DEGRADATION OF ERGOTHIONEINE BY CELL-FREE EXTRACTS OF *ALCALIGENES FAECALIS*[1]

II. Production of Glutamic Acid

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Abstract

Booth, James S. (University of Southern California, Los Angeles) and Milo D. Appleman. Degradation of ergothioneine by cell-free extracts of *Alcaligenes faecalis*. II. Production of glutamic acid. J. Bacteriol. **85**:654–657. 1963.—On the basis of oxidation and paper chromatographic procedures, glutamic acid was identified as the end product of ergothioneine degradation by cell-free extracts of *Alcaligenes faecalis*. Hydrogen sulfide and ammonia yields were determined. Several differences between the metabolism of whole cells and cell-free extracts were noted. Cleavage of the imidazole ring by cell-free extracts appeared to be hydrolytic rather than oxidative.

Yanasugondha and Appleman (1957) reported that *Alcaligenes faecalis* is capable of adaptively degrading ergothioneine with the production of thiolurocanic acid and trimethylamine. Disappearance of the thiolurocanic acid is presumed to be due to its degradation to unknown products.

Cell-free enzyme preparations of this strain of *A. faecalis* were shown by Kelly and Appleman (1961) to be effective in degrading ergothioneine and thiolurocanic acid with the evolution of hydrogen sulfide, a variation from whole-cell metabolism.

In the present study, the end product of ergothioneine degradation by cell-free extracts was elucidated.

Materials and Methods

Ergothioneine·HCl·H_2O was obtained from the California Corporation for Biochemical Research, Los Angeles.

[1] Confirmation of the identification of this organism was recently made by Rudolph Hugh, The George Washington University, Washington, D.C. Dr. Hugh is maintaining this organism as *Alcaligenes faecalis* 1848.

The phosphate buffer used was a mixture of 0.067 M KH_2PO_4 and 0.067 M Na_2HPO_4. A pH of 8.0 was used in these experiments.

Cell suspensions and extracts. Cells grown in nutrient broth (Difco) and adapted in phosphate buffer containing 1 μmole of ergothioneine per ml were suspended in phosphate buffer. The density of the suspension was adjusted so that a reading of 700 was obtained on a Klett-Summerson colorimeter at 540 mμ.

Cell-free extracts were obtained by sonic treatment as described by Kelly and Appleman (1961).

Manometric procedures. Standard Warburg techniques were employed (Umbreit, Burris, and Stauffer, 1959). Flasks contained 1.0 ml of cell suspension or 0.5 ml of cell-free extract, phosphate buffer containing the appropriate amount of substrate, and sufficient phosphate buffer to adjust the volume to 2.1 ml.

Ammonia production. Series of 25-ml Erlenmeyer flasks were prepared by mixing 2.0 ml of phosphate buffer, containing 5 μmoles of ergothioneine, and 1.0 ml of cell suspension in each flask. The flasks were incubated at 30 C on a "Precision" Dubnoff metabolic shaking incubator. Flasks were removed at intervals and assayed for ammonia by the method cited by Umbreit and Burris (1959) for free ammonia. Controls included cells in buffer without substrate and cells plus acetate as substrate.

Reaction mixtures for chromatography. Aerobic and anaerobic mixtures were prepared in an identical manner. Series of 25-ml Erlenmeyer flasks containing 3.0 ml of phosphate buffer, 10 μmoles of ergothioneine, and 1.0 ml of cell-free extract were incubated in a "Precision" Dubnoff metabolic shaking incubator at 30 C. One compartment of the incubator was continually flushed with nitrogen gas for anaerobic conditions and the other continually aerated for the duration of the experiment. Flasks from each compartment were removed at hourly intervals for analysis.

Chromatography. Chromatograms were developed on Whatman no. 1 paper by the ascending technique in butanol-acetic acid-water (250:60:-250) using the upper phase for development and adding a sample of the lower phase to the chromatography jar. Schleicher & Schuell no. 589 "Blue Ribbon" paper, buffered at pH 12.0, was developed in saturated phenol, pH 12.0 (McFarren, 1951).

Mixtures were deproteinized with 30% trichloroacetic acid before development in the buffered phenol; however, this step was often omitted in preparation for the butanol-acetic acid-water system without loss of separation efficiency.

Ergothioneine and thiolurocanic acid spots were detected on chromatograms developed in the butanol-acetic acid-water system with ultraviolet light (Mineralight model SL 2537), under which ergothioneine appears as a dark spot and thiolurocanic acid as a bright fluorescent spot.

Chromatograms from both systems were sprayed with ninhydrin (0.25% in acetone) for detection of glutamic acid. The paper was heated 10 min at 80 C before reading. Authentic glutamic acid was always developed along with the reaction mixtures.

Portions of 20 μliters of the samples were applied to the paper, with care taken to insure spots of nearly uniform size. Crude estimations of comparative quantities of glutamic acid in the samples were made by visually comparing the size and intensity of the developed spot with that of a known quantity of glutamic acid developed along with the sample.

