DEGRADATION OF ERGOTHIONEINE BY *ALCALIGENES FAECALIS*

DAVI YANASUGONDHA AND MILO D. APPLEMAN

Department of Bacteriology, University of Southern California, Los Angeles, California

Received for publication March 29, 1957

Ergothioneine was first isolated from ergot and later demonstrated in red blood cells of mammals and birds (Hunter and Eagles, 1925; Benedict *et al.*, 1926; Gulland and Peters, 1930), in boar seminal fluid (Mann and Leone, 1953), and in many other tissues of mammals (Melville *et al.*, 1954). Many fungi are capable of synthesizing ergothioneine (Melville *et al.*, 1956). The physiological action of ergothioneine in animals is not known.

No information concerning the action of bacteria on ergothioneine has been available. In the present investigation it is shown that the ability to degrade ergothioneine is not a universal property of bacteria. In the course of study, a bacterial strain, identified as *Alcaligenes faecalis*, was isolated from soil by the enrichment culture techniques. This organism is capable of utilizing ergothioneine as a sole source of carbon and energy. Two intermediate products, trimethylamine and thiolurocanic acid, have been detected.

MATERIALS AND METHODS

Ergothioneine was prepared from hog whole blood by the method of Hunter and Eagles (1927) without the use of H_2S. Deproteinization was with $Zn(OH)_2$ (Mann and Leone, 1953) by treating with $(CH_3COO)_2Zn$ and neutralizing with NaOH. A yield of 1.5 g of pure ergothioneine was obtained from 30 L of blood. Chemically prepared thiolurocanic acid was made from ergothioneine by the method of Barger and Ewins (1911). Trimethylamine hydrochloride was purchased from Eastman Kodak (Rochester, New York). Paper chromatography was one-dimensional and ascending on Whatman no. 1 filter paper and the solvent used was *n*-butanol, acetic acid and water (4:1:5 by vol, upper layer). The ultraviolet light source was a Mineralight lamp model SL2537 efficiency peaked at 2537 A (Ultra-violet Products, Inc., San Gabriel, California).

Washed cell suspension. Nonadapted cells of ergothioneine metabolizing *A. faecalis* were grown aerobically at 30 C in 100 ml of nutrient broth (Difco) in a 1 L Erlenmeyer flask for 24 hr. Ergothioneine adapted cells were grown similarly for 36 hr in 0.1 per cent ergothioneine solution containing 0.01 g of dehydrated nutrient broth per 100 ml. Cells were spun down, washed 4 times with water and resuspended in 0.1 M phosphate buffer to give a turbidity reading of 200 with a Klett-Summerson photoelectric colorimeter equipped with a 620 mμ filter.

Analytical methods. Ergothioneine and thiolurocanic acid were determined quantitatively by the method of Melville and Lubschez (1953). The color was read in a Klett-Summerson photoelectric colorimeter with a 540 mμ filter. Ergothioneine in mixture with thiolurocanic acid was separated by paper chromatography, eluted in water and determined. The following procedure was used. A 0.04 ml sample was applied on a sheet of filter paper in a horizontal band (1.5 cm by 3.5 cm). The band was allowed to dry, the sheet was subjected to chromatography in fresh solvent for 9 hr, and the ergothioneine determined within 24 hr. R_f of ergothioneine was 0.2, and thiolurocanic acid, 0.7. Under Mineralight ergothioneine spots appeared dark and thiolurocanic acid, fluorescent and greenish. The least amount of ergothioneine detectable was 2 to 3 μg while that of thiolurocanic acid was even less. The ergothioneine spot (2 cm by 4 cm) was marked with a pencil and then cut off, torn into 8 pieces and put in a test tube with 6 ml of water. Elution was carried on for 4 hr, shaking once every hour. A volume of 3 ml of eluate was tested for ergothioneine content. This required an ergothioneine calibration curve prepared by paper chromatography with known ergothioneine standards.

For qualitative detection, the chromatograms were viewed in Mineralight and also sprayed with alkaline diazo reagent of Mann and Leone (1953).

