excess. Lieberman and Kornberg have reported the fermentation products to be NH_3 , CO_2 , acetate and an unidentified dicarboxylic acid (6). However, no balance or methods were described. Since the products proposed are difficult to recover, they may have been missed under the methods for chemical determinations available at that time.

Under conditions of growth where the original NADH from glutamic dehydrogenase is not required to reduce orotate to dihydroorotate, (for example, when cells are grown on either ureidosuccinate or aspartate)

Figure 10. A complete scheme for the degradation of orotate by Cl. oroticum.

the degradative pathway becomes more complicated. One ATP could be produced during the ureidosuccinase reaction but the remainder of the scheme as shown in Figure 10 produces two net NADH molecules. An excess of NADH molecules (reducing power) can not be tolerated under anaerobic conditions. The net NADH molecules from growth on aspartate or ureidosuccinate could be used to reduce oxaloacetate to malate in the proportion necessary to relieve the excess NADH supply. A scheme like this would reduce net ATP production due to removal of oxaloacetate. The unidentified dicarboxylic acid reported to be present in fermentative products of Cl. oroticum grown on orotate could have been malate formed from reduction of oxaloacetate.

Since the fermentation products of Cl. oroticum cells grown on glucose include ethanol and acetate, the organisms must be capable of synthesizing alcohol dehydrogenase. Thus, the excess NADH encountered during fermentation on ureidosuccinate or aspartate could be relieved by the formation of ethanol and/or malate.

Although ATP has only been implicated as a product of ureidosuccinase activity, its formation is important to explaining the growth patterns on orotate, glucose and aspartate, the high level of carbamyl phosphate kinase in orotate grown cells, and the stimulation of ureidosuccinase activity by ADP. The scheme presented in Figure 10 provides for the energy (ATP) and reducing power (NADH) balance required during fermentation of orotate by Cl. oroticum.

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