Hydrogen sulfide production. Under the conditions described for chromatography, H_2S production was determined qualitatively by suspending lead acetate paper in the necks of the flasks. In several experiments, a flask containing 20 μmoles of ergothioneine was equipped with a rubber stopper and glass tubing, and the atmosphere of the flask was continually flushed with nitrogen gas into a test tube (15 × 130 mm) containing 2 ml of 1.0 M cadmium acetate. The CdS collected was assayed by a modification of the method of Smythe (1955). After 6 hr of incubation, 0.4 ml of 0.2 N iodine solution and 1.0 ml of 1.0 N HCl were added to the test tube, and excess iodine was titrated against 0.01 N $Na_2S_2O_3$. No controls other than the titration blank were used in the quantitative determination.

TABLE 1. *Oxygen uptake by whole cells of Alcaligenes faecalis on a variety of substrates at the completion of oxidation*

Substrate	Substrate concn	Oxygen uptake	Oxygen-substrate ratio
	μmoles	*μliters*	
Ergothioneine	1	69	3.1:1
	5	280	2.5:1
Acetate	5	230	2:1
Glutamic acid	5	240	2.1:1
Glutaric acid	1	10	0.4:1
Urocanic acid	1	44	2:1
Trimethylamine	5	0	—

Using this modification, about 90% of the added sodium sulfide, treated with H_2SO_4 under test conditions, was recovered as H_2S.

RESULTS

The oxidation of ergothioneine by adapted whole cells reached completion in 2 hr when 1 μmole of substrate was used. Numerous experiments were run in which the substrate concentration was varied over a range of 1 to 5 μmoles. The oxidation in these experiments varied from 43 to 71% of the theoretical 5 μmoles of O_2 per μmole of ergothioneine with an average, of 25 experiments, of 55%.

A decrease in the percentage of oxidation was observed when 5 μmoles of substrate were used as compared with the oxygen uptake on 1 μmole of ergothioneine (Table 1). Data from ten experiments involving concentrations of 1 to 5 μmoles indicated an average depression in oxygen-ergothioneine ratio of 19% at the higher concentration.

Several experiments were run to test the oxidation of possible intermediates in ergothioneine degradation. The data (Table 1) represent determinations with whole cells which oxidized ergothioneine in a "normal" manner, i.e., an oxygen-ergothioneine ratio approximating 3:1. It should be noted that trimethylamine was not oxidized.

Two experiments were made to determine the effect of various concentrations of trimethylamine on the oxidation of 5 μmoles of ergothioneine by whole cells. The data (Table 2) indicated that trimethylamine concentration has essentially no effect on the oxidation of ergothioneine.

Oxidative activity was not retained by the cell-free extracts (Table 3). The data represent

six determinations in the case of ergothioneine and three for the other substrates.

Ammonia production. Ammonia production from ergothioneine was essentially the same in three determinations. The data (Fig. 1) show that approximately 35% of the theoretical ammonia was recovered from 5 μmoles of ergothioneine in 4 hr. This is equivalent to 60 μg recovered from a theoretical 170 μg or 3.5 μmoles of ammonia from 5 μmoles of ergothioneine.

Hydrogen sulfide production. By use of the quantitative test, approximately 400 μg of H_2S were recovered from a theoretical 680 μg in a 6-hr period. This represents 58% of the H_2S of the 20-μmole sample. Since the accuracy of this method of recovery has not been critically tested, these results should not be accepted without reserve; however, they are more informative for purposes of interpretation than are the results with lead acetate paper.

Hydrogen sulfide was never detected in any of the controls or with whole cells, but was always detected after 10 to 15 min of incubation of active extracts with ergothioneine.

Chromatography. Thiolurocanic acid accumulated in the reaction mixture with what appeared to be steady rate during a 6-hr incubation period with cell-free extracts. Ergothioneine was not detected at the end of this time.

TABLE 2. *Oxidation of 5 μmoles of ergothioneine by whole cells of Alcaligenes faecalis in the presence of various concentrations of trimethylamine*

Trimethylamine	Oxygen uptake	Oxygen-substrate ratio
μmoles	*μliters*	
0	286	2.6:1
2	270	2.5:1
5	291	2.6:1

TABLE 3. *Oxidation of several substrates by cell-free preparations of Alcaligenes faecalis*

Substrate	Substrate concn	Oxygen uptake	Oxygen-substrate ratio
	μmoles	*μliters*	
Ergothioneine	5	0	0
Glutamic acid	5	0	0
Lactic acid	10	71	0.3:1
Glycerol	10	33	0.15:1