Trimethylamine was determined by the method of de la Huerga and Popper (1951) using the phenol reagent of Folin and Ciocalteu (1927). The color was read in a Klett-Summerson photoelectric colorimeter with a 620 mμ filter. As subsequent work involved collection of trimethyl-

amine in 0.5 N HCl, trimethylamine standards were made by dissolving known weights of trimethylamine hydrochloride in 0.5 N HCl, and for each determination, 4.5 ml of standard solution were neutralized with 0.5 ml of 4.5 N NaOH prior to addition of 2 ml of diluted phenol reagent and 3 ml of 10 per cent Na_2CO_3.

Screening test for ergothioneine degrading ability. The following strains of bacteria were tested for their ability to degrade ergothioneine: *Bacillus subtilis*, *Bacillus polymyxa*, *Escherichia coli*, *Escherichia freundii*, *Aerobacter aerogenes*, *Streptococcus faecalis*, *Streptococcus liquifaciens*, *Micrococcus pyogenes* var. *aureus*, *Serratia marcescens*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Neisseria catarrhalis*, and two *Alcaligenes faecalis* strains, one of which was the Department stock culture and the other, the soil isolate known to metabolize ergothioneine.

Cultures of these organisms were maintained on nutrient agar slants. Each organism was grown at 37 C in nutrient broth containing 5 μmoles of ergothioneine per ml which had been sterilized by Seitz filtration and added aseptically. At various times of incubation, 0.01-ml samples of the medium were tested qualitatively for the presence of ergothioneine by paper chromatography.

Incubation of washed cells with ergothioneine. Equal volumes of washed cell suspension and ergothioneine solution (10 μmoles/ml) were mixed and incubated at 30 C and, at various stages, the mixture was assayed for ergothioneine, thiolurocanic acid and trimethylamine. The first two compounds were determined by the following procedure. A 0.04 ml sample was made up to 6.0 ml with water in a test tube immersed in an ice water bath. From this tube, 3 ml were taken and tested directly for the content of diazo-reacting substances which represented the amount of ergothioneine and thiolurocanic acid present. This was valid because of the complete absence of any diazo-reacting compound following the disappearance of thiolurocanic acid. Simultaneously, another 0.04-ml sample was applied on a filter paper sheet, dried, and the ergothioneine content found by paper chromatography as described previously. The amount of thiolurocanic acid could be found by difference. For all practical purposes, the removal of cells prior to analysis was not necessary.

For the trimethylamine determination, a 0.4-ml sample was pipetted into a test tube and acidified immediately with 0.4 ml of 0.25 N HCl. The tube was heated in a boiling water bath for 2 min and then cooled to room temperature. Three ml of saturated Na_2CO_3 solution were added and the mixture aerated at 25 to 30 C for 2 hr. The trimethylamine which was driven off was caught in a collecting tube containing 4.5 ml of 0.5 N HCl. The collecting tube was detached and the contents neutralized with 0.5 ml of 4.5 N NaOH and the amount of trimethylamine determined. Trimethylamine hydrochloride incubated with cells was used as a control.

Isolation of thiolurocanic acid. One hundred and fifteen mg of ergothioneine dissolved in 50 ml of water and 50 ml of nonadapted cell suspension in 0.1 M phosphate buffer pH 8 were mixed and incubated at 30 C until no ergothioneine could be detected by paper chromatography. The mixture was kept in a refrigerator while the test was being performed. The total incubation time was about 12 hr. The cells were removed by centrifugation. The supernatant was made acid (to Congo red) with acetic acid to precipitate yellow crystals of thiolurocanic acid. The mixture was kept 48 hr in a refrigerator to insure complete crystallization. The precipitate was filtered, redissolved in 40 ml of water with the aid of a small amount of 0.1 N NaOH, and then recrystallized. The final precipitate of pure thiolurocanic acid after being washed with absolute ethanol and dried at 120 C weighed 47 mg. In a capillary tube, the preparation started charring at 275 C and was completely charred with indications of fusion at 300 C.

RESULTS AND DISCUSSION

Thiolurocanic acid gave the same color reaction as ergothioneine and the same calibration curve (figure 1) could be used for both. The only difference observed was that the color development in the case of thiolurocanic acid was faster. Another thiolimidazole derivative known to give this color reaction was 4-phenyl-2-thiolimidazole (Lawson *et al.*, 1950). Because of loss and probably interfering substances in filter paper, the recoveries of ergothioneine from paper chromatograms were not 100 per cent, but were quite uniform (figure 1).

Except for the ergothioneine metabolizing *A. faecalis* isolated from soil, none of the organisms tested caused any disappearance of ergothioneine added to the medium even after 96 hr of incubation. Although the detection method employed was not quantitative and a disappearance of a

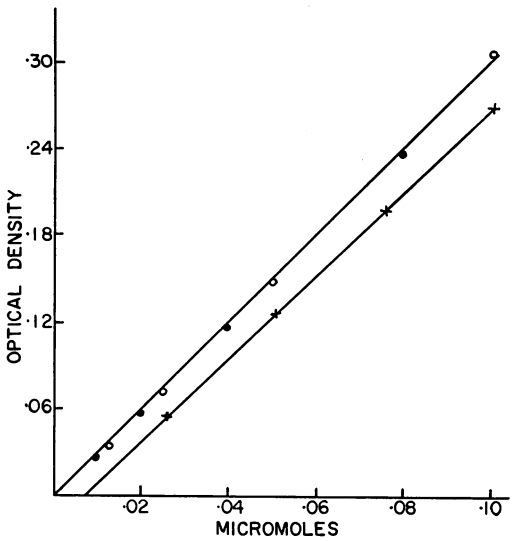


Figure 1. Calibration curves. O——O Ergothioneine determined directly; X——X ergothioneine by paper chromatography; ●——● thiolurocanic acid.

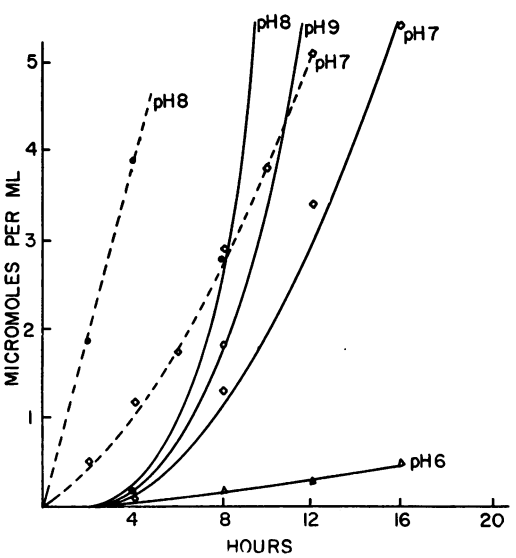


Figure 2. Ergothioneine disappearance in incubation mixture containing washed cells and 5 μmoles of ergothioneine/ml. —— Nonadapted cells; - - - ergothioneine adapted cells.

small amount of ergothioneine might have occurred, it would appear that the organisms were not capable of degrading this compound rapidly. In the case of the ergothioneine metabolizing *A. faecalis*, the chromatograms showed a gradual decrease of ergothioneine with a temporary

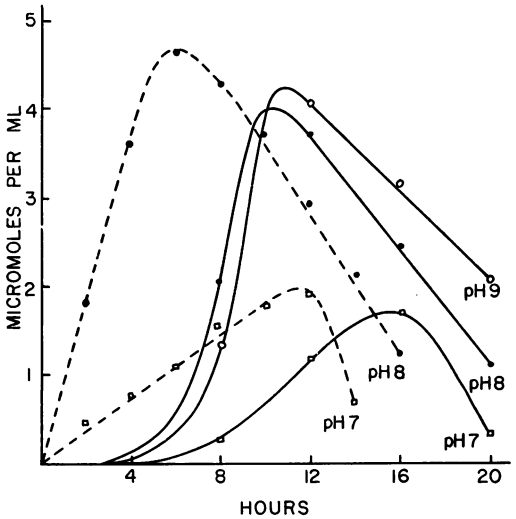


Figure 3. Thiolurocanic acid accumulation in incubation mixture containing washed cells and 5 μmoles of ergothioneine/ml. —— Nonadapted cells; - - - ergothioneine adapted cells.