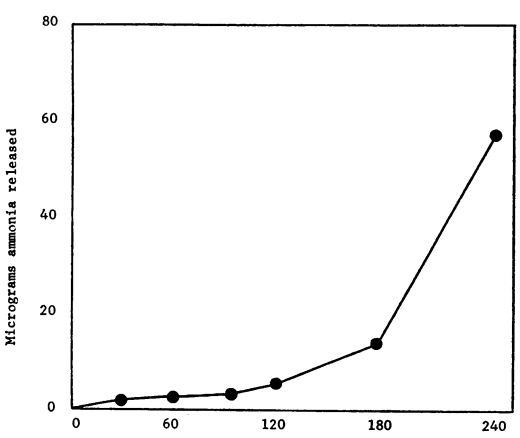

FIG. 1. *Release of ammonia from 5 μmoles of ergothioneine by whole cells of Alcaligenes faecalis.*

TABLE 4. *Chromatographic comparison of the product of ergothioneine degradation by cell-free extracts with authentic L-glutamic acid*

	Butanol-acetic acid-water (250:60:250)	Saturated phenol (pH 12.0)
	R_F	R_F
Product of ergothioneine degradation	0.25	0.33
Authentic glutamate	0.26	0.34
Reaction mixture with 5 μmoles of added glutamic acid*	0.25	0.33

* Glutamic acid was added to the reaction mixture which had been incubated for 6 hr.

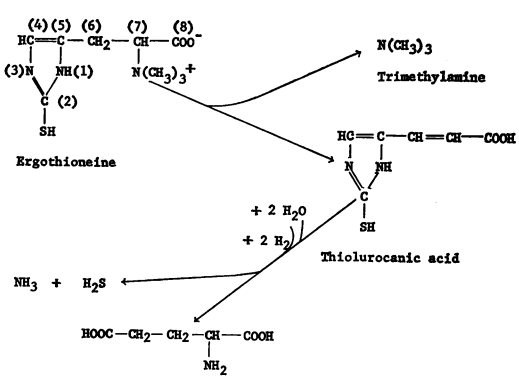

FIG. 2. *Formation of glutamic acid from ergothioneine by Alcaligenes faecalis.*

A substance which reacted strongly with ninhydrin was isolated on chromatograms of reaction mixtures containing cell-free extracts and ergothioneine. The position of the spot corresponded closely to that of authentic glutamic acid (Table 4). When glutamic acid was mixed with the diluted cell-free extract, the resultant spot corresponded exactly with that of the ninhydrin-reactive spot of the reaction mixture. This substance did not appear in any of the controls but began to accumulate from the ergothioneine substrate at 30 min (approximately 0.3 μmole per ml) and gradually increased to a maximum of approximately 5 μmoles per ml at 6 hr. No spots other than those appearing in the controls were evident on the chromatograms.

Discussion

It would seem that cleavage of the imidazole ring of ergothioneine is hydrolytic. Two atoms of oxygen are required in the formation of the second carbonyl group of glutamic acid, and, since the cell-free extracts did not utilize atmospheric oxygen, this oxygen must be derived from water.

If this hydrolytic cleavage mechanism is also true of whole cells, several factors must be considered. Whole cells utilized 3.1 μmoles of O_2 per μmole of ergothioneine but only 2.1 μmoles of O_2 per μmole of glutamic acid. The ergothioneine molecule offers other atoms which may be oxidized by the whole cell. The 2 carbon of the imidazole ring is possibly oxidized to CO_2 or to formamide in conjunction with the 3 nitrogen of the ring. In the degradation of histidine (Magasanik and Bowser, 1955), cell-free preparations of *Aerobacter aerogenes* were reported to degrade histidine with the formation of glutamic acid and formamide, whereas whole cells cleaved the formamide to CO_2 and ammonia. *Pseudomonas fluorescens* degraded histidine to glutamic acid, ammonia, and formic acid.

Formic acid was not detected by paper chromatography, although it would theoretically be produced in quantities sufficient for detection by such methods. The paper chromatographic procedures reviewed by the authors were not sufficiently sensitive for detection of formamide in this system.

The fate of the sulfhydryl group in the whole cell was not investigated; however, the whole cell is possibly capable of disposing of the sulfur in a nontoxic manner since it does not evolve H_2S.

The depressed oxygen-ergothioneine ratio at the higher concentrations has not been satisfactorily explained. Trimethylamine·HCl added to the reaction mixtures did not affect either the rate or total uptake of O_2 by whole cells. It is probably safe to assume that trimethylamine is an inert by-product of the reaction.

The 50 to 60% yield of H_2S by cell-free extracts compares favorably with the crude estimates of approximately 50% recovery of glutamic acid and thiolurocanic acid by paper chromatography.

None of the observations made during the course of these experiments would conclusively rule out glutamic acid as an intermediate in ergothioneine degradation, and, since the unknown product which accumulated in the reaction mixture resembled glutamic acid in its chromatographic characteristics, glutamic acid was thought to be a true intermediate in this pathway.

A schematic summary of the degradative pathway (Fig. 2) would indicate that the reaction requires hydrogen ions. The source of this hydrogen has not been determined; however, elucidation of the fate of the 2 carbon of the ring should give some indication of the source.

Acknowledgment

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