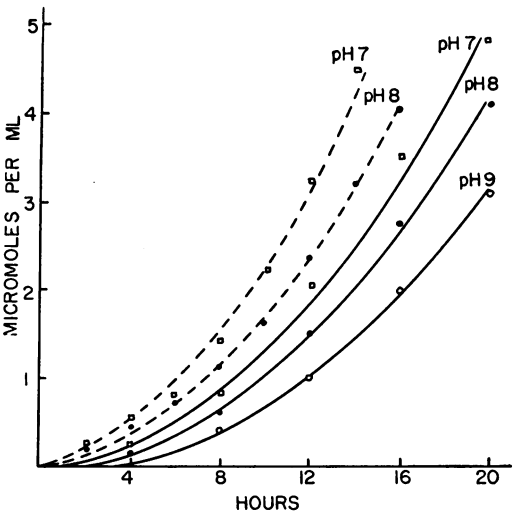


Figure 4. Thiolurocanic acid disappearance in incubation mixture containing washed cells and 5 μmoles of ergothioneine per ml. —— Nonadapted cells; - - - ergothioneine adapted cells.

accumulation of thiolurocanic acid. The former compound disappeared totally at the twelfth hour and the latter, about the twenty-fourth.

The incubation of washed cells of ergothioneine metabolizing *A. faecalis* with ergothioneine showed that the disappearance of ergothioneine was rapid at pH 8 to 9 and negligible at pH 6 (figure 2). A 2-hr lag period observed in the case

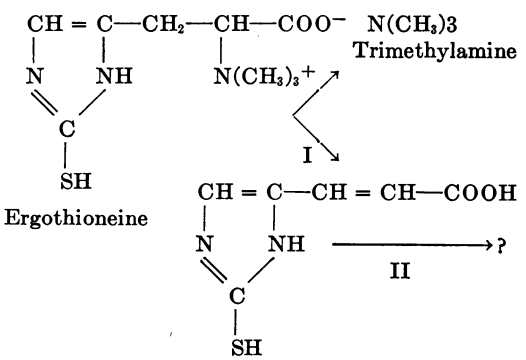
TABLE 1

Trimethylamine found in incubation mixtures (μmoles/ml)

Substrate	pH 7		pH 8	
	0 Hr	20 Hr	0 Hr	20 Hr
Ergothioneine	0	5.00	0	5.03
	0	5.03	0.02	4.85
	0.02	5.03	0	4.73
Trimethylamine	4.95	5.35	4.90	5.03
	5.20	5.28	5.12	5.03
	5.10	5.20	5.25	5.03

Incubation mixture contained nonadapted cells and 5 μmoles of substrate/ml.

of nonadapted cells disappeared when ergothioneine adapted cells were used. Temporary accumulation of thiolurocanic acid existed in all cases (figure 3). The decrease of the diazo-reacting substances at any stage could be attributed to the disappearance of thiolurocanic acid (figure 4) which was most rapid at pH 7 and consequently great accumulation of the compound could not be expected at this pH. Table 1 shows the amounts of trimethylamine from ergothioneine through the enzymatic action of cells and from trimethylamine controls. Ergothioneine was quite stable in a saturated Na_2CO_3 solution for at least 2 hr at 25 to 30 C, and in 0.1 M buffer pH 9 for at least 24 hr at 30 C. From this evidence, it appears that the organism metabolizes ergothioneine according to the following scheme:



Thiolurocanic acid

The enzymes responsible for reaction I are different from those for II. The organism evidently has no ability to metabolize trimethylamine.

It was thought that this organism might deaminate L-2-thiolhistidine, a compound structurally similar to ergothioneine, forming thiolurocanic acid in a similar fashion to the deamination of histidine to urocanic acid (Tabor *et al.*, 1952; Wickremasinghe and Fry, 1954; Hall, 1952; Mehler and Tabor, 1953). However, preliminary experiments involving growing or resting cells showed that L-2-thiolhistidine was not decomposed.

Thiolurocanic acid prepared from ergothioneine by the use of this organism was identical with that prepared chemically. This enzymatic method was comparatively simple and involved no drastic treatments with heat or alkali and was, in the authors' opinion, as good as or superior to the chemical method.

SUMMARY

Fourteen laboratory strains of bacteria did not possess the ability to degrade ergothioneine. An ergothioneine metabolizing strain of *Alcaligenes faecalis* isolated from soil degraded ergothioneine by first splitting the molecule to trimethylamine and thiolurocanic acid. Trimethylamine did not undergo further change. Thiolurocanic acid accumulated rapidly but then disappeared gradually through further degradation. The steps of this latter process are unknown. The organism did not metabolize L-2-thiolhistidine.

A method for the preparation of pure thiolurocanic acid from ergothioneine enzymatically and a method for the determination of ergothioneine in the presence of thiolurocanic acid were described.

REFERENCES

BARGER, G. AND EWINS, A. J. 1911 The constitution of ergothioneine: a betaine related to histidine. J. Chem. Soc., 2336–2341.

BENEDICT, S. R., NEWTON, E. B., AND BEHRE, J. A. 1926 A new sulfur-containing compound (thiasine) in the blood. J. Biol. Chem., **67**, 267–277.

DE LA HUERGA, J. AND POPPER, H. 1951 Urinary excretion of choline metabolites following choline administration in normals and patients with hepatobiliary diseases. J. Clin. Invest., **30**, 463–470.

FOLIN, O. AND CIOCALTEU, V. 1927 On tyrosine and tryptophane determinations in proteins. J. Biol. Chem., **73**, 627–650.

GULLAND, J. M. AND PETERS, R. A. 1930 Observations upon the reducing substances of pigeons' blood. Biochem. J. (London), **24**, 91–103.

HALL, D. A. 1952 Histidine α-deaminase and

the production of urocanic acid in the mammal. Biochem. J. (London), **51**, 499–504.

HUNTER, G. AND EAGLES, B. A. 1925 The isolation from blood of a hitherto unknown substance, and its bearing on present methods for the estimation of uric acid. J. Biol. Chem., **65**, 623–641.

HUNTER, G. AND EAGLES, B. A. 1927 Non-protein sulfur compounds of blood. I. Sympectothion. J. Biol. Chem., **72**, 123–132.

LAWSON, A., MORLEY, H. V., AND WOOLF, L. I. 1950 The determination of ergothioneine. The non-occurrence of ergothioneine in urine. Biochem. J. (London), **47**, 513–518.

MANN, T. AND LEONE, E. 1953 Studies on the metabolism of semen. 8. Ergothioneine as a normal constituent of boar seminal plasma. Purification and crystallization. Site of formation and function. Biochem. J. (London), **53**, 140–148.

MEHLER, A. H. AND TABOR, H. 1953 Deamination of histidine to form urocanic acid in liver. J. Biol. Chem. **201**, 775–784.

MELVILLE, D. B., GENGHOF, D. S., INAMINE, E., AND KOVALENKO, V. 1956 Ergothioneine in microorganisms. J. Biol. Chem., **223**, 9–17.

MELVILLE, D. B., HORNER, W. H., AND LUBSCHEZ, R. 1954 Tissue ergothioneine. J. Biol. Chem., **206**, 221–228.

MELVILLE, D. B. AND LUBSCHEZ, R. 1953 A method for the determination of ergothioneine in blood. J. Biol. Chem., **200**, 275–285.

TABOR, H., MEHLER, A. H., HAYAISHI, O., AND WHITE, J. 1952 Urocanic acid as an intermediate in the enzymatic conversion of histidine to glutamic and formic acids. J. Biol. Chem., **196**, 121–128.

WICKREMASINGHE, R. L. AND FRY, B. A. 1954 The formation of urocanic acid and glutamic acid in the fermentation of histidine by *Clostridium tetanomorphum*. Biochem. J. (London), **58**, 268–